Fibroblast Growth Factor-16 And Acute Doxorubicin Cardiotoxicity: A Target For Early Protection

by

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ABSTRACT

Background: Doxorubicin is an anti-cancer drug that is widely used in chemotherapy. However, doxorubicin-induced cardiotoxicity is a major risk factor for cancer patients and survivors, and can lead to heart failure. Dexrazoxane is the only approved drug to offer protection against doxorubicin-induced cardiotoxicity, but its use is limited. Strategies are needed to protect the heart against doxorubicin-induced cardiotoxicity and still allow its effective treatment of cancer.

Fibroblast growth factors (FGFs) are a family of 23 multifunctional proteins, with properties that include effects on cell growth, survival, efflux transport, and cytoprotection. FGF-16 is the only member of the family that is produced preferentially by postnatal cardiac myocytes. Production of cardiac-specific proteins, including α-actin, troponin I, and myosin light chain 2, are often targeted negatively by doxorubicin due to effects at the transcriptional level. In addition, evidence including from FGF-16 null mice that were stressed through chronic high blood pressure, suggests FGF-16 contributes to the maintenance of a healthy myocardium and may have cardioprotective properties.

Hypothesis: FGF-16 synthesis (transcription) is an early target of doxorubicin, and decreased endogenous FGF-16 levels will decrease cardiac myocyte survival and, as a result, may contribute to a decreased resistance to heart damage. Thus, maintaining and/or increasing cardiac FGF-16 levels will increase resistance to doxorubicin-induced cardiotoxicity. This is due, at least in part, to a specific effect on efflux drug transport consistent with a decrease in intracellular doxorubicin concentration in cardiac myocytes.

Approaches and Results: Using quantitative polymerase chain reaction, FGF-16 messenger RNA levels were significantly decreased within 6 hours of doxorubicin treatment in both 8-week-old rat hearts and neonatal rat cardiac myocytes. The latter was linked to decreased transcription factor Csx/Nkx2.5 association with the FGF-16 gene promoter, based on the results of transient gene transfer, protein binding, and RNA stability assays. Together with the relatively short FGF-16 mRNA half-life (~1.75 hours), FGF-16 is an early target of doxorubicin-induced cardiotoxicity. Furthermore, a decrease in FGF-16 production using FGF-16 siRNA "knockdown" was linked to reduced cardiac myocyte survival, while an increase in FGF-16 levels using adenoviral delivery was associated with resistance to doxorubicin-induced cardiac dysfunction and cardiac myocyte death; the latter corresponded to an increase in efflux transport of calcein AM and doxorubicin.

Discussion: Observations made demonstrate that postnatal cardiac-specific FGF-16 synthesis is an early target of acute doxorubicin-induced cardiotoxicity due to a negative effect on the cardiac transcription factor Csx/Nkx2.5 and the relatively unstable FGF-16 transcripts. Endogenous FGF-16 helps maintain neonatal cardiac myocyte viability, while exogenous FGF-16 is protective by, at least in part, upregulation of efflux drug transporters. These observations are consistent with a cardioprotective role for FGF-16.

Dedicated to my grandmother 柳桂珍 Liu Guizhen (1931-2016)

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ABBREVIATIONS

μM	micromolar
nM	nanomolar
+dp/dt	the rate of rise of left ventricular pressure
-dp/dt	the rate of decline of left ventricular pressure
7-AAD	7-Amino-Actinomycin D
AAV	adeno-associated virus
ABC transporters	ATP-binding cassette transporters
ABCB1	ATP-binding cassette sub-family B member 1
ABCC1	ATP binding cassette subfamily C member 1
ABCG2	ATP-binding cassette sub-family G member 2
ACE	angiotensin converting enzyme
Act D	actinomycin D
AdV	adenovirus
Akt	protein kinase B
ALD	adrenoleukodystrophy
AngII	angiotensin II
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
ATRX	alpha thalassemia/mental retardation syndrome X
bc	*bulbus cordis*
Bax	Bcl-2-like protein 4

Bcl-2	B-cell lymphoma 2
$Ca_v1.2$	calcium channel, voltage–dependent, L type, alpha 1C subunit
CCON	Canadian Cardiac Oncology Network
ChIP	chromatin immunoprecipitation
CO	cardiac output
CsA	cyclosporine A
Ct	cycle threshold
CT-1	cardiotrophin-1
CV	common ventricle
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DOX	doxorubicin
DP	developed pressure
E′/ A′	peak early/late diastolic velocity ratio
EDP	end-diastolic pressure
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GSK-3	glycogen synthase kinase-3
HCN1	potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1
FOXP1	forkhead box P1

HF	heart failure
HFpEF	heart failure with preserved ejection fraction
HO-1	heme oxygenase-1
HR	heart rate
HSPGs	heparan sulfate proteoglycans
IgG	immunoglobulin G
IGF	insulin-like growth factor
IVCT	isovolumic contraction time
IVRT	isovolumic relaxation time
kDa	kilodalton
LA	left atrium
LDH	lactate dehydrogenase
LVEF	left ventricle ejection fraction
LVFS	left ventricle fractional shortening
LV MPI IV	left ventricle isovolumic myocardial performance index
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance protein
MEF2	myocyte enhancer factor 2
MF4	fusion between the 4^{th} and the 5^{th} metacarpals and hypoplasia of the 5^{th} digit
MKP	MAPK phosphatase
MLC2	myosin light chain 2
MOs	morpholinos
MOI	multiplicity of infection

MRP	multidrug resistance-associated protein
mTOR	mammalian target of Rapamycin
NCL-H460	human non-small lung cancer cell line
NE	nuclear extract
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
NF-κB	nuclear factor-kappa B (p50/65)
Csx/Nkx 2.5	NK2 homeobox 5, CSX, Csx
Nrf-2	nuclear factor erythroid-2-related factor
NRG-1	neuregulin-1
NS	not significant
OABP	organic anion-binding transporter
p.Luc	promoter firefly luciferase reporter
PBS	phosphate-buffered saline
p-gp	p-glycoprotein
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PVDF	polyvinylidene fluoride
RAS	rat sarcoma protein
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
ROS	reactive oxygen species
qPCR	quantitative polymerase chain reaction

SDS- PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
si RNA	small interfering RNA
SEF	similar expression to FGF genes
SOX17	Sex Determining Region Y box 17
SPRY	Sprouty
STAT	signal transducer and activator of transcription
SU5402	fibroblast growth factor receptor (FGFR) tyrosine kinase inhibitor
SV	stroke volume
TGF- β	transforming growth factor- β
TKI	tyrosine kinase inhibitors
TKRs	tyrosine kinase receptors
TnI	troponin I
Untr17	gene desert on chromosome 17
VEGF	vascular endothelial growth factor
WT	wild type

Chapter 1: Introduction

1.1 Chemotherapy-induced cardiotoxicity

The word chemotherapy ("chemo", antineoplastic, or cytotoxic therapy) originally meant the use of any chemical or drugs to treat any diseases, including cancer, or those related to viral, bacterial and other microorganism infection [1, 2]. To the public, chemotherapy usually refers to the use of specific chemical drugs to selectively treat different types of cancer [1, 3]. Cancer is the second leading cause of death in the world [4]. Successful chemotherapy plays an important role in cancer patient survival and quality of life in cancer survivors [5].

The German biochemist Paul Ehrlich laid the groundwork for the concept of chemotherapy, and the use of chemicals to fight infectious diseases, for which he received the Nobel Prize in Physiology or Medicine in 1908 [3]. The first type of cancer chemotherapy drug was nitrogen mustard, which was derived from (sulphur) mustard gas and used as a chemical weapon during World War I and II [6, 7]. Exposure to nitrogen mustard gas during World War II led to very low white blood cell counts in a group of people during a military operation [8]. As a result, doctors treated a few advanced lymphoma patients with intravenous mustard injection and obtained reasonable but temporary positive results in managing cancer progression [8]. This had doctors and researchers looking for other drugs or substances that have similar effects on rapidly growing cells such as cancer cells [6, 8]. From then, more than 200 drugs (listed on National Cancer Institute website: *https://www.cancer.gov/about-cancer/treatment/drugs*) have been discovered or developed, and some continue to be used for cancer treatment today.

Cancer chemotherapy drugs can be divided into different groups based on the chemical structure and how they work in treating cancer: alkylating agents (nitrogen mustard, cyclophosphamide, cisplatin), antimetabolites (5-fluorouracil), anti-tumor antibiotics (doxorubicin, mitomycin), topoisomerase inhibitors (topotecan, etoposide), mitotic inhibitors (paclitaxel, vinblastine), corticosteroids (dexamethasone), and miscellaneous chemotherapy drugs (L-asparaginase) [9-11]. These chemotherapy drugs work by blocking one or more phases of the cell cycle, specifically G0, G1, S, G2, and M phases [11, 12]. Combinational cancer therapy is often given to patients to block multiple phases of the cellscycle at the same time for maximum effect in order to cure cancer, to control cancer progression or offer palliative treatment in advanced cancer [13].

Chemotherapy is often used systematically by intravenous administration, which is different from radiation and surgery that are used locally [14]. Chemotherapy does not distinguish rapidly dividing cancer cells from "normal" cells trying to replace the dead cells in healthy tissue [15]. This means that chemotherapy may cause severe side effects by killing or damaging normal cells in our body [15, 16]. If too little chemotherapy is given, cancer cells will survive; if too much is given, cancer cells will be killed but so will our normal healthy functioning cells, tissues, and organs. To maximize the effects of cancer chemotherapy, a fine balance must be found between killing the cancer cell and sparing normal healthy cells. For example, hair follicle cells can grow back at the end of chemotherapy, but damage to other tissues such as bone marrow may promote the secondary development of cancer [14]. In addition, cardiac myocytes are largely post-mitotic after birth, so the damage to cardiac myocytes from chemotherapy is permanent and irreversible [10, 11]. As a result, heart failure that develops from cancer therapy-induced cardiotoxicity is a leading cause of death in

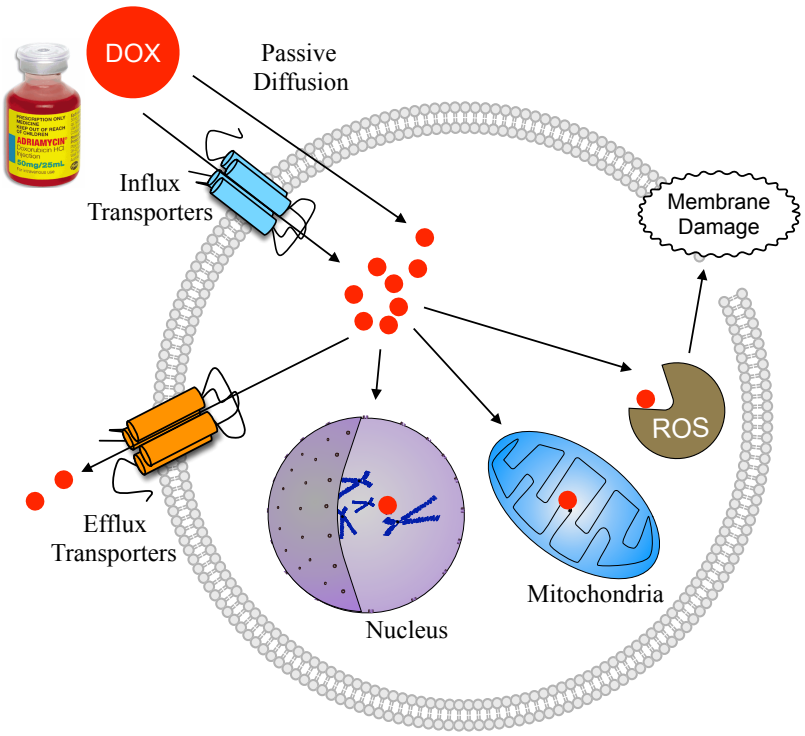
cancer survivors, and second only to cancer recurrence [11, 16].

1.2 The emergence of "Cardio-Oncology"

All cancer treatments can have various cytotoxic effects in different parts of our body [15]. Among all the chemotherapy drugs, anthracyclines are well known for their severe cardiotoxicity [9-11]. The anthracyclines (doxorubicin, daunorubicin, and epirubicin) are strong anti-tumor antibiotics that have been used widely to treat cancers in both adults and children [17, 18]. However, anthracycline chemotherapy can induce severe cardiotoxicity that leads to interference with the chemotherapy drug treatment regimen and affects the life quality of cancer patients or survivors [9, 11, 18].

Doxorubicin is the 14-hydroxylated version of daunorubicin (the first drug discovered in the anthracycline family) and is still widely used today in the treatment of cancer patients [18]. It is produced by the bacteria *Streptoycete peucetius* and has antibacterial properties [18]. The severe side effects of cardiotoxicity can lead to heart failure up to 10 years following treatment and the distinct red color have earned doxorubicin the name the "red devil" [18-20]. The major mechanisms involved in doxorubicin-induced cardiotoxicity are: 1) production of high levels of free radicals or reactive oxygen species, which promotes DNA, RNA, protein damage and lipid peroxidation [21, 22]; 2) inhibition of topoisomerase II, an enzyme critical to DNA unwinding and repair [23]; and 3) interaction and intercalation with DNA and transcriptional inhibition of cardiac muscle gene expression [24, 25]. This includes genes encoding enzymes that are important for energy production in the heart or genes that are important to cardiac contractility [24, 25] (**Figure 1**).

Figure 1. The major mechanisms involved in doxorubicin-induced cardiotoxicity



Doxorubicin (DOX) enters the cardiac myocytes by passive diffusion across membranes or through influx drug transporters (blue). Once in the cell, doxorubicin causes damage by DNA intercalation and transcriptional inhibition in the nucleus; increase iron and reactive oxygen species (ROS) accumulation to induce mitochondria and membrane damage [21-25]. Increased membrane damage by ROS further increases the intake of DOX and causes more intracellular cardiac myocyte damage. In addition, more than 50% of DOX is removed from the cell by efflux drug transporters [26].

Doxorubicin-induced cardiotoxicity is cumulative and dose-dependent [18-20, 23]. This means cancer patients are at risk of developing acute, sub-acute or chronic cardiac events at any time from the starting of the first chemotherapy treatment until years after the chemotherapy has ended [18-20, 27]. The risk of developing acute and sub-acute cardiotoxicity, such as arrhythmias, acute coronary syndromes, pericarditis, myocardial edema and acute left ventricular failure a few hours after the first dose of chemotherapy administration or any time up to two weeks after chemotherapy was completed is about 11% [9-11, 27]. This means the cardiac damage is already done before the cardiotoxicity was diagnosed based on the patient's symptoms. Early detection of cardiac dysfunction for diagnosis, as well as appropriate cardiovascular drug treatment, such as the use of vasodilators to treat the early symptoms, may reverse the cardiotoxicity induced by doxorubicin at the acute stage [27]. The chance of developing chronic cardiotoxicity such as cardiomyopathy, hypertrophy, fibrosis, and congestive heart failure is lower at 1.7%, within 30 days following the last dose of chemotherapy, but may occur even up to 10 years after the treatment [27]. The typical signs of chronic cardiotoxicity are often subclinical, this includes asymptomatic systolic and/or diastolic myocardial dysfunction that can lead to heart failure and death [9-11]. In addition, about 60% of children with cancer are being treated with anthracycline chemotherapy drugs, and up to 10% of cancer survivors will develop symptomatic cardiomyopathy even up to 15 years after completion of the chemotherapy and into their adulthood [28-30]. Thus, the chronic cardiotoxicity induced by chemotherapy drugs is often irreversible and the symptoms tend to progress even when managed with cardiovascular drugs for heart failure [27]. When congestive heart failure develops, the mortality rate in cancer survivors is 50% in the first year [27]. Thus, it is important to identify early biomarkers and recognize early cardiac changes before acute or chronic cardiac symptom develops.

With the advancement of both basic and clinical research in imaging and the health sciences, the number of cancer survivors has increased significantly due to better treatment plans, with better patient care and follow up [31]. Consequently, evidence of death caused by irreversible and relentless cardiac side effects from chemotherapy drugs has been revealed [31]. Cardiovascular disease caused by chemotherapy treatment is the second leading cause of death in cancer survivors [11, 16 31]. Thus, it is important for healthcare providers to identify cardiovascular risk factors in cancer patients with close monitoring of the progression of heart dysfunction before, during and after chemotherapy [9, 32-34]. However, the limited definition and diagnostic standard of doxorubicin-induced cardiotoxicities, specifically, the controversy that still remains regarding what is acute versus chronic, and asymptomatic versus symptomatic, makes it hard for clinicians to determine when or how to provide intervention [31].

Changes in left ventricle ejection fraction are still the gold standard for diagnosing of chemotherapy-induced cardiotoxicity [9]. Based on clinical trials, chemotherapy-induced cardiotoxicity is defined as or includes one or more of the following: " 1) cardiomyopathy in terms of a reduction of left ventricle ejection fraction, either global or specific in the interventricular septum; 2) symptoms or signs associated with heart failure; 3) reduction in left ventricle ejection fraction from baseline ≥ to 5% to <55% in the presence of signs or symptoms of heart failure; or 4) a reduction in left ventricle ejection fraction ≥10% to <55% without signs or symptoms of heart failure [9]." However, there are many broader effects of doxorubicin on the cardiovascular system, such as on diastolic function, myocardial strain (stretching), fibrosis, and cardiac response to injury and stress [31]. Thus, more research is needed to better understand the different aspects of chemotherapy-induced

cardiac side effects and to better predict or monitor the occurrence and progression of chemotherapy-induced cardiotoxicity [9, 33, 35, 36]. This has prompted cardiologists and oncologists to collaborate and develop the new sub-discipline referred to as "Cardio-Oncology" [32, 34, 35]. The Canadian Cardiac Oncology Network started in the year 2011 with the intention of bringing cardiologists and oncologists together to improve medical practice and life quality to cancer survivors [37]. To date, many Cardio-Oncology programs are being developed and offered through our health care system for training cardiologists to be specialized in the better management of cardiovascular complications in cancer patients [36, 38-40]. With more advanced non-invasive imaging technology (e.g., strain imaging) and specific cardiac biomarkers (e.g., troponin I [TnI] and atrial natriuretic peptide [ANP]), the Cardio-Oncology program offers earlier and more accurate detection of chemotherapy-induced cardiotoxicity, which is very promising [17, 33, 41, 42].

In this regard, improving or new forms of chemotherapy and/or reducing the cardiotoxicity of available chemotherapeutic agents would be a positive development [17, 33]. These goals are not, however, without difficulty. The cost of developing a new pharmaceutical drug and gaining marketing approval now exceeds $2.6 billion, even without considering its relative effectiveness when compared to the current drugs available [43, 44]. Among 2000 analogues of doxorubicin and daunorubicin that were originally synthesized and evaluated, doxorubicin remains the most effective anti-cancer drug that has been discovered [45]. Drug modification has been considered. Liposomal doxorubicin is favorably accumulated in cancer cells with less cardiac side effects, but its use is controversial because of the cost-effectiveness ($2,851/50mg compared to $67/50 mg for unmodified doxorubicin) and the risk to cancer patients of other side effects due to the

nature of the modification [46]. Combinational therapy has been the most effective approach to reduce cardiotoxicity in Cardio-Oncology, by using different classes of chemotherapy drug in order to decrease the dose of each drug, but increase the effectiveness of killing cancer cells by targeting different stages of cell death [47, 48]. However, it does not change the nature of the drug-induced cardiotoxicity. In addition, if patients show any signs of chemotherapy drug-induced cardiotoxicity, the chemotherapy is likely to be stopped or even withdrawn [49]. Thus, a successful cancer treatment that improves the life quality of cancer survivors is defined by reaching a balance between maximizing the efficacy of killing cancer cells but minimizing the toxic side effects [50].

1.3 Current gaps in Cardio-Oncology

Unfortunately, there is limited evidence regarding the management of cardiovascular side effects of chemotherapy, and current clinical practices are largely based on opinions of cardiologists or oncologists without a specific prevention or treatment guideline [33]. The effectiveness of strategies like maintaining a healthy life style and good cardiovascular history also varies from individual to individual [33]. Early detection using imaging or blood biomarkers, seems to be important in the diagnosis and treatment of chemotherapy-induced cardiotoxicity [51]. However, novel imaging such as strain rate imaging based echocardiography assessment lacks standard criteria and is time/cost consuming. Thus, it is still not used routinely for monitoring chemotherapy-induced cardiotoxicity [52-54]. Also, imaging and/or biomarkers are often too late to rescue the damage done because doxorubicin-induced cardiotoxicity is irreversible and progressive [55]. Thus, prevention of doxorubicin-induced cardiac damage by offering cardioprotection before or

during chemotherapy is a very important and efficient approach to provide better cardiovascular health outcomes in cancer patients [56].

Cardioprotection includes "all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage" [57]. Cardioprotection methods have been used for many cardiovascular conditions such as pre-conditioning in ischemia-reperfusion, and primary prevention of coronary artery disease by controlling cholesterol levels [58, 59]. In chemotherapy-induced cardiotoxicity, many drugs have also been tested for their ability to protect the heart: for example, beta-blockers (Acebutolol, Metoprolol, Carvedilol and Propranolol), angiotensin-converting enzyme (ACE) inhibitors (Benazepril, and Enalapril), angiotensin receptor inhibitors (Losartan, and Valsartan), statin (Atorvastatin), as well as Dexrazoxane (Zinecard) [60-66]. Meta-analysis of different clinical trials indicate a controversial effect on the effectiveness of the current cardiovascular drugs versus its addictive cytotoxic side effects during chemotherapy, including carvedilol, atorvastatin, enalapril and dexrazoxane [55, 65, 66]. Dexrazoxane is the only United States Food and Drug Administration (FDA) approved drug that is given to cancer patients as a treatment to reduce the incidence and severity of doxorubicin-induced cardiotoxicity. However, dexrazoxane is also a topoisomerase II inhibitor [67]. Thus, when given in combination, dexrazoxane competes or interferes with doxorubicin, and showed an increased rate of doxorubicin associated secondary malignancy [60, 68, 69]. As a result, dexrazoxane use as a cardioprotective agent is restricted to late-stage breast cancer patients who have received >300 mg/m^2 doxorubicin or >540 mg/m^2 epirubicin [60]. All these limitations on current available cardioprotective methods strongly indicate that additional cardioprotective agents need to be identified that have less cytotoxic/side effects when

combined with chemotherapy drugs [56, 64, 65].

1.4 Exploration of additional cardioprotective agents

Unlike synthetic drugs, cytokines and growth factors that are endogenously produced by the heart have shown great potential in offering protection to the heart with fewer cytotoxic effects under different cardiac injury settings when administrated exogenously [70, 71]. Endogenous factors like the vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), cardiotrophin-1 (CT-1), insulin-like growth factors (IGFs), transforming growth factor-β (TGF-β) have all been linked to cardioprotection during ischemia-reperfusion injury with respect to their ability to confer myocardial protection through activation of cell-surface receptors and underlying pro-survival intracellular cardioprotective signaling pathways, such as phosphoinositide 3-kinase (PI3K) – protein kinase B (AKT), and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) 1/2 [26, 71-74]. In addition, the survival related signaling pathways including PI3K-Akt and MAPK/ERK1/2 all play important roles in the regulation of doxorubicin-induced apoptosis [75, 76]. Thus, targeting these intrinsic cardioprotective factors, receptors and/or their signaling pathways may protect against or rescue doxorubicin-induced cardiotoxic damage.

Among all the intrinsic factors mentioned above, members of the FGF family have been studied intensively regarding their importance to heart development and function as well as in postnatal cardioprotection [77-88]. More importantly, many FGFs are synthesized in the heart and have been linked to cardiac cell or tissue survival [89-92]. As such, they represent

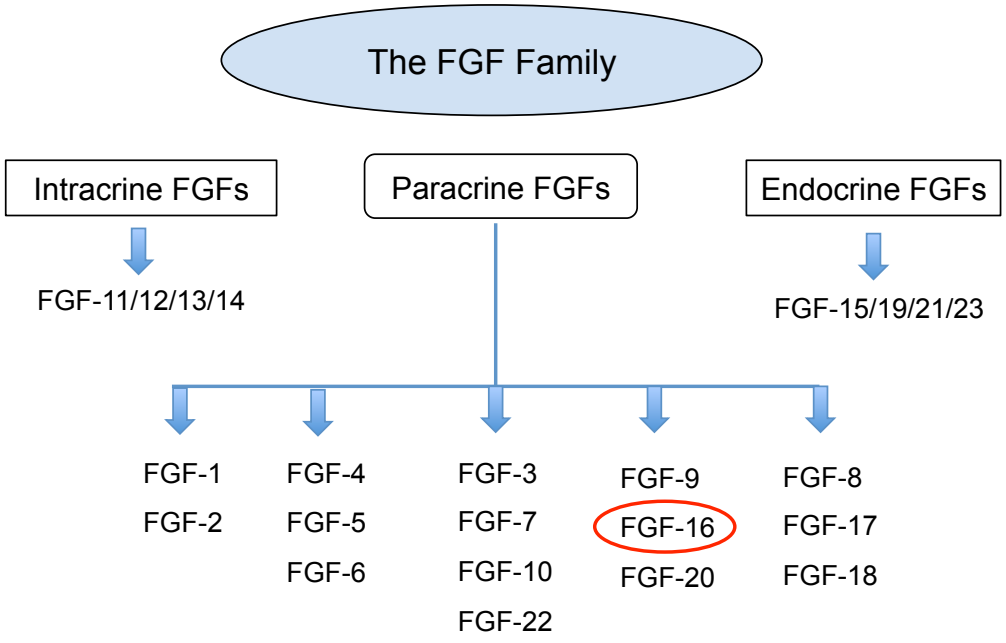
candidates to pursue as part of a potential therapeutic strategy to increase resistance to cardiac injury, including chemotherapy-induced cardiotoxicity[93-97].

1.5 FGFs and signaling pathways linked to cardioprotection

The FGF family have 23 family members, with mouse FGF-15 as the ortholog of human FGF-19 [98]. There are seven different FGF gene (*Fgf*) subfamilies: *Fgf* 1/2, *Fgf* 4/5/6, *Fgf* 3/7/10/22, *Fgf* 8/17/18, *Fgf* 9/16/20, *Fgf* 11/12/13/14, and *Fgf* 15/19/21/23 [98, 99] (**Figure 2**). FGFs are a family of proteins that consist of ~150–300 amino acids with ~30–60% identity[98]. FGFs have a conserved internal core region of ~120 amino acids that include 28 highly conserved and 6 identical amino acids that are functional as FGF receptor (FGFR) and heparin binding domains [98]. Based on their structural similarity, the FGF family has presumably evolved through a series of gene duplications and losses [85, 98]. Gospodarowicz isolated a protein from bovine pituitary extract and named it "fibroblast growth factor" based on its ability to stimulate 3T3 fibroblast cell proliferation [100]. Although FGFs are involved in a diverse variety of effects in embryonic development, angiogenesis, wound healing and endocrine signaling, the name "Fibroblast Growth Factor" has persisted due to the substantial conserved protein sequence homology with the first FGF that was discovered [85, 98]. FGFs can act as autocrine, paracrine, endocrine and even intracrine signaling factors in development, health, and disease in different organs such as brain, liver, kidney, bone, as well as the heart [88]. The varied roles of different FGFs likely reflect the presence of multiple FGF receptors (FGFR1-4) and their differing affinities for FGF family members [85, 98]. The immunoglobulin (Ig)-like III domain of the FGFR (1-3) can be alternative spliced to b or c isoforms [101, 102], producing FGFR1b, FGFR1c, FGFR2b,

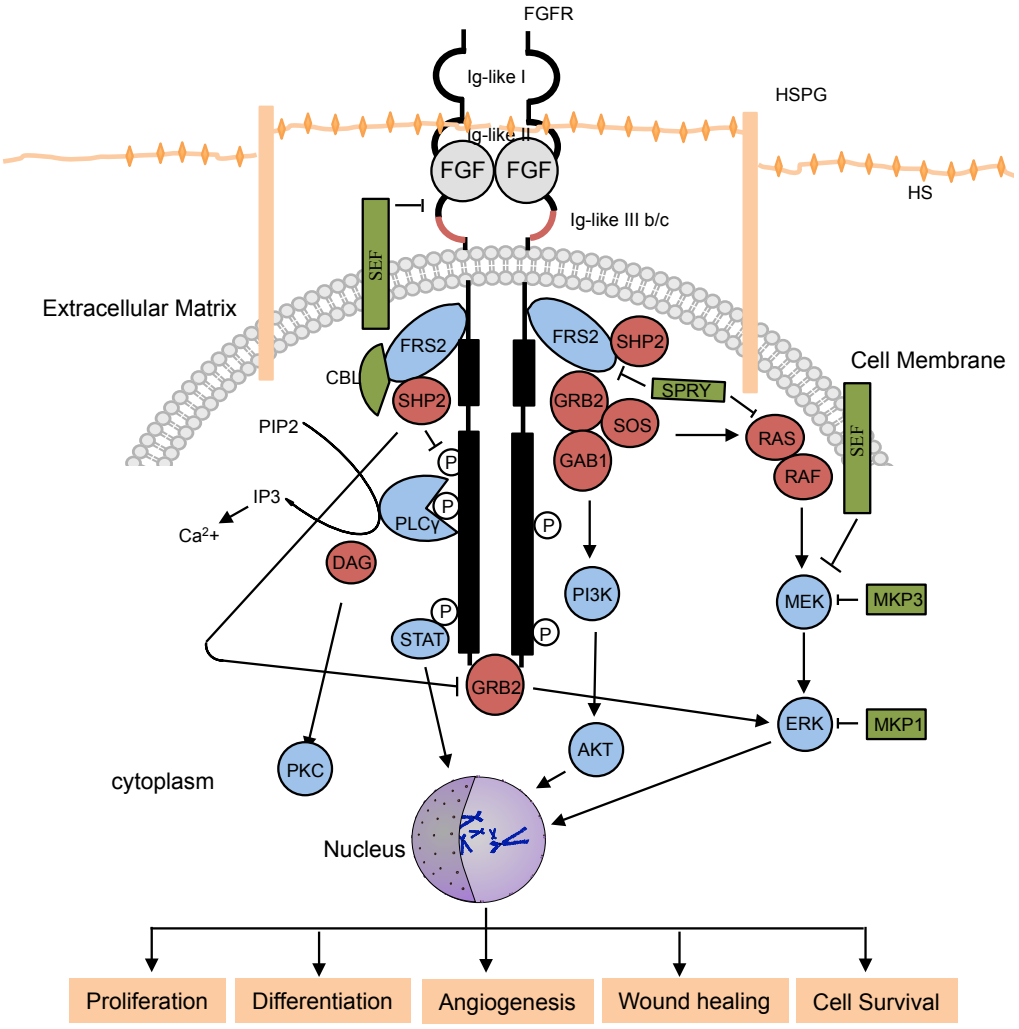
FGFR2c, FGFR3b, FGFR3c and FGFR4. Within the same FGF subfamily, however, the binding specificity tends to be similar because of the higher amino acid sequence similarity [83, 88, 103]. FGFs can also bind to heparan sulfate proteoglycans, α-Klotho (FGF-23), and β-Klotho (FGF-15/19/21) as co-receptors for FGF and FGFR binding and signaling [104] [88]. Engagment of FGFs drives the formation of FGFR dimerization and the intracellular tyrosine kinase domains are activated by autophosphorylation, followed by activation of a few key downstream signaling pathways that collectively influence the majority of intracellular events [85, 87, 105]. These include the rat sarcoma protein (RAS)- MAPK, PI3K-AKT serine/threonine kinase (AKT), phospholipase Cγ (PLCγ), protein kinase C (PKC), and signal transducer and activator of transcription (STAT) pathways [83, 101, 106, 107] (**Figure 3**).

Figure 2. The FGF family and subfamilies



There are seven different FGF gene subfamilies. Paracrine FGFs: *Fgf* 1/2, *Fgf* 4/5/6, *Fgf* 3/7/10/22, *Fgf* 8/17/18, *Fgf* 9/16/20; Intracrine FGFs *Fgf* 11/12/13/14; and Endocrine FGFs: *Fgf* 15/19/21/23. FGF-16 belongs to the FGF9 subfamily. FGF-15 is the mouse ortholog of human FGF-19.

Figure 3. The FGF-FGFR mediated signaling pathways



FGF receptors (FGFRs) are tyrosine kinase receptors (TKRs) with an ectodomain of three Ig-like domains (I–III), a *trans*-membrane domain, a juxtamembrane domain and a tyrosine kinase domain [101, 102]. The Ig-III domain of FGFR1-3 has both b and c isoform due to alternative splicing [101, 102]. The FGF-FGFR complex in this figure is formed by two FGFs, two heparan sulfate proteoglycan (HSPG) chains and two FGFRs [101, 102]. FGFs binding to FGFRs cause dimerization and *trans*phosphorylation of the two tyrosine kinase residues in the kinase activation loop in the intracellular domain of the FGFRs and subsequently cause activation of other tyrosine kinases as well as downstream signaling cascades, including RAS- Mitogen activated protein kinase (MAPK), phospholipase C-γ (PLCγ)- protein kinase C (PKC), phosphatidylinositol 3,4,5-tri-phosphate (PI3)-Akt, and signal transducer and activator of transcription (STAT) [101, 102]. Negative regulators of the FGF signaling pathway includes similar expression to FGF (SEF), Sprouty (SPRY), and MAPK phosphatase 1 and 3 (MKP1/3) [102]. *(This figure is re-created based on the literature [84, 101])*

The heart is the first organ to form during embryonic development and is vital to our survival [108]. Several FGF members are involved in heart development, maintenance and diseases [87, 88, 109, 110]. FGFs such as FGF-3, 8, 9, 10 15/19 and 16 are all known to play an important role during embryonic development in the specification of early cardiac mesoderm and morphogenesis [84, 88, 109-115]. As autocrine or paracrine signals, FGFs stimulate cell survival and cardiogenic cell division, as well as cardiac myocyte proliferation and differentiation [109].

In 1992, the first report appeared that FGF-2 is cardioprotective in the canine heart against myocardial infarction due to its angiogenic or proliferative properties [116]. However, studies by Kardami and colleagues in 1995 and 1998, and subsequently confirmed by others, indicated a cardioprotective effect involving a series of signaling pathways with cardioprotective mediators such as protein kinase C-α/ε and Erk1/2, which are independent of the mechanisms linked to the angiogenic properties of FGFs [97, 117, 118]. Kardami and colleagues also showed that FGF-2 offers protection against acute doxorubicin-induced cardiac myocyte damage [92]. This protection has been linked to the mammalian target of Rapamycin (mTOR) activation, and maintaining the endogenous anti-oxidant/detoxification nuclear factor erythroid-2-related factor (Nrf-2) and heme oxygenase-1 (HO-1) axis [92] as well as, at least in part, increasing efflux drug transport [26].

Upon discovering the cardioprotective signaling pathways, FGFs such as FGF-1, 2, 9, 10, 16, 20, 21 and 23 have all been studied in the context of cardiac remodeling and postnatal cardiac protection [26, 77-88, 90, 119]. Although endocrine FGF-23 and its co-receptor α-Klotho were not expressed in the heart, induction of FGF-23 is associated with cardiac

hypertrophy through α-klotho-independent signaling pathways by activating FGFR4 in patients with chronic kidney disease [120]. These studies opened up the field, and the desire to increase our understanding of FGFs in the context of maintaining normal heart function and offering cardiac protection and/or repair during or after cardiac injury. By extension, the hope is to provide a basis for investigating the therapeutic potential of FGFs in cardiovascular injury and/or diseases [87, 110].

There are, however, multiple FGFs produced by the heart [87], and not all are equally good candidates to offer cardioprotection in the context of a doxorubicin-induced cardiotoxicity model, specifically when the systematic application and adjuvant therapy with doxorubicin are taken into consideration. For example, it is assumed that factors that are synthesized and/or widely distributed in different tissues may have multiple and varied cell/tissue signaling and effects [121, 122]. Thus, if there were an endogenous factor preferentially synthesized and secreted by the heart, and perhaps a cardiac myocyte-specific factor with protective properties, this might be advantageous when considering delivery of specific cardioprotection [71]. As such, in terms of prioritizing an FGF(s) for investigation as an endogenous cardioprotective agent, a good candidate would:

1) possess a highly-related gene and protein structure (as well as chromosome location) between species, including human, suggesting a conserved and important function;
2) be expressed preferentially in the heart, and more specifically, be synthesized and secreted by cardiac cells;
3) play a role in maintaining endogenous cardiac cell and/or heart function;
4) offer increased resistance to postnatal doxorubicin-induced cardiac myocyte and/or

cardiac damage; and

5) offer little or no interference with the negative effect of doxorubicin on tumour cell survival (under conditions where protection of cardiac cells is observed).

Among all FGFs, FGF-16 is unique given its structural conservation, preferential postnatal cardiac-production and its potential as an endogenous maintenance factor for a healthy heart [79, 80, 123, 124].

1.6 FGF-16

1.6.1 Structural conservation between species and cardiac synthesis

The FGF-16 gene (*Fgf-16*) is highly conserved between species, including human, rat, mouse, chicken, and zebrafish [79, 105] (**Table 1**). When compared, human and mouse, and human and rat, share 95% and 94% nucleotide sequence identity, respectively [105, 125]. Human *Fgf-16* was mapped to the X chromosome (q21.1), and is located at the equivalent region and closely associated with the alpha thalassemia/mental retardation syndrome X-linked (ATRX) gene in human and murine species [105, 115] (**Figure 4**). *Fgf-16* includes three exons and two introns that predict a 1.8 kb transcript [105, 125]. Human FGF-16 protein shares 98.6% and 99% amino acid sequence homology with the rat and mouse proteins, respectively, and all three predict a 207 amino acid protein of ~23.7 kDa [105]. In terms of other family members, FGF-16 shows the greatest structural similarity with FGF-9 (~73%) and FGF-20 (~67%), compared to less than 50% with other FGFs; as such they constitute an FGF-9 subfamily [105, 110, 126]. FGF-9, FGF-16, and FGF-20 production are restricted to the endocardium and epicardium in the embryonic heart, and expression of both FGF-16 and

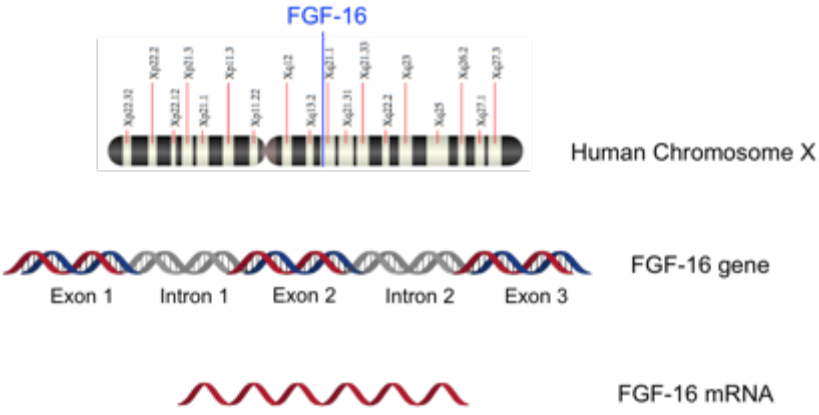
FGF-20 can induce Wnt signaling in cardiac development [105, 111, 126, 127].

Table 1: Conservation of *Fgf-16* gene and protein in different species

FGF-16	Human*	Rat	Mouse	Chicken	Zebra fish
Chromosome location	X	X	X	4	14
mRNA identities	100% 624 bp	94% 624 bp	95% 624 bp	83% 624 bp	78% 612 bp
Protein sequence identities	100% 207 aa	98.6% 207 aa	99% 207 aa	90% 207 aa	79% 203 aa

* Human FGF-16 sequence was set as 100% for comparison with other species; bp, base pairs; aa, amino acids.

Figure 4. The human FGF-16 gene



Human *Fgf-16* was mapped to the X chromosome (q21.1) [105]. *Fgf-16* has 3 exons and 2 introns, with one 1.8 kb FGF-16 transcript

Multiple FGFs are synthesized both in the embryonic and postnatal heart, including FGF-1, 2, 3, 8, 9, 10 15, 16, 19, 20, 21, and 23 [87, 88, 128, 129]. However, only FGF-16 has been reported to be synthesized preferentially by cardiac myocytes in the postnatal heart [79, 105, 114]. Evidence from a rat model indicates that FGF-16 is expressed predominantly in brown adipose tissue during embryonic development (E17.5–19.5), although cardiac expression is also detected [105, 114, 130, 131]. Similarly, during mouse embryonic development, FGF-16 expression has been detected in ear, as well as developing structures related to the jaw, thymus, parathyroid, and face [132]. However, mouse FGF-16 is also synthesized and secreted at low levels by the endocardium and epicardium at embryonic day (E) 10.5 and E12.5 during heart development, as well as developing structures related to the cardiac outflow tract [105, 108, 111, 114, 123, 132]. A mechanism for FGF-16 protein secretion has been described involving a bipartite signal sequence including the N-terminal and central hydrophobic regions using cDNAs encoding FGF-16 expressed in the mammalian kidney COS-1 fibroblast-like cell line [79, 105, 124].

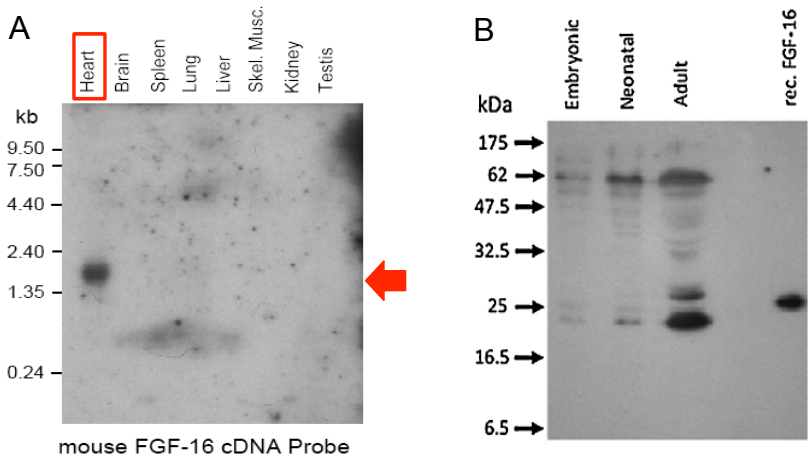
A unique characteristic of cardiac FGF-16 production is the change in spatial and temporal pattern of expression in the (murine) heart during the perinatal period [79, 105]. Expression of *Fgf-16* switches from the epicardium and endocardium to the myocardium during the perinatal period and levels increase into adulthood [77,107,120]. In addition, elevated FGF-16 mRNA and protein can be detected [79, 105, 108, 111, 133] (**Figure 5**). FGF-16 mRNA is also detectable at low levels in the postnatal human heart, including left atrium and ventricle, as well as endothelial cells and aortic smooth muscle [105, 119, 134]. By contrast, the closest FGF members structurally, FGF-9 and FGF-20, are produced in the kidney and brain, respectively [105, 135 136]. Thus, *Fgf-16* displays both cardiac cell and development-

specific expression, at least in the murine system [105]. FGF-16 protein is also secreted by postnatal cardiac myocytes when disaggregated and placed in primary cultures [79]. FGF-16 can be isolated (enriched) from the medium using heparin-agarose beads, and then detected by immunoblotting using FGF-16 antibodies [79, 105]. Thus, FGF-16 is expected to bind locally to the extracellular matrix and act on cardiac cells in the postnatal murine heart [79]. Increases in FGF-16 protein correlate with the temporal increases in FGF-16 mRNA seen during heart growth and development into the postnatal period [79, 105].

In summary, in terms of satisfying the specific characteristics of a "good candidate" for investigation as an endogenous cardioprotective agent, FGF-16 does possess a highly-related mRNA and protein structure (as well as chromosome location for the gene) between human, rat and mouse species, suggesting a conserved and important function. *However, a comparison of Fgf-16 promoter sequences from human and murine species had not been reported and was explored through this thesis.*

Similarly, FGF-16 also satisfies the characteristic of being synthesized preferentially in the heart, and more specifically, is synthesized and secreted by postnatal cardiac myocytes. *However, the mechanism, including the cardiac transcription factor(s) involved, is poorly understood and was explored through this thesis.*

Figure 5. FGF-16 expression during the embryonic, perinatal and postnatal period in the heart



(A) Detection of FGF-16 transcripts in adult mouse tissues by RNA blotting [105]. Poly-adenylated RNA (2µg) isolated from various adult mouse tissues, separated by gel electrophoresis and blotted, was probed with radiolabeled FGF-16 cDNA [105]. A prominent signal corresponding to FGF-16 with an apparent size of 1.8 kb relative to molecular markers was detected only in the postnatal heart [105]. (B) Detection of FGF-16 protein in the embryonic, neonatal, adult mouse heart by western blotting using FGF-16 antibodies [105]. Proteins were extracted and concentrated using heparin-coated beads from embryonic day 17, postnatal day 4 and adult 8 week-old mouse [105]. Multiple bands were detected at each developmental stage, including prominent signals in the adult heart with apparent molecular weights of 19.5 kDa (non-secreted, non-glycosylated, and amino-terminal truncated) and 26.5 kDa (secreted and glycosylated) [79, 105, 137, 138]. Human recombinant FGF-16 (rec. FGF-16, 23.7 kDa) was used and detected as a positive control. Possible formation of FGF-16 protein dimer of 45 kDa and 50 kDa may be present resulting from both non-glycosylated and glycosylated forms. A dimer for human recombinant FGF-16 protein (~45-50 kDa) has also been reported [137]. Both FGF-9 and FGF-20 protein have a tendency to form homodimers with or without heparin extraction [139, 140]. *(Reproduced with permission from Elsevier, Cytokine & Growth Factor Reviews [105])*

1.6.2 Comparison of human and murine Fgf-16 promoter sequences identifies a conserved putative cardiac-specific Csx/Nkx2.5 transcription factor binding site

The unique spatial and temporal expression pattern of FGF-16 before and after birth, as well as the postnatal cardiac-specific expression, indicates a complex regulation of the FGF gene at different developmental stages [79]. It has been suggested that FGF-16 synthesis is under the 'indirect' control of the transcription factor Forkhead box P1 (FOXP1) in the embryonic endocardium, through its ability to directly repress the transcription factor SRY (Sex Determining Region Y)-Box 17 (SOX17) [105, 133]. SOX17 is produced at high levels in the embryonic endocardium, and is linked to both gene activation and repression [105, 133, 141, 142]. SOX17 is suggested to compete with β-catenin for co-transcription factor association thereby interfering with the Wnt/β-catenin signaling pathway [105, 133, 143]. As such repression of SOX17 by FOXP1 is expected to stimulate Wnt signaling and FGF-16 (as well as FGF-20) production in the endocardium, as well as myocardium proliferation [105]. However, unlike the embryonic endocardium, the mechanism controlling *Fgf-16* expression in the postnatal myocardium is less clear [105].

The complex pattern of embryonic versus postnatal mouse *Fgf-16* gene expression might also be related, at least in part, to the presence of two TATA-like elements (TATA1 and TATA2) identified upstream of the methionine (ATG) translation start site; the adenine residue of which is designated as nucleotide position +1, as previously described [105, 119, 144]. Multiple promoter regions or alternative promoter usage of FGF genes, including FGF-2, FGF-4 and FGF-8, has been reported to be associated with tissue, cell, developmental stage, or pathophysiological conditions (cancer) [145-148]. However, the biological function of

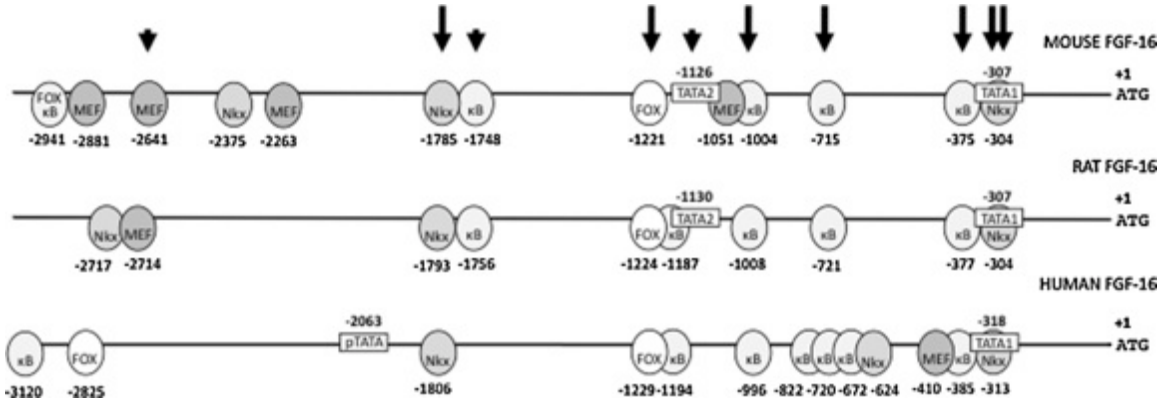
alternative promoter usage is not well understood. Interestingly, only TATA1 is conserved in the human FGF-16 gene [119, 105] (**Figure 6**). Examination of *Fgf-16* flanking sequences also reveals putative binding sites for transcription factors that play essential roles in cardiac cell growth and development. These include FOXP1, myocyte enhancer factor 2 (MEF2), NK2 homeobox 5 (Csx/Nkx2.5 also known as Nkx2-5 and Csx), and the stress-related transcription factor nuclear factor-kappa B (NF-κB) [105] (**Figure 6**). FOXP1, MEF2 and NF-κB have all been implicated in *Fgf-16* regulation [105, 119, 144]. However, the level of FOXP1 expression is different depending on the location and developmental stage in the heart [133]. FOXP1 is highly expressed in the embryonic myocardium and acts as a repressor of a cardiac transcription factor Csx/Nkx2.5 [105, 133]. After birth, however, FOXP1 levels decrease and there is a corresponding increase in Csx/Nkx2.5 levels in the heart [105, 133]. Csx/Nkx2.5 is expressed abundantly in the postnatal heart and can interact with MEF2 as well as with its co-activators p300 and GATA4, which have been linked to cardiac expression and development [105, 149-152]. Certainly, mutation of the Csx/Nkx2.5 gene is associated with congenital cardiovascular disease [105, 153-155]. Thus, based on the conservation of putative regulatory DNA elements across human and murine species, and their availability during heart development, FOXP1 and Csx/Nkx2.5 likely play roles in the spatial and temporal control of *Fgf-16* expression. More specifically, the switch in the cellular site of *Fgf-16* expression in the embryonic and postnatal heart might be related to the increase in Csx/Nkx2.5 either alone or in balance with FOXP1[105, 119, 133, 144]. Thus, access or use of one or more TATA sequences, as well as different levels of associated transcription factors at different stages of development may contribute to the distinct spatial and temporal expression pattern of the (murine) FGF-16 gene.

A MEF2 site located 2,263 nucleotides upstream of the mouse FGF-16 ATG start codon is associated with a region of nuclease hypersensitivity in the mouse heart; hypersensitive sites are often an indication of a more 'open' local chromatin structure [105, 144] (**Figure 6**). MEF2C would be expected to bind this site and if so confer preferential cardiac expression of *Fgf-16* [105]. Certainly, mutation of this MEF2 site in the context of a hybrid 1.2-kilobases (kb) mouse FGF-16 (TATA2) promoter/luciferase gene resulted in a significant decrease in activity in transfected neonatal rat cardiac myocytes [105, 144]. This site is not conserved, however, in either rat or human sequences, but putative MEF2 sites were identified in the upstream flanking DNA of human and rat genomic sequences based on a search for the consensus MEF2 binding site [105, 144]. MEF2 contains both MADS-box and MEF2 protein DNA binding domains [105, 156]. MEF2 is essential for embryonic heart development and cardiac myocyte differentiation [157], which is consistent with the expression of *Fgf-16* during cardiac development [108, 114]. In addition, MEF2 plays an important role in pathological growth and adaptation of the adult heart to stress as with cardiac hypertrophy [156, 158, 159], and is implicated in cardioprotection via vascular endothelial growth factor-A (VEGF-A) activation [105, 160].

Six putative NF-κB binding sites were identified in the first 6 kb of upstream flanking mouse *Fgf-16* promoter sequences [105, 119] (**Figure 6**). Of note, TATA1 and the upstream and adjacent NF-κB putative element are conserved in human and murine sequences, and association of NF-κB p50 and p65/RelA subunits with the proximal *Fgf-16* promoter region was observed in mouse heart chromatin *in situ* [105, 119]. Both human and mouse proximal *Fgf-16* promoter regions were responsive to isoproterenol, a β-adrenergic agonist and known activator of NF-κB, in transfected neonatal rat cardiac myocytes; this

activity was blunted in the presence of NF-κB inhibitors [105, 119]. Activation of the NF-κB transcription factor complex has been implicated in many stress-related processes in the heart including atherosclerosis, ischemia reperfusion-related cell death and injury, as well as response to injury through remodeling and hypertrophy [105, 161-164].

Figure 6. Comparison of known and putative TATA and cardiac transcription factor binding sites relative to the adenine residue (nucleotide +1) of the methionine (ATG) start site in the upstream promoter of mouse, rat and human FGF-16 genomic sequences



Sequences were obtained from the Ensembl database and assessed using Genomatix MatInspector software, based on consensus sequences for: FOXP1 (FOX) = 5′-AA(C/t)A(C/t)AAATA-3′; MEF2 (MEF) = 5′-YTA(A/T)$_4$TAR-3’; NF-κB (κB) = 5′-GGGRNNYYCC-3′; and Csx/Nkx2.5 (Nkx) = 5′-TNAAGTG-3′ [105]. The arrows indicate the presence of equivalent binding sites in mouse, rat and human sequences, and the arrowheads indicate comparable sites in mouse and rat but not human sequences [105]. A possible (p) second upstream TATA site in human sequences, based on TATA1, is also indicated [105]. *(Reproduced with permission from Elsevier, Cytokine & Growth Factor Review [105])*

Although not in the promoter region, a transcription factor GATA-binding motif (5′- AGATAG -3′) located in its second intron has been linked to *Fgf-16* regulation in the mouse [86]. GATA4 is an important transcription factor for both embryonic and neonatal heart development, including cardiac myocyte proliferation and cardiac hypertrophy, and is also essential for cardiac myocyte viability [86]. Sequence comparison indicates this site is conserved in the rat and human FGF-16 genes (**Figure 7**), and a second possible GATA4-related DNA element can also be identified in the second intron of human *Fgf-16*. In addition, Csx/Nkx2.5 is known to regulate GATA4 gene expression by binding directly to a consensus DNA binding site in the GATA4 gene promoter region.

Thus, based on: (1) conservation of *Fgf-16*-related sequences between mouse, rat and human species, related (2) specifically to consensus transcription factor binding sites located in proximal promoter or intronic regions (**Figures 6** and **7**), which are (3) directly or indirectly implicated in *Fgf-16* regulation and (4) postnatal cardiac expression, the d*irect involvement of GATA4 and Csx/Nkx2.5 in postnatal cardiac myocyte FGF-16 gene expression was explored through this thesis. In addition, and specifically, the predicted binding of Csx/Nkx2.5 to conserved TATA1-related sequences was investigated.*

Figure 7. Sequence alignment of Csx/Nkx2.5 and GATA4 binding sites on mouse, rat and human FGF-16 DNA sequence

A

GATA4 binding site

MOUSE: ACATTTTTCCCTGACATCTGGGAGATAGGCCTGTCCCCAGCACTCAGTATCGGGCACCTCAGCAAACAGTATGGCaGCCCGg

+8482

RAT: ACATTTTTCCCTGACACCTGGGAGATAGGCCTGTCCCCAGCACTCAGTATCaGGCACCCCAGCAAACAGAATGGtGcCCCGA

+8781

HUMAN: ACATTTTTCCaTGACACCTaaGAGATAGGCCTGTCCCCAGCACTCAGTATtGGcaACtCCAGCAAGtAGTATGGCGGttgGA

+7966

B

Csx/Nkx2.5 binding site

MOUSE: AATATGTCAATGGAAGGGGAGTGGGTACGTATATAAAGTGCCATTTGCATCTGATAGCTCCAGGAAGCCAGCTATGAGAGGC -

307

RAT: AATATGTCAATGGAAGGGGAGTGGGTACGTATATAAAGTGCCATTTGCATCTGATAGCTCCACGAAGCCAGCTATGAGAGGC -

307

HUMAN: AATATGTCAATGtAAGGGGAGTGGGTAtGTATATAAAGTGCCgTTTGCATCTGATAGCTggACGAAGCCAGCTATGAGAGGC -

318

Sequences were obtained from the NCBI and Ensembl databases. (A) The GATA4 binding site previously identified in the mouse [86], was also detected in human and rat *Fgf-16* and all are based on the consensus sequence 5′-AGATAG -3′ [105]. (B) A possible binding site for Csx/Nkx2.5 (B) was identified in the proximal *Fgf-16* promoter region based on the consensus sequence 5′-TNAAGTG-3′ sequence [105, 165]. Nucleotide positions (+ or -) are given relative to the adenine residue (nucleotide +1) of the methionine (ATG) start codon for mouse rat and human FGF-16, respectively.

1.6.3 FGF-16 plays a role in maintaining endogenous cardiac cell and/or heart function during embryonic development

Endocardial and epicardial production of FGF-16 has been linked to the development of the myocardium and cardiac myocyte proliferation during embryogenesis [105, 133]. FGF-16 is also associated with the FGF signaling network acting downstream of Wnt/β-catenin pathway that is involved in the expansion of cardiovascular progenitor cells that are anterior heart field insulin gene enhancer protein IsL-1 positive, from which cardiac myocytes are derived [105, 111, 127].

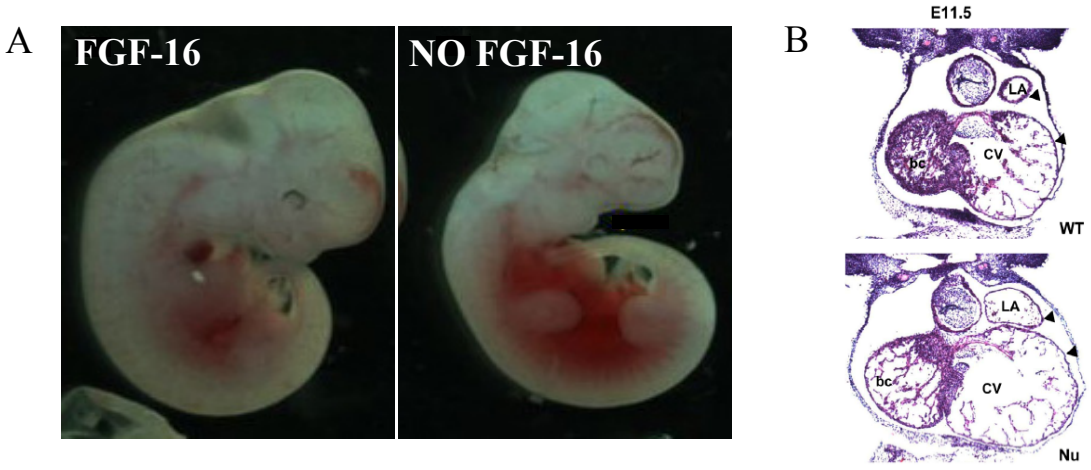
Phenotypic differences have been described in FGF-16 null mice from different genetic backgrounds, but there is a consensus that FGF-16 plays an important role in cardiac myocyte growth/proliferation and embryonic heart development [105, 108, 114]. Loss of FGF-16 in outbred Black Swiss FGF-16 null mice (**Figure 8**) via disruption of exon 1 results in embryonic lethality and evidence of cardiac and skeletal or craniofacial defects as early as day E11.5 [105, 132]. The former includes chamber dilation, thinning of the atrial and ventricular walls, and poor trabeculation [105, 132]. These effects are visible at E10.5 but more pronounced at E11.5, and a decrease in FGF-8 mRNA levels was also noted [105, 114]. FGF-8 is linked to anterior heart development and is expressed at E10.5 [105, 132]. By contrast, FGF-16 null mice on an inbred C57BL/6 mouse strain genetic background through disruption of exons 2 and 3, survive fetal development [105, 108]. There is, however, a significant decrease in embryonic cardiac myocyte proliferation, which is consistent with a role for FGF-16 during embryonic heart development [105, 132]. Heart weight and cardiac myocyte number were also decreased at six months of age after birth [105, 108]. This embryonic heart phenotype is similar

to that of the FGF-9 null mouse, which would be consistent with FGF-9 and FGF-16 acting synergistically to support embryonic cardiac myocyte proliferation [105, 132]. These data also suggest that genetic background can modify the phenotype and affect the embryonic survival of FGF-16 null mice [105, 132]. Breeding of FGF-16 null mice on a Black Swiss genetic background (heterozygotes) onto a C57BL/6 mouse background increased survival of null offspring within three generations [132]. Basal neuregulin, ErbB2, and ErbB4 as well as FGF-8, FGF-9, and FGF-16 mRNA levels varied in Black Swiss versus C57BL/6 mice [105, 132]. Neuregulin as well as signaling via members of the epidermal growth factor receptor family, ErbB2 and ErbB4, have been linked to trabeculae formation and cardiac development around E10.5 [105, 132]. In addition, given the structural and potential functional relationship between FGF-9, FGF-16, and FGF-20, the severity of the cardiac phenotype in FGF-16 null mice may depend on compensation by other subfamily members [105]. Certainly, overlapping expression patterns for FGF-9, FGF-16, and FGF-20 are reported in the endocardium and epicardium during murine heart development [105, 108, 111, 114].

An increased risk of developing cardiovascular disease in human families with nonsense mutation in exons 1, 2 or 3 of *Fgf-16* has been suggested [166]. Knockdown of FGF-16 in zebrafish using splicing blocking morpholinos (MOs) to mimic the abnormal splicing of FGF-16 RNA, resulted in 76% of zebrafish embryos developing heart edema [166]. In addition, FGF-16 has also been linked to cell motility and anti-apoptosis in human teratocarcinoma [167]. At birth, the change in tissue/cell patern of FGF-16 expression raises the possibility of a different (non-proliferative) role for FGF-16 in the postnatal myocardium, given adult cardiac myocytes are post-mitotic [79 88, 105, 168]. Subsequent studies using the C57BL/6 FGF-16 null mouse model suggests that postnatal cardiac FGF-16 plays

a role in maintaining a healthy myocardium [105, 169].

Figure 8. A role for FGF-16 in embryonic heart development



*Lu SY, Sheikh F, Sheppard PC, Fresnoza A, Duckworth ML, Detillieux KA, Cattini PA. FGF-16 is required for embryonic heart development. Biochem Biophys Res Commun 2008;**373**:270-274.*

(A) FGF-16 null embryos at E12.5. FGF-16 knockout mice embryos show severe hemorrhage in and around the cardiac and ventral body regions, as well as craniofacial defects [114]. (B) Histological assessment of hearts in FGF-16 null mice (Nu) and wild type (WT) mice at E11.5. Heart defects were seen with both atrial and ventricular enlargement in Nu mice compared to WT [114]. Poor trabeculation was shown in the bulbus cordis (bc) area that will develop into the right ventricle [114]. Thinning of the ventricular and atrial walls in the Nu mice compared to WT is indicated by the arrow heads [114]. Abbreviations: CV, common ventricle; LA, left atrium. *(Reproduced with permission from Elsevier, Biochemical and Biophysical Research Communication [114])*

1.6.4 Endogenous FGF-16 helps maintain a healthy postnatal myocardium

Various FGFs play a fundamental role during embryonic development and continue to be expressed in the postnatal heart [84, 87, 110]. Instead of proliferation or division, postnatal cardiac myocytes and by extension the heart will grow in size through a process of cardiac hypertrophy to satisfy the pumping and circulatory needs of the growing individual [170]. However, pathological hypertrophy can occur, resulting in thickening of the heart muscle, a reduction in chamber size and decreased ability of the heart to pump blood to the tissues and organs systemically [171]. Often, this remodeling is the result of high blood pressure (hypertension) and heart valve stenosis [171]. Additionally, increased fibroblasts and their secreted extracellular proteins such as collagen contribute to fibrosis during cardiac remodeling [88]. FGFs as autocrine, paracrine, and endocrine factors in the postnatal heart, have been shown to play physiological and pathophysiological roles as growth and protective factors in cardiac health and disease [87, 88, 110].

The preferential synthesis and release of FGF-16 in the myocardium in the perinatal period suggests a specific role in the postnatal heart [79, 105]. FGF-16 can bind to FGFR1, but also FGFR3c with high affinity [79, 103, 105]. FGF-16 does not appear, however, to have a strong effect on postnatal cardiac myocyte proliferation based on the exogenous treatment of neonatal rat cardiac myocyte cultures and the cardiac phenotype of FGF-16 null C57BL/6 mice [79, 105, 169]. The fact that FGF-16 can bind multiple FGFRs raises the possibility of both shared but also an antagonistic function of, or modulation of signaling by other FGF family members [79, 105]. In support, common but also distinct effects on cell cycle-related gene expression were observed in neonatal rat cardiac myocyte cultures

treated with FGF-2 versus FGF-16, and versus co-treatment with FGF-2 and FGF-16 [79, 105]. There is also evidence that FGF-16 has a higher affinity for FGFR1 than FGF-2 [114]. Pretreatment with FGF-16 can interfere with PKCα and PKCε activation as well as cardiac cell proliferative potential induced by FGF-2 [79, 105]. The lack of MAPK/ERK1/2 activation is also consistent with FGF-16 being relatively non-proliferative in the postnatal heart [79, 105]. In addition, FGF-2, but not FGF-16, induced transforming growth factor (TGF) β1 expression significantly in cultured neonatal rat cardiac myocytes and non-cardiac myocytes [105, 169]. Interestingly, when FGF-16 was added before FGF-2, the induced TGF-β1 expression was inhibited [105, 169]. In this context, FGF-2 null mice that lack expression of both high and low molecular weight FGF-2 isoforms, display increased resistance to hypertrophy [105, 172, 173]. Furthermore, overproduction of FGF-2 is associated with increased cardiac fibrosis in transgenic mice [105, 172, 173]. By contrast, when cardiac injuries were induced in FGF-16 null C57BL/6 mice, using angiotensin II treatment to abnormally increase blood pressure, a significant increase in susceptibility to hypertrophy and cardiac fibrosis was observed [105, 169]. Thus, the reported blunting of the TGF-β1 response to FGF-2 by FGF-16 pre-treatment suggests a mechanism by which FGF-16 may limit cardiac hypertrophy and fibrosis by competing for FGF-2 binding, and likely the high molecular weight isoforms, to FGF receptors [105, 169, 174]. These observations support a role for FGF-16 in maintaining a healthy myocardium, and a potential mechanism involving direct and/or antagonistic effects on FGFR signaling [79, 105, 169].

In summary, in terms of satisfying the specific characteristics of a "good candidate" for investigation as an endogenous cardioprotective agent, there is evidence that postnatal FGF-16 may serve as an endogenous cardiac maintenance factor by limiting the response to

stress or injurious stimuli over a physiological range, e.g., changes in blood pressure. However, *the effect of stress or injury associated with acute exposure to the anti-cancer agent doxorubicin on FGF-16 availability in vivo, had not been reported and was explored through this thesis. It is noted that if FGF-16 is a cardiac maintenance factor that is required for normal function or cell survival, then a negative effect on FGF-16 production and availability may contribute to doxorubicin-induced injury.*

1.6.5 Evidence that FGF-16 possesses cardioprotective activity

If FGF-16 functions to help maintain a healthy myocardium in the postnatal heart, it may also possess cardioprotective activity when supplemented or supplied exogenously. Evidence from a murine cryo-injury model and post myocardial infarction in a GATA4-ablated heart injury model suggests that FGF-16 is cardioprotective [86]. Based on cardiac-specific deletion, GATA4 plays a role in cardiac myocyte replication, coronary angiogenesis, and heart repair after cryo-injury in neonatal mice [86]. In addition, FGF-16 protein levels were significantly reduced in 1-week-old GATA4 null mice when compared to their non-transgenic littermates [86]. However, the authors reported that FGF-16 supplementation in these GATA4 null mice for 8 weeks using adeno-associated virus (AAV9) delivery: (a) rescued ejection fraction and fractional shortening; (b) inhibited cardiac myocyte hypertrophy and apoptotic cell death (supported by a <1.3-fold increase in cell number); as well as (c) reduced scar tissue formation in a cryo-injury infarcted mouse heart [86].

Further evidence of a cardioprotective role for FGF-16 comes from studies using the

db/db mouse, a known model of obesity, diabetes, and dyslipidemia [80]. More specifically, this mouse is deficient in leptin receptor activity because the mice are homozygous for a point mutation in the leptin receptor gene [175]. In a coronary artery ligation-induced model of myocardial infarction in the adult db/db mouse, post infarction FGF-16 treatment significantly decreased the size of infarcted myocardium, inhibited myocardial hypertrophy, prevented apoptotic cardiac myocyte death and improved left ventricular function [80]. In addition, FGF-16 treatment also attenuated post-myocardial infarction interstitial fibrosis and myocardial inflammation [80]. Together these observations are consistent with the FGF-16 cardioprotective activity.

1.7 A potential role for FGF-16 in doxorubicin-induced cardiotoxicity

As indicated above, there is evidence that FGF-16 possesses properties consistent with a role in the maintenance of a healthy postnatal myocardium, and the ability to offer increased resistance to heart damage. However, there is no report on the role of FGF-16 in the context of Cardio-Oncology, and specifically the effect of doxorubicin-induced cardiotoxicity. In this section, the potential for FGF-16 synthesis to be targeted negatively by acute doxorubicin treatment will be discussed. By extension, this would support the possibility that decreased FGF-16 contributes to doxorubicin-induced damage to cardiac myocytes, and by extension myocardial function [105, 119, 150, 176].

1.7.1 Doxorubicin targets cardiac-specific gene expression

There is evidence to suggest that doxorubicin selectively targets muscle-specific

gene expression, including the α-actin (ACTA1), troponin I, and myosin light chain 2 (MLC2) genes [25]. The cardiac transcription factors MEF2C and Csx/Nkx2.5 upregulate each other and induce levels of α-actin expression in P19 embryonic carcinoma cells [177]. Troponin I is also a downstream target of both GATA4 and Csx/Nkx2.5 [178]. In addition, data support that cardiac MLC2 is regulated by Csx/Nkx2.5 in neonatal cardiac myocytes [179]. This negative effect on cardiac-specific gene expression is related to the rapid depletion of cardiac-specific transcription factors in response to doxorubicin treatment. This includes GATA4, MEF2, and Csx/Nkx2.5 as well as p300 protein, a known co-factor, and all are implicated in the regulation of cardiac *Fgf-16* expression [105, 119, 150, 176, 180, 181]. Thus, transcription of the FGF-16 gene is expected to decrease in response to acute doxorubicin treatment. Furthermore, unless *Fgf-16* transcripts possess a "long" half-life, mRNA levels would be expected to decrease rapidly with doxorubicin treatment. Finally, in addition to conservation across human and murine species of the putative GATA4 and Csx/Nkx2.5 DNA element associated with FGF-16 gene sequences (**Figure 7**), it is also noted that restoration of GATA4 and Csx/Nkx2.5 is known to increase protection against doxorubicin-induced cardiac myocyte death [180, 181].

As such, in addition to pursuing a role for Csx/Nkx2.5 in the regulation of postnatal *Fgf-16* expression, *the possibility that Fgf-16 mRNA levels are an early target of (acute) doxorubicin treatment, including an effect on mRNA stability, was explored through this thesis*.

Furthermore, given the evidence for FGF-16 as a cardiac maintenance factor in the postnatal heart, and the probable negative effect of doxorubicin on FGF-16 synthesis, *the*

effect of a reduction in FGF-16 expression on cardiac myocyte viability was also explored through this thesis.

1.7.2 A possible effect of FGF-16 on efflux drug transport in the heart

There is evidence to support a role for FGF-16 in the maintenance of a healthy myocardium, which presumably is the result of signaling through one or more FGFRs alone or through modulating, positively or negatively, the effects of other FGFs (as discussed in section 1.6.4). A possible mechanism includes contributing to cell and tissue health through effects on efflux substrate or drug transport. Stress stimuli such as UV-irradiation, heat shock protein, and growth factors that trigger stress related signaling pathways, such as PLC and MAPK, are known to regulate efflux transporter production [182]. In addition, exogenous FGF-2 supplementation was shown to upregulate efflux drug transporters and offer protection against doxorubicin-induced damage in neonatal rat cardiac myocyte cultures, through binding to FGFRs and activation of protein kinase C [26]. Thus, as FGF-16 and FGF-2 share "some" receptor binding [79], an effect of FGF-16 on efflux transport is also possible, but has not been reported. Increased removal of doxorubicin from the cell interior offers another potential approach to protect cardiac myocytes and myocardial function [26].

The presence of multidrug resistance protein (MDR) or P-glycoprotein, multidrug resistance-associated protein (MRP), and breast cancer resistance protein are all reported to be part of the multidrug resistance system in cancer cells[183-185]. Drug resistance is one of the major obstacles in cancer treatment besides severe systematic side effects from chemotherapy [186]. For example, after entering the cells, more than 50% of doxorubicin is

"pumped" out by members of a transmembrane protein family that function as efflux drug transporters [187]. Thus, cancer patients who develop cancer multidrug resistance have a higher mortality rate due to the increased survival of cancer cells [186, 188]. Multidrug resistance inhibitors have been developed to block their action and concentrate doxorubicin inside the cell to enhance anti-cancer activity [183, 189]. However, recent studies discovered that efflux drug transporters are also expressed in cardiac cells and are associated with chemotherapy drug distribution and chemotherapy-induced cardiotoxicity [188, 190, 191]. Thus, identification of modulators or agents that increase the level of these efflux drug transporters specifically in the heart would be beneficial [192].

Efflux transporters use the energy of ATP binding and hydrolysis to transport different substrates across the membrane of the cell [188]. The name, ATP-binding cassette (ABC) transporters, is based on the sequence and organization of the nucleotide-binding domain (binding and hydrolysis of cytoplasmic ATP) as well as sequence similarity (30-50%) [188]. The presence of ABC efflux drug transporters was first demonstrated in tumor cells in relation to multidrug resistance, but are also expressed in normal tissues including the heart later on [188, 190, 191]. Under normal conditions, these transporters play an important role in the maintenance of health through efflux of endogenous substrates as well as toxic foreign substances [183, 193, 194].

To date, more than 50 different efflux drug transporter family members have been discovered [188]. There are seven subfamilies of the ABC transporters, ABC1, multidrug resistance proteins (MDR/TAP), multidrug resistance-associated proteins (MRP/CFTR),

adrenoleukodystrophy (ALD), organic anion-binding transporter (OABP), GCN 20 and White. Two subfamilies MDR/TAP and MRP/CFTR are particularly involved in the efflux transport of xenobiotics and multidrug resistance [188]. P-glycoprotein 1 (permeability glycoprotein), also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1), is encoded by the MDR1 gene (*Mdr1*) in humans and by the MDR1a and 1b genes in rodents [188]. In 1987, *Mdr1* expression and thus P-glycoprotein, was shown to be increased in tumor cells that are associated with multidrug resistance [188]. However, *Mdr1* is also highly expressed in cardiac myocytes and endothelial cells of capillaries and arterioles [188]. Multidrug resistance-associated protein 1 (MRP1) is encoded by the MRP1 gene (*ABCC1*) in humans, and is also highly expressed in cardiac myocytes in both rodent and human hearts [188]. Other transporters such as MRP2 and ABCG2/BCRP are also expressed in the heart but at relatively low levels in normal tissue [188]. Use of knockout mouse models (MDR1a$^{-/-}$, MDR1a/1b$^{-/-}$ and MRP1$^{-/-}$) in combination with the anti-cancer drug vinblastine treatment revealed an increased drug concentration in several tissues including the heart [188, 195].

Blocking drug efflux using multidrug-resistance-reversing agents, appears to be more effective in treating cancer because of the increased drug concentration in cancer cells, however, the cardiotoxicity worsens with an additional increase in drug concentration in heart cells [188, 196]. By contrast, when MDR1 was overexpressed in the hearts of transgenic mice, the degenerative effects of doxorubicin-induced cardiotoxicity were absent [188, 197]. This supports an important role for efflux drug transporters in the protection of the heart against anti-cancer drugs [188]. Specifically, increased production of efflux drug transporters in the heart in response to the anti-cancer drug therapy would increase the resistance of

heart cells to the damage caused by the anti-cancer drug itself [188]. There is support for this effective self-protection system in the heart, as P-glycoprotein levels increase within a few days of anti-cancer drug treatment, and are associated with removing (pumping out) the anticancer drug from the heart [198].

The question arises then, if there is effective efflux anti-cancer drug transport in the heart, why is the heart susceptible to early anti-cancer drug-induced toxicity. One explanation is the impact of polymorphisms in ABC transporter genes. Patients with a silent mutation from a cytosine to a thymine in the MDR gene at position 3435 in exon 26 (C3435T), when compared to patients with no mutations, have lower MDR1 expression levels and higher digoxin plasma concentrations when administrated [199]. Patients with low or high P-glycoprotein expression in the heart will have increased or reduced levels of substrates, including anti-cancer drugs, due to transporter availability and the effect on substrate or drug removal [188, 190, 191]. Thus, cardiotoxicity is more severe in the hearts with less functional P-glycoprotein [188, 190, 191].

A second explanation relates specifically to the "early" nature of the "anti-cancer drug-induced toxicity". In this context, "early" relates to the window immediately after anti-cancer drug treatment, and before the multidrug resistance self-protection develops. Evidence that doxorubicin targets cardiac transcription factors and/or muscle-specific genes, strongly supports the notion that the heart is a specific target of doxorubicin-induced cytotoxicity [25]. However, there is no report on efflux drug transporter levels, or more specifically activity at this early stage before multidrug resistance is induced by doxorubicin. In addition, the function of efflux drug transporters has been widely studied

but not the regulation of the gene products. Thus, understanding the early effects of doxorubicin on efflux drug transporters, as well as regulators of efflux drug transporters, including potentially FGFs and specifically FGF-16, may reveal mechanisms of doxorubicin-induced cardiotoxicity. Furthermore, in cancer patients receiving other medications that are inhibitors of efflux drug transporters, such as calcium-channel blockers (verapamil or diltiazem), the intracellular level of chemotherapy drugs may increase, thus, potentially increasing the risk of chemotherapy-induced cardiotoxicity [200, 201].

In summary, efflux drug transporters could also be regulated, modulated and activated in a tissue or cell-specific manner [202]. The predominant cardiac expression and the associated FGFR signaling suggests a potential role for FGF-16 in the regulation of efflux drug transporters, and thus, drug distribution and chemotherapy-induced cardiotoxicity [188, 190, 191]. *The possibility that FGF-16 can increase efflux drug transporter gene expression and doxorubicin efflux in cardiac cells, is also explored through this thesis*.

Chapter 2: Rationale, Hypothesis, and Objectives

RATIONALE:

There is a need for additional agents to protect the heart from damage related to the cardiotoxicity of effective anti-cancer drugs including the anthracycline doxorubicin. Fibroblast growth factors (FGFs) are heparan sulfate proteoglycans binding proteins that signal multiple functions through binding tyrosine kinase receptors, including angiogenesis, fibrosis, hypertension, hypertrophy, and cell survival. FGF-16 is relatively understudied compared to other members of the FGF family. It does, however, possess a number of characteristics that support an essential role in normal heart development and function, including maintenance of a healthy postnatal myocardium. These include:

- FGF-16 is a highly-conserved member of the FGF family; human, rat and mouse *Fgf-16* share equivalent locations on the X chromosome, and their respective proteins share greater than 98.5% amino acid sequence identity [105]
- FGF-16 is required for normal embryonic mouse heart development [105, 108, 114]
- *Fgf-16* is expressed by the embryonic endocardium and epicardium, but switches to the myocardium, beginning around the time of birth, and continue to increase into adulthood [79, 105]
- While multiple members of the FGF family are produced in the murine heart, only FGF-16 is produced preferentially by the postnatal cardiac myocytes [79, 105].
- The *Fgf-16* promoter contains highly conserved putative binding sites for the cardiac transcription factors FOXP1 and Csx/Nkx2.5 (**Figure 6**) [105]

- Endogenous mouse FGF-16 is anti-hypertrophic and anti-fibrotic in the stressed heart [105, 169]
- FGF-16 is cardioprotective in models of postnatal cryo-injury and ischemia injury [80, 86] [80, 86, 105]
- Human *Fgf-16* mutations are linked to increased cardiovascular risk [105, 166]

Thus, FGF-16 is unique as a candidate of the FGF family to potentially: (i) contribute to cardiac damage as a result of a decrease in endogenous FGF-16 levels in response to doxorubicin treatment; but (ii) also increase resistance to doxorubicin cardiotoxicity through exogenous supplementation. The following *hypothesis* was developed for this thesis.

HYPOTHESIS:

It is hypothesized that *Fgf-16* is an early response gene for acute doxorubicin-induced cardiotoxicity and that an increase in FGF-16 levels can stimulate resistance to doxorubicin-induced damage, in part, through an effect on efflux drug transport.

OBJECTIVES:

The hypothesis will be addressed through the following three objectives and each objective will be further addressed in separate Chapters:

Objective 1: To examine the regulation of endogenous cardiac *Fgf-16* expression in response to acute doxorubicin-induced cardiac injury and the potential underlying

mechanisms by identifying candidate cardiac transcription factors (*Chapter 4: Fgf-16 is an early response gene for acute doxorubicin-induced cardiotoxicity*)

Objective 2: To assess the effect of a decrease in FGF-16 levels on cardiac myocyte survival (*Chapter 5: FGF-16 is an Endogenous and Exogenous Cardiac Survival Factor*)

Objective 3: To characterize the effect of FGF-16 overexpression or supplementation in doxorubicin-induced cardiac injury. In addition, potential regulation of efflux drug transport will be explored. (*Chapter 5: FGF-16 is an Endogenous and Exogenous Cardiac Survival Factor, and Chapter 6: FGF-16 regulates multidrug resistance protein 1a (MDR1a), an underlying mechanism of FGF-16 protection against acute doxorubicin-induced cardiotoxicity*)

The results and observations made by addressing all three objectives are discussed in the context of doxorubicin cardiotoxicity and FGF-16 as a potential maintenance and cardioprotective factor. In addition, possible future studies/directions are also described.

Chapter 3: Material and Methods

Animals

All animals (CD-1 mice and Sprague-Dawley rats) were treated according to standards and guidelines set by the Canadian Council for Animal Care and conform to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition. Revised 2011). The animal protocols were approved by the Bannatyne Campus Animal Protocol Management and Review Committee at the University of Manitoba [90].

Adult rat models

Eight-week-old Sprague–Dawley male rats (~250g) were treated with 15 mg/kg body weight doxorubicin (Sigma-Aldrich, ON, CA) or saline (0.9%) vehicle by intraperitoneal (i.p.) injection for 6 or 24 hours [59, 203]. Heart function to monitor the doxorubicin-induced cardiac side effects was performed using echocardiography (IVIS Spectrum optical imager) in collaboration with Ms. Bo Xiang in the SAM Imaging Core Facility at the University of Manitoba*. The echocardiography was used to assess the heart function in anesthetized rats at baseline (0), 6 or 24 hours post-injection. The parameters such as left ventricle ejection fraction (LVEF), fractional shortening (FS), stroke volume (SV), cardiac output (CO), diastolic/systolic volume, diastolic/systolic diameter, heart rate (HR), isovolumic contraction time (IVCT), isovolumic relaxation time (IVRT), left ventricle isovolumic myocardial performance index (LV MPI IV) and peak early/late

* http://umanitoba.ca/faculties/health_sciences/medicine/units/cacs/sam/index.html

diastolic velocity (E′/ A′) were recorded and analyzed using computer program VEVO 2100 (Version1.6.0). Rats were then euthanized at 6 or 24 hours after doxorubicin injection and hearts were harvested for RNA and protein isolation.

Isolated mice hearts

Retrograde heart perfusion

Heart perfusion was conducted in collaboration with Dr. David Sontag as previously described [106, 199]. Adult CD-1 mice (8-9 weeks, 36-46 g) were euthanized by cervical dislocation. Hearts were excised and perfused using a retrograde (Langendorff) method [204]. Briefly, the ascending aorta was cannulated with a 21-gauge needle and tied with a 6-0 silk suture and then perfused with perfusate [90]. The perfusion pressure was restored within 5 minutes of euthanization under non-recirculating conditions at a constant pressure of 80 mmHg; the perfusate consisted of a modified Krebs-Henseleit solution containing 118 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM mono-potassium phosphate, 1.2 mM magnesium sulfate, 2.2 mM calcium chloride, 10 mM glucose, 24 mM sodium bicarbonate, 3% bovine serum albumin (Sigma-Aldrich), and saturated with 95% O_2-5% CO_2 (pH 7.4, 37°C). The atria were removed and a silicone balloon filled with perfusion buffer inserted into the left ventricle through the mitral valve. This allows monitoring of systolic left ventricular pressure, defined as developed pressure (DP), and left ventricular end-diastolic pressure (EDP) using a Digimed Heart Performance Analyzer (Micro-Med, KY, USA) [90]. In addition, a thermocouple was inserted into the right ventricle to monitor the temperature of the heart, which was kept at 37 °C [90]. All hearts were paced electrically using platinum electrodes on the surface of the right ventricle with 3-volt 1-ms pulses at 8.3 Hz throughout

the experiment. Preload in all hearts was gradually increased until an EDP of 2–5 mmHg or a systolic pressure of 80 mmHg was achieved. DP was calculated by subtracting the average EDP from the average systolic pressure during a 10-second interval [90]. Following balloon insertion, hearts were subject to an equilibration period of 20 minutes, after which only hearts demonstrating a DP of at least 55 mmHg and stable contractions were included for study [90].

Treatment procedure (for isolated heart studies)

Recombinant human FGF-16 (Peprotech, NJ, USA) was reconstituted and stored in vehicle (phosphate buffered saline containing 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.5% bovine serum albumen) as frozen aliquots at -80 °C and thawed within 20 minutes of use as per the manufacturer's instructions [90]. Doxorubicin (Sigma-Aldrich) was dissolved in sterile water and stored as a stock (10 mM) at 4 °C until required [90].

Following a 20-minute equilibration period, hearts were perfused with a 2-ml bolus of Krebs-Henseleit solution containing either 10 μg FGF-16 or vehicle (100 μL) followed by 7 minutes of equilibration [90]. Hearts were then perfused with Krebs-Henseleit solution or filtered (0.45 μm) 10 μM doxorubicin in Krebs-Henseleit solution for 120 minutes [90]. For protein kinase C (PKC) signlaing inhibition, 5 μM chelerythrine was added to Krebs-Henseleit solution before FGF or vehicle. Measurements of perfusion rate and contractility were performed 1 minute prior to addition of FGF-16 or vehicle [90]. Perfusion rate was

calculated by measuring the time required for 10 drops of effluent, and then extrapolating based on the volume of 60 drops [90].

Lactate dehydrogenase (LDH) assay (for isolated heart studies)

LDH activity in the perfusate was assessed using an In Vitro Toxicology Assay Kit (Lactic Dehydrogenase-based) according to the manufacturer's instructions (Sigma-Aldrich) [90]. As a positive control and source of LDH, perfusate was obtained from a mouse heart re-perfused after 20 minutes of 'no-flow' ischemia [90].

Neonatal rat cardiac myocyte primary cultures

Sprague-Dawley neonatal rats (one-day old) were decapitated and then heart ventricles were isolated by excision and digested with collagenase (5,180 units), trypsin (2,590 units) and DNase I (15,960 units, Worthington Biochemical Corporation, Lakewood, NJ, USA) in phosphate buffered saline (PBS) -Glucose Buffer (10g/L glucose) [26]. Total cells were then centrifuged and fractionated (2,000 x g using an Avanti JE, Beckman Coulter, ON, CA) at room temperature for 30 minutes on a Percoll (GE Healthcare, SE-75184, Uppsala Sweden) gradient with two separate layers: an upper layer with cardiac non-myocytes and a lower layer with cardiac myocytes, 55% and 45% of the total volume, respectively [26].

Cardiac myocyte number was then counted using a cell counter (TC20^{TM} automated cell counter, Bio-Rad, Mississauga, ON, CA) and plated on culture plates that were coated with 0.25% w/v rat tail collagen, type I (BD Sciences, Mississauga, ON, CA) at a density of

1.3 million cells/60-mm diameter plate, 0.75 million cells/35-mm plate, or 0.45 million cells/22-mm plate [26].

Cardiac myoctyes were then plated in culture medium consisting of Ham's F-10 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (GIBCO, Life Technologies, Burlington, ON, CA), and 1% Penicillin streptomycin (Penstrep, GIBCO) for 24 hours [26], before being changed to defined low serum medium consisting of DMEM/F-12 (GIBCO), supplemented with 0.05% FBS, 0.66% Albumin (Sigma-Aldrich), 1% Penstrep (GIBCO), 0.02% Ascorbic Acid (Sigma-Aldrich), and 1% Insulin-Transferrin-Selenium (GIBCO) [26].

For doxorubicin treatment alone, cultures were refed with medium supplemented with 1 μM doxorubicin for 2, 6, 12 or 24 hours. For FGF receptor inhibition, 20 μM SU5402 (Sigma-Aldrich) was added for 15 minutes before without or with FGF-16 and doxorubicin. For treatment with efflux drug transporter inhibitors, cyclosporine A (CsA, 1:1000, 20 μM), or verapamil (2 μM) were added 15 minutes before, with or without FGF-16 and doxorubicin.

Transient transfection and the luciferase (Luc) reporter gene assay

Generation of hybrid Luc genes driven by different lengths of mouse (m) *Fgf-16* gene promoter (p) sequences is described elsewhere [138, 144]. This includes -5771/-12, -3772/-12, -1267/-12 and -759/-12 FGF-16p.Luc, where the nucleotide designation for each promoter fragment is given relative to the adenine residue (nucleotide +1) of the methionine

(ATG) translation start site of mFGF-16. Neonatal rat cardiac myocytes were seeded at $3.5x10^5$ cells/well (12-well culture plates) for one day before gene transfer using the TransIT-2020 transfection reagent (Mirus, WI, USA) [138]. A total of 2 μg of test plasmid, or empty vector as a negative control (pcDNA 3.1), was used per well. Cells were also co-transfected with the Renilla-luciferase (R-Luc) gene (20 ng) as a control for DNA uptake [119, 138]. After 18 hours, the cultures were refed with the defined medium before 1 μM doxorubicin treatment for 2, 6 or 24 hours. The cells were lysed in 100 mM Tris and 0.1% Triton-X-100, and collected with a cell scraper. Luciferase activity was read in a luminometer (Lumat LB 9507) using a dual luciferase reporter assay (Promega, Dual-Luciferase® Reporter Assay System, cat # E1960) [138].

Electrophoretic mobility shift assay (EMSA)

Neonatal rat cardiac myocyte nuclear extract (NE) was isolated using the EpiSeeker Nuclear Extraction kit (Abcam Inc, ON, CA) according to the manufacturer's instructions. EMSA was performed using the cardiac myocyte nuclear extract (NE) and incubated with 2 μg of poly (dI-dC) and [γ^{32}P]ATP-labeled oligonucleotide (5′-ACGTATATAAAGTGCCATTT-3′) probe containing TATA1 sequences and a putative Csx/Nkx2.5 binding site [138, 144]. These sequences (nucleotides –312/-292) are identical in both the rat and mouse *Fgf-16* promoter regions. Reactions were done in binding buffer (10 mM HEPES-NaOH, pH 7.9, 50 mM KCl, 2.5 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) for 30 min at room temperature [205]. For competition assays, Csx/Nkx2.5 antibody (Ab) and mouse immunoglobulins (IgG) were added and pre-incubated on ice for 10 minutes at room temperature and then radiolabeled probe for 20 min [138, 205]. DNA-

protein complexes were resolved in non-denaturing 5% (w/v) polyacrylamide gels and visualized by autoradiography [138, 205].

Small interfering (si) RNA – mediated knockdown

Cellular transfection to knockdown Csx/Nkx2.5 and FGF-16 expression was performed using HiPerFect Transfection Reagent according to the manufacturer's instructions in neonatal rat cardiac myocytes (Qiagen, cat # 301705) [138]. Csx/Nkx2.5 siRNA (FlexiTube siRNA, cat # SI01920338, Rn_Nkx2-5-2, 5′-CGCCTACGGCTACAACGCCTA-3′), FGF-16 siRNA (5′-CACCAGAAATTCACTCACTTT-3′ [138], GeneSolution siRNA, cat#1027416, hFGF-16-4), and control siRNA (cat#SI03650318) were purchased from Qiagen. The knockdown was done in 12-well culture plates at a cell density of $3.5x10^5$ cells/well using 50 nM or 25 nM siRNA and control siRNA (Qiagen, cat # SI03650318) [138].

Adenovirus (AdV) concentration qPCR titration and AdV-mediated gene delivery

Adenoviral DNA was purified using the NucleoSpin Virus kit (Macherey-Nagel, PA, USA, cat#740977.10) [138]. Briefly, 150 μL of rat cardiac myocyte lysate was harvested after adenoviral transduction and was treated with deoxyribonuclease I (DNase I) to remove any residual host cell or plasmid DNA carried over from cell packaging, and viral DNA isolated after Proteinase K treatment [138]. Serial dilution combined with qPCR was done to determine the threshold cycle for each dilution using Adeno-X qPCR Titration Kit (Clontech, CA, USA, cat# 632252) [138]. DNA copy number and multiplicity of infection (MOI) were then

calculated from a standard curve generated by plotting the Ct values of the serial diluted (6 orders of magnitude) Adeno-X DNA control template[138].

Pre-made Csx/Nkx2.5 (Human-Csx/Nkx2.5-AdV, cat # 00454A), CMV Null (CMV-Null-AdV, cat # 000047A), FGF-16 (FGF-16 AdV, cat #091605A) and HA-GATA4 (a gift from Dr. Mona Nemer, University of Ottawa, Rat-GATA4-AdV) overexpression adenoviruses, were amplified in HEK293 cells according to the manufacturer's protocol (Applied Biological Materials Inc, BC, CA) [138]. Adenovirus transduction (overexpression) was performed in 12-well multiple culture plates at a cell density of $3.5x10^5$ cells/well at an MOI of 1 or 10 for 1 hour. After washing, the cells were refed with medium and incubated for 48 hours before treatment with 1 μM doxorubicin for 12 hours prior to isolation and assessment of RNA or protein by qPCR or western blots, respectively. For FGFR inhibition, 20 μM SU5402 (Sigma-Aldrich) was added to cardiac myocytes before adenovirus transfection [138].

RNA isolation

Total RNA from neonatal rat cardiac myocytes was isolated using the RNeasy Plus Mini Kit (Qiagen Inc, Mississauga, Ontario, Canada) and total RNA from rat and mouse heart was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen).

Briefly, cardiac myocyte cultures were washed three times with phosphate-buffered saline (PBS) (GIBCO) [26] and 500 μL of Lysis Buffer RLT Plus with 1% beta-mercaptoethanol (Sigma-Aldrich) was added to disrupt the cells into lysates [26]. The total

lysates was then transferred into the QIAshredder spin column provided from the kit and centrifuged for 2 minutes at maximum speed (>10,000 xg) [26]. The QIAshredder was used to homogenize cell lysates to reduce viscosity by incorporating a unique biopolymer shredding system into a micro-centrifuge spin-column format and shearing the high-molecular weight genomic DNA and other high-molecular-weight cellular components to create a homogenous lysate [26]. The homogenized lysate was then transferred to a genomic DNA Eliminator spin column before being centrifuged for 30 seconds at maximum speed to eliminate genomic DNA [26]. The column was discarded, and the flow-through was saved before adding 500 µL of the 70% ethanol [26]. The lysate was then transferred to a RNeasy spin column that can bind to mRNA larger than 200 nucleotides and centrifuged for 15 seconds at maximum speed [26]. The flow through was discarded, and the RNeasy spin column was then washed once with 700 µL of buffer RW1 and twice with Buffer RPE to get rid of buffer contaminants [26]. The RNeasy spin column was placed in a new collection tube and spun for 1 minute to eliminate any possible carryover of Buffer RPE [26]. The RNeasy spin column was placed in a new 1.5 ml collection tube and 25 µL of RNase-free water added directly to the spin column membrane [26].

For heart tissue, less than 30mg heart was used and 300 µL Lysis Buffer RLT was added before homogenizing the tissue into lysates. Ten µL of Proteinase K, 590 µL of RNase-free water were added before incubation for 10 minutes at 55 ^{o}C. Then 0.5 volume of 100% ethanol was added and mixed before transfer to an RNeasy Mini Column for spinning. The column was then washed using RW1 buffer before adding DNase solution for 15 minutes at room temperature to digest the genomic DNA. The column was then washed by RW1 and RPE buffer before elution of RNA using 50 µL of RNase-free water.

RNA quality and concentration were assessed using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific Inc., ON, CA) using 1.5 µL of eluted RNA solution. A 260/280 ratio of greater than 1.8 was considered to be acceptable.

Quantitative real-time reverse transcriptase-polymerase chain reaction (qPCR)

Total RNA (1 µg) from cardiac myocytes or heart tissue was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) [26]. One µg total RNA template, plus 2 µL gDNA Wipeout Buffer, make up to 14 µL total volume with RNase-free water was incubated at 42°C for 2 minutes to eliminate any genomic DNA [26]. Then 1µL Quantiscript Reverse Transcriptase (comprised of Quantiscript Reverse Transcriptase and RNase inhibitor), 1 µL RT primer Mix (oligo-dT and random primers) and 4 µL Quantiscript RT Buffer (containing dNTPs) were added to a total of 20 µL [26]. Mixed samples were then incubated for 45 minutes at 42 °C for the reverse transcription reaction, and 3 minutes at 95 °C to inactivate Quantiscript Reverse Transcriptase [26].

Amplifications were performed in triplicate in 20 µL using the SYBR green PCR Master Mix Kit (10 µL; A&B Applied Biosystems, UK), with 33 ng of template cDNA and 5µM each of forward and reverse primers (Invitrogen) in an ABI 7500 fast Real-Time PCR System; cycle conditions were: incubation 95 °C/10 minutes, then 40 cycles with denaturation 95 °C/15 seconds, annealing 60 °C/30 seconds, and extension 72 °C/1 minute [26].

RNA levels were analyzed using the comparative cycle threshold (Ct) method [26].

The average Ct values were determined for both the target gene and control gene RNA polymerase II (RNA pol II) in each sample from the treated and untreated (control) groups [26]. The average delta Ct value (ΔCt) was determined for each group by subtracting the average Ct value for the housekeeping gene from the average Ct value for the target gene [26]. The $\Delta\Delta$Ct value was then determined by subtracting the ΔCt value for the untreated group from the DCt value for the treated group [26]. Relative quantification (RQ) of gene expression = $2^{-\Delta\Delta Ct}$. The relative quantity was determined for each group using the RQ Study feature within the 7500 fast Real-Time PCR System Sequence Detection Software, version 2.0.5 (A&B Applied Biosystems) [26]. All mRNA qPCR results were analyzed and normalized relative to RNA pol II expression.

Chromatin immunoprecipitation (ChIP)-qPCR assay

ChIP-qPCR assay (FactorPath™) was performed by Active Motif (Carlsbad, CA, USA). ChIP reactions were done using 20 μg of neonatal rat cardiac myocyte chromatin and 20 μL of Csx/Nkx2.5 or NF-κB (p65) antibodies (Santa Cruz, cat# sc-8697 and sc-109) [138]. Antibodies were validated and tested for non-specific binding by Active Motif. Primers for specific regions of interest as well as a gene desert region on chromosome 17 (Untr17) as a negative control were used for qPCR [138] (**Table 2**). Atrial natriuretic factor gene (Nppa-310) and nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (Nfkbia+1002) sequences, contain known binding sites for Csx/Nkx2.5 and NF-κB (p65) binding, respectively, [206, 207], and were thus assessed as positive controls using specific primers (**Table 2**) [138].

Table 2: Primer sequences used for qPCR and ChIP-qPCR [138]

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	size (nt)
RNA pol II	GGCTCTCCAGATTGCGATGTG	GCAGGTAACGGCGAATGATGATG	126
FGF-16	GTTTTCCGGGAACAGTTTGA	ATGGAGGGCAACTTAGAAGGA	196
Csx/Nkx2.5	CTTCAAGCAACAGCGGTACCT	CGCTGTCGCTTTCACTTGTA	122
FGF-2	TCTTCCTGCGCATCCATCCAGA	CAGTGCCACATACCAACTGGAG	265
HCN1	CCTCAAATGACAGCCCTGAATTG	TCGGTGTGGAACTACCAGGTGT	463
$Ca_v1.2$	AGCAACTTCCCTCAGACGTTTG	GCTTCTCATGGGACGGTGAT	97
Untr17	AGGCACATAGGAGGTAAAAGTTC	GGAGGTCACAGGAGGACTTC	92
-396/-315 *Fgf-16* ChIP-qPCR	ACCCACACACCAATCTCTGA	CACTCCCCTTCCATTGACAT	81
Nppa-310 ChIP-qPCR	GTTGCCAGGGAGAAGGAATC	CCCCACTTCAAAGGTGTGAG	87
Nfkbia+1002 ChIP-qPCR	GGGTCAAAGAGGGCACAG	TTGGTCAGGTGAAGGGAGAC	90
FGF-9	TTCGGCATTCTGGAATTTATC	CTTCTCGTTCATGCCGAGGTA	69
MDR1a	AGCGGTCAGTGTGCTCACA	CTTGGCATATATGTCTGTAGCA	180

MDR1b	GAAATAATGCTTATGAATCCCAAAG	GGTTTCATGGTCGTCGTCTCTTGA	325
MRP1	AAGGAGTCCAGTCCTCAGG	AGAGGTCACTGCTCTTCAGG	335
MRP2	GAAGGCATTGACCCTATCT	CCACTGAGAATCTCATTCATG	317
ANP	ATGGGCTCCTTCTCCATCAC	TCTACCGGCATCTTCTCCTC	157
GATA4	CTGTGCCAACTGCCAGACTA	AGATTCTTGGGCTTCCGTTT	164

Protein extraction and immunoblotting

For total RNA extraction, neonatal rat cardiac myocytes or heart tissues were extracted after cell lysis [137, 138]. For nuclear extraction, neonatal rat cardiac myocyte nuclear extract (NE) was isolated for the detection of Csx/Nkx2.5 using the EpiSeeker Nuclear Extraction kit (Abcam Inc, ON, CA) according to the manufacturer's instructions. FGF-16 was isolated from the culture medium using heparin sepharose beads (GE Healthcare, ON, CA, cat# CL-6B), prepared by adding an equal volume of 0.5M NaCl/10 mM Tris [138]. Twenty-five µL of bead solution was added to each 1.5 mL of medium and mixed overnight at 4 ^{o}C. Beads were washed with 200 µL 0.5M NaCl/10 mM Tris-Cl. Then 30 µL of sodium dodecyl sulfate (SDS) protein sample buffer were added before boiling the samples and centrifugation to collect the protein in the supernatant using a Hamilton syringe[138]. Proteins (5 µg of nuclear protein and all extractions from the culture medium) were separated by 10% or 15% SDS-polyacrylamide gel electrophoresis (PAGE),

respectively. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane and immunoblotted with either anti-Csx/Nkx2.5 (Santa Cruz, TX, USA, cat# sc-8697) or anti-FGF-16 antibodies (A61 [138], A60 [138], PeproTech #500-P160BT [138] and Abcam, #ab170515). Lamin B (Santa Cruz, cat # sc-6217) was used as a control for nuclear protein loading and β-tubulin (Santa Cruz, # sc-9104) was used as a control for total protein loading. Recombinant FGF-9 (PeproTech cat#100-23) and FGF-16 (PeproTech cat#100-29) were used to test the specificity of the FGF-16 antibodies. The proteins were visualized using horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) secondary antibody and ECL plus immunoblotting detection reagents (Thermo Fisher Scientific Inc.) and X-ray film (GE Healthcare high performance chemiluminescence film, cat#45001507) after film machine development [138]. Quantification of specific protein band density was performed using Image J.

Cell death quantification

Cell death quantification was assessed using a Lactate Dehydrogenase Assay Kit (In Vitro Toxicology Assay Kit, Sigma), and PE -V Apoptosis Detection Kit I (BD Pharmingen™, ON, Canada).

Lactate dehydrogenase assay (for cell culture)

The lactate dehydrogenase assay was performed using the In Vitro Toxicology Assay Kit (lactate dehydrogenase-based) from Sigma as previously described [26]. For cardiac mycoyte cultures, one plate was treated with 1/10 volume of the Lysis Solution and incubated for 45 minutes to lyse all cells to release a large amount of LDH as a positive

control [26]. Briefly, 50 μL of the culture medium from the positive control group, 50 μL of the medium from a treatment group, and a negative control containing only culture medium was used as the blank sample, were assessed for lactate dehydrogenase activity in 96-well culture dishes [26]. One hundred μL of the Lactate Dehydrogenase Assay Mixture (equal volumes of Lactate Dehydrogenase Assay Substrate Solution, Lactate Dehydrogenase Assay dye solution, and 1x Lactate Dehydrogenase Assay Co-factor Preparation) was then added to start the reaction after plates were covered for 30 minutes with aluminum foil at room temperature for protection from light [26]. The reaction was ended with the addition of 15 μL of 1 M hydrochloric acid [26]. The absorbance was measured at a wavelength of 490 nm using a spectrophotometer [26]. The background absorbance was measured at a wavelength of 690 nm and this value was subtracted from the 490-nm reading [26]. The amount of lactate dehydrogenase release from both the treatment and control groups was then compared and analyzed for significant changes [26].

PE Annexin-V apoptosis detection kit I (for cell culture)

Flow cytometry combined with biomarkers for apoptotic and dead cells using the PE Annexin-V Apoptosis Detection Kit I was done as previously described [26]. Briefly, cardiac myocytes ($1x10^5$ cells) were pelleted and then re-suspended in 100 μL 1xBinding Buffer before adding both 5 μL of PE Annexin-V and 5 μL 7-Amino-Actinomycin (7-AAD) for staining. An unstained control sample, a PE Annexin-V single positive control sample, and a 7-AAD single positive control sample were also included as gating controls. Cells were then vortexed and incubated for 15 minutes at room temperature (25 ^oC) in the dark and then 400 μL of 1xBinding Buffer was added to each tube and analyzed by flow

cytometry (operated by Dr. Monroe Chan from the Department of Pathology) within 1 hour according to the manufacturer's instructions (MoFloXDP, Beckman Coulter, ON, CA). Data were analyzed using the Summit v.5.2 program (Beckman).

Efflux drug transporter function quantification

Doxorubicin autofluorescence quantification

Doxorubicin is autofluorescent and the fluorescence intensity can be detected due to the fluorescent hydroxy-substituted anthraquinone chromophore structure [26]. Doxorubicin is both a regulator and a substrate for efflux drug transporters [26]. Thus, the intracellular concentration of doxorubicin can be compared between each group using the fluorescent intensity [26, 208].

Cells were processed to a different experimental treatment regimen with positive controls treated with CsA (1:1000, 20 μM) or verapamil (2 μM) [26]. Cells were then centrifuged and resuspended with no phenol red DMEM/F-12 medium before analysis by flow cytometry according to the manufacturer's instructions (MoFloXDP, Beckman Coulter, Mississauga, Canada) within 1 hour of harvesting[26]. Data were analyzed using the Summit v.5.2 program (Beckman). Gating for the analysis was based on determinations for no doxorubicin treatment cells [26].

Multi-drug resistance assay kit (calcein-AM quantification)

To study efflux drug transporter activities, neonatal rat cardiac myocytes were assessed using a Multi-Drug Resistance Assay Kit (Calcein-AM) according to the manufacturer's instructions (Cayman Chemical Company, MI, USA). Cardiac myocyte cultures were treated with cell-permeable non-fluorescent substrate calcein-AM solution 25 minutes prior to being pelleted and resuspended in 400 μL of Assay Buffer and analyzed with a flow cytometer within 1 hour of harvesting. After entering the cell, the intracellular esterase activity removes the acetomethoxy (AM) group from the calcein-AM and converts it into fluorescent calcein, which is retained in the cytoplasm [209]. Positive controls reflecting the treatment with cyclosporine A (CsA, 1:1000, 20 μM) or verapamil (2 μM) to block efflux transport were also included. Data were analyzed using the Summit v.5.2 program. Gating for the analysis was based on determinations for no calcein-AM treated cells [26].

Statistical analysis

For single comparisons, an unpaired t-test was used to determine the significance of any differences. For single group (treatments) analyses and multiple (treatments and time), one-way analysis of variance (ANOVA) with a *post hoc* Tukey test and two-way (ANOVA) with a *post hoc* Bonferroni test were used, respectively. Results are normally expressed as mean plus or minus standard error of the mean [138]. In figures, comparisons were made relative to the untreated "control" group and arbitrarily set to 1.0 (for fold increase) or 100% (for percent reduction) [138]. Mean values were considered significantly different if $p<0.05$; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ [138]. For comparisons made between treatment groups in figures (and not arbitrarily set to 1.0 or 100%), these are represented as #/‡ $p<0.05$, ##/‡‡ $p<0.01$ and ###/‡‡‡ $p<0.001$ [138]

Chapter 4: *Fgf-16* is an Early Response Gene for Acute Doxorubicin-Induced Cardiotoxicity

4.1 The effect of doxorubicin on endogenous FGF-16 mRNA levels in rat hearts (*in vivo*)

Rationale*:** Doxorubicin is a widely used anti-cancer drug but is limited by its cardiotoxicity [19]. Doxorubicin is known to rapidly and negatively target cardiac-specific gene expression, including muscle genes, and has earned the name "red devil" because of the red color and the severe cardiotoxic effects on heart muscle function and survival [19, 20]. In addition, cardiac transcription factor GATA4 and Csx/Nkx2.5 are also downregulated by doxorubicin treatment [181, 210]. Murine *Fgf-16* is expressed preferentially in the postnatal heart and cardiac myocytes [169]. The GATA4-ablated mouse model indicates a down-regulation of FGF-16 expression in the heart. Sequence analysis using consensus binding sites, also identified multiple putative cardiac transcription factor DNA elements, including a Csx/Nkx2.5 site in the proximal promoter (TATA1) region of the human, rat and mouse *Fgf-16* (**Figure 6**). Thus, it was ***predicted that endogenous cardiac FGF-16 RNA levels would be rapidly decreased in response to acute doxorubicin treatment. *The **objective** was to establish a doxorubicin-induced acute cardiotoxicity model (in vivo) and assess the effect of doxorubicin on heart function as well as endogenous FGF-16 mRNA levels.*

***Approaches*:** An acute doxorubicin-induced toxicity model was previously described using a single doxorubicin intraperitoneal (i.p.) injection (15 mg/kg) in male Sprague-Dawley rats [211]. The model was established, and heart function was closely monitored with echocardiography. Cardiac mRNA levels for FGF-16 and atrial natriuretic

peptide (ANP), as a stress marker [212], were determined by qPCR. Unless stated otherwise in figure legends, the experimental design, generation, analysis and interpretation of experimental data were done by Jie Wang. Parts of the data have now been published [138], and copyright permission was obtained and is indicated in each individual figure legend as appropriate.

Results:

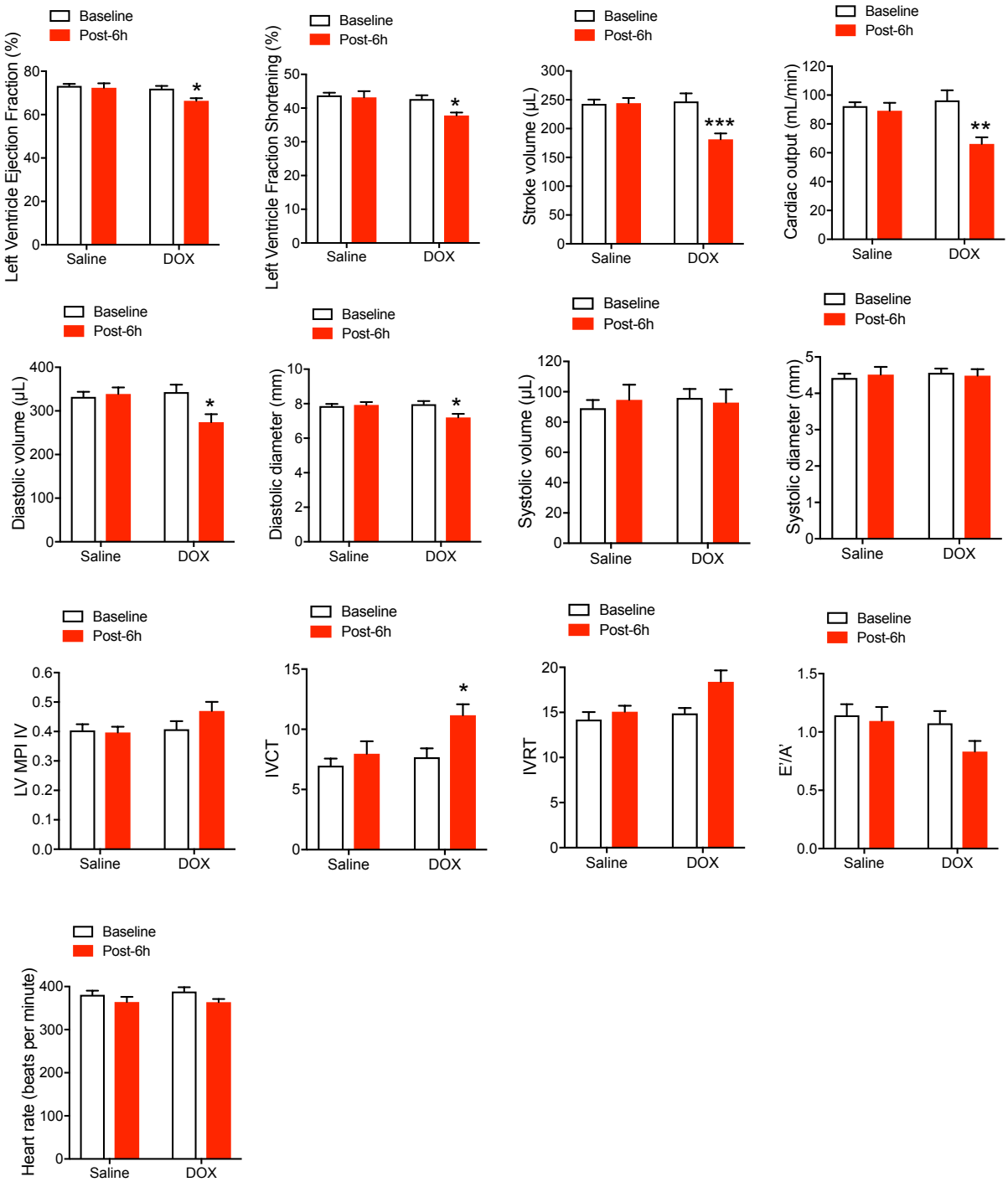
4.1.1 Doxorubicin induces acute cardiac dysfunction at both 6 and 24 hours in rat hearts

Eight-week-old Sprague–Dawley rats were treated with 15 mg/kg body weight doxorubicin or saline vehicle by i.p. injection [59, 138, 203]. Heart function was assessed at 0, 6 (**Figure 9**) or 24 hours (**Figure 10**) after injection using echocardiography to monitor the progress of doxorubicin-induced cardiac dysfunction [138]. Rats were then euthanized at 6 or 24 hours right after echocardiography and hearts were harvested for RNA isolation [138]. At 6 hours (**Figure 9**), doxorubicin significantly decreased systolic function such as left ventricle ejection fraction (LVEF), fractional shortening (FS), stroke volume (SV), and cardiac output (CO) in rat hearts compared to saline treatment at the same time. This is also associated with a significant increase in isovolumic contraction time (IVCT) and a significant decrease in left ventricle diastolic volume and diameter. No significant effect on systolic diameter, systolic volume, left ventricle isovolumic myocardial performance index (LV MPI IV), isovolumic relaxation time (IVRT), and the ratio of the early-to-late annular velocity (E′/A′) were seen with doxorubicin treatment. At 24 hours (**Figure 10**), systolic function such as left ventricle ejection fraction (LVEF), fractional shortening (FS), stroke

volume (SV), and cardiac output (CO) were still significantly decreased by doxorubicin treatment in rat hearts compared to saline treatment. The diastolic volume and diameter were back to normal at 24 hours with doxorubicin treatment. However, a significant increase in isovolumic contraction time (IVCT), isovolumic relaxation time (IVRT) and left ventricle isovolumic myocardial performance index (LV MPI IV), together with a significant decrease in E′/A′ after 24 hours doxorubicin injection indicate a diastolic dysfunction induced by doxorubicin treatment. No significant effect was observed with systolic volume and heart rate with doxorubicin treatment at either 6 or 24 hours. In addition, all echocardiography parameters tested by in saline only group were unchanged at either 6 or 24 hours.

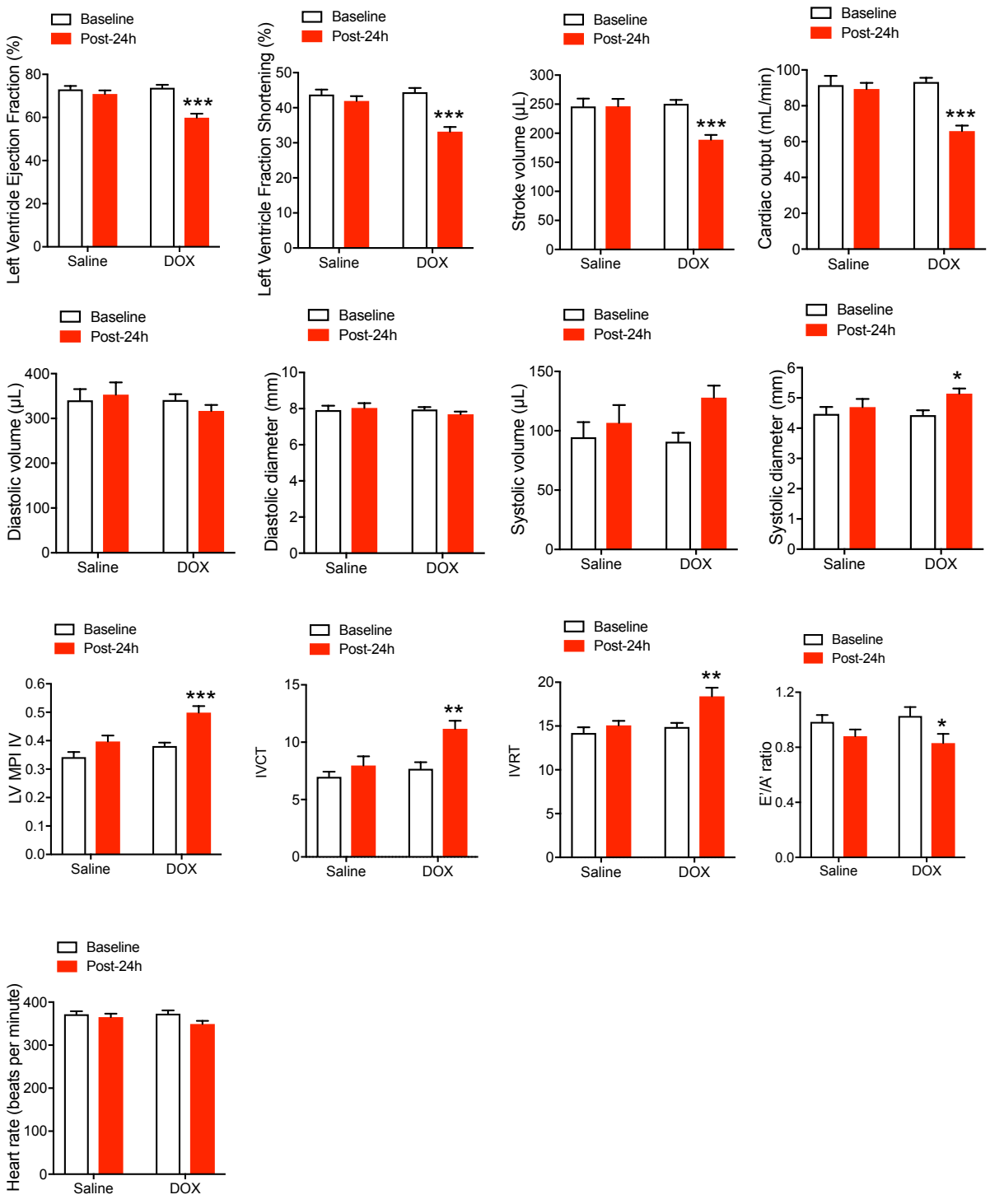
Thus, the doxorubicin treatment regimen successfully induced detectable acute cardiac dysfunction (*in vivo*). The effect of doxorubicin-induced cardiotoxicity on endogenous FGF-16 and other cardiac markers transcript levels were investigated.

Figure 9. Rat heart function assessed by echocardiography before and after 6 hours (h) doxorubicin (i.p.) injection



Eight-week-old Sprague-Dawley rats were injected (i.p.) with 15 mg/kg doxorubicin (DOX) and assessed after 6 hours (h). Heart function was assessed by echocardiography at baseline (0) and 6 h post-DOX or post-saline using the Vevo 2100 (Version 1.6.0) program (n=6 rats for each group). Results at each time point are compared to Baseline values, which are arbitrarily set to 100. Unaired t-test was applied for single comparison at each time point. Mean values for DOX treatment were considered significantly different compared to Saline group if $p<0.05$; $p<0.05$*, $p<0.01$**, and $p<0.001$***. (* compared to Baseline values). Contribution: Echocardiography was performed by Dr. Bo Xiang (from Dr. Vernon Dolinsky's laboratory, Department of Pharmacology & Therapeutics, University of Manitoba) with the assistance of Jie Wang. Generation, analysis and interpretation of the echocardiography data were done by Jie Wang.

Figure 10. Rat heart function assessed by echocardiography before and after 24 hours (h) doxorubicin (i.p.) injection



Eight-week-old Sprague-Dawley rats were injected (i.p.) with 15 mg/kg doxorubicin (DOX) and assessed after 24 hours (h). Heart function was assessed by echocardiography at baseline (0) and 24 h post-DOX or post-saline using the Vevo 2100 (Version 1.6.0) program (n=6 rats for each group). Results at each time point are compared to Baseline values, which are arbitrarily set to 100. Unaired t-test was applied for single comparison at each time point. Mean values for DOX treatment were considered significantly different compared to Saline group if $p<0.05$; $p<0.05$*, $p<0.01$**, and $p<0.001$***. (* compared to Baseline values). Contribution: Echocardiography was performed by Dr. Bo Xiang (from Dr. Vernon Dolinsky's laboratory) with the assistance of Jie Wang. Generation, analysis and interpretation of the echocardiography data were done by Jie Wang.

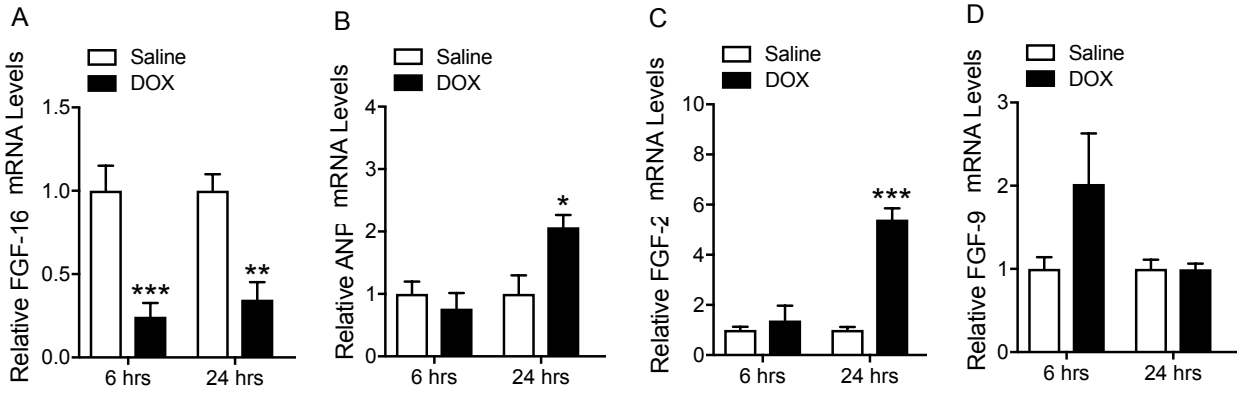
4.1.2 FGF-16 mRNA levels are rapidly decreased in the doxorubicin-induced acute cardiac injury model (*in vivo*) at both 6 and 24 hours

To assess the effect of doxorubicin on endogenous FGF-16 mRNA levels, rats were treated with or without a single i.p. injection of doxorubicin (15 mg/kg body weight) and assessed at 6 or 24 hours post-doxorubicin [138]. This dose is reported to induce acute cardiomyopathy in the rat [59, 203]. RNA was isolated from the hearts and FGF-16 transcript levels were determined by qPCR (**Figure 11A**) [138]. A significant >70% decrease in FGF-16 mRNA levels with doxorubicin relative to saline injection was detected at both 6 and 24 hours post-doxorubicin, relative to post-saline, injection [138].

4.1.3 The effect of doxorubicin on cardiac stress marker gene ANP as well as FGF-2 and FGF-9 transcript levels

ANP is a cardiac stress biomarker [212]. ANP mRNA levels were significantly increased following doxorubicin injection in rat hearts at 24 hours but not at 6 hours when normalized against control gene RNA pol II (**Figure 11B**). Furthermore, FGF-2 mRNA levels remained unchanged at 6 hours but were increased at 24 in the doxorubicin- compared to saline-injected hearts (**Figure 11C**). In addition, transcripts for FGF-9, another member of the FGF-9/16 subfamily, were unchanged at both 6 and 24 hours (**Figure 11D**). These observations are consistent with FGF-16 representing an early and specific target of doxorubicin, and potentially more sensitive target than ANP.

Figure 11. Endogenous FGF-16 mRNA levels are decreased in rat hearts following doxorubicin injection



FGF-16 (A), ANP (B), FGF-2 (C), and FGF-9 (D) mRNA levels were assessed by qPCR in hearts harvested from rats treated for 6 or 24 hours (hrs) without (saline) or with 15 mg/kg doxorubicin (DOX) (n=6 rats for each group). All mRNA expression levels were normalized to control gene RNA Pol II. Results at each time point are compared to Saline control values, which are arbitrarily set to 1. Unpaired t-test was applied for single comparison at each time point. Mean values for DOX treatment groups were considered significantly different compared to the Saline group if $p<0.05$; $p<0.05$*, $p<0.01$**, and $p<0.001$***. (* compared to Saline treatment group). *(Figure 11A, 6 hours data was reproduced and modified with permission from DNA and Cell Biology [138])*

4.2 The effect of doxorubicin on endogenous FGF-16 mRNA levels in neonatal rat cardiac myocytes (*in vitro*)

Rationale*:** The *in vivo* rat heart model used in section 4.1 is useful to study whole system cell and tissue interactions as well as general heart function, however, primary cultures were pursued to study the effects of doxorubicin on *Fgf-16* expression at the level of the cardiac myocyte. Neonatal rat cardiac myocytes express endogenous *Fgf-16*, and provide an accessible system to study both the mechanism for rapid downregulation in response to doxorubicin, and the consequences of a loss or reduction in FGF-16 [79]. Thus, the effect of acute doxorubicin treatment on endogenous FGF-16 mRNA levels was now tested in primary neonatal rat cardiac myocyte cultures. Viable neonatal rat cardiac myocytes can be obtained in relatively high numbers, and consistently, and have been widely used to assess the physiological, pharmacological, and morphological properties of postnatal cardiac myocytes [213]. These cultures have been established in our laboratory and are used here to study the effects of doxorubicin in cardiac myocytes without the interference of the other cell types [26, 138, 213]. Gaining insight into the mechanism behind the rapid downregulation of FGF-16 mRNA levels, may provide support for FGF-16 as a potential biomarker, like ANP, for cardiac stress. Thus, based on the *in vivo* data, it was ***predicted that doxorubicin treatment will rapidly decrease endogenous FGF-16 mRNA levels within 6 hours in primary neonatal rat cardiac myocyte cultures. *If validated in neonatal rat cardiac myocytes (in vitro), the* ***objective*** *was to investigate the mechanism of this downregulation in terms of RNA synthesis and degradation.*

***Approaches*:** Doxorubicin-induced cardiac myocyte cardiotoxicity is both time- and concentration-dependent and is cumulative [214-216]. Neonatal rat cardiac myocyte cultures were exposed to 1 μM doxorubicin for 24 hours to induce cell damage and death, followed by assessment of endogenous FGF-16 mRNA levels by qPCR [216]. Actinomycin D treatment was also used to distinguish between effects on transcription versus RNA degradation. Actinomycin D is a widely-used DNA-dependent RNA polymerase inhibitor and acts as a non-selective general eukaryotic transcription inhibitor [25, 217]. When treating the cardiac myocytes with Actinomycin D before doxorubicin, the effect of doxorubicin on *Fgf-16* transcription is blocked, and only the effects on FGF-16 mRNA degradation are observed [217]. This provides the basis for an approximate assessment of the stability of FGF-16 transcripts in neonatal rat cardiac myocytes in the presence and absence of doxorubicin treatment. In addition to FGF-16 mRNA levels, the effect of doxorubicin treatment on FGF-16 protein levels in the primary culture medium was assessed by immunoblotting of heparin-binding protein.

***Results*:**

4.2.1 FGF-16 mRNA levels of neonatal rat cardiac myocytes are rapidly decreased relative to ANP transcripts following 1 μM doxorubicin treatment

Neonatal rat cardiac myocyte cultures were treated with 1 μM doxorubicin for 2, 6 and 24 hours, and total RNA was isolated and assessed by qPCR [138]. There was a significant >70% decrease in FGF-16 mRNA levels at 2 hours that was reduced further (>90%) at 6 hours and 24 hours (**Figure 12A**) [138]. This rapid decrease or depletion of FGF-

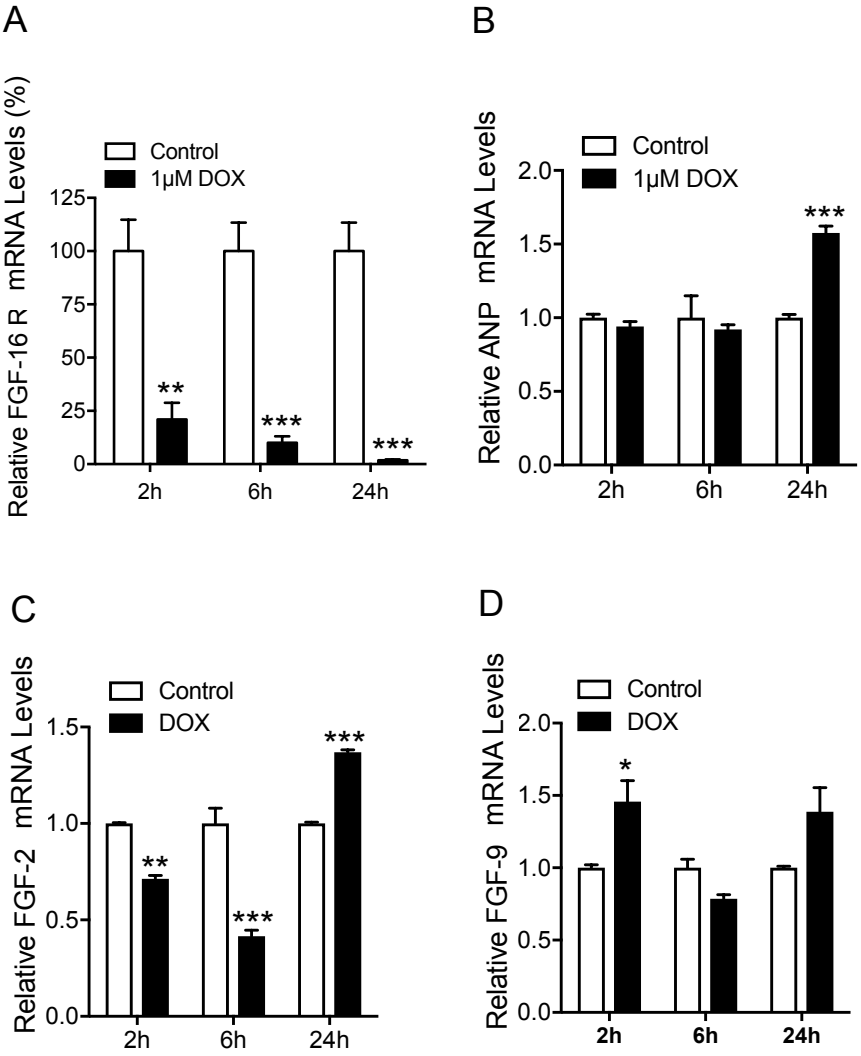
16 mRNA levels by doxorubicin treatment is consistent with the *in vivo* rat study (**Figure 11**).

Doxorubicin will also act eventually as a non-specific transcriptional inhibitor at late stages of cardiotoxicity, when the damage is severe [19, 214]. The effect of doxorubicin treatment on ANP as well as FGF-2 and subgroup member FGF-9 was also assessed by qPCR. There was no significant change in the ANP mRNA levels at 2 and 6 hours (**Figure 12B**). A significant increase was observed at 24 hours with 1 μM doxorubicin treatment, and this is consistent with the *in vivo* rat study (**Figure 11**).

Like FGF-16, FGF-2 mRNA levels showed a significant although more modest decrease at 2 (~25%) and 6 hours (~50%) with doxorubicin treatment, but unlike FGF-16 an increase was detected at 24 hours (**Figure 12C**). The rapid decrease of FGF-2 at 2 and 6 hours is consistent with the results of our previous study in neonatal rat cultures, but not those from the *in vivo* rat heart, where FGF-2 mRNA levels were unchanged at 6 hours (**Figure 11**) [26]. It is noted, however, that the increased level of FGF-2 mRNA at 24 hours with doxorubicin treatment is consistent with the *in vivo* adult rat heart study.

FGF-9 mRNA levels were significantly increased at 2 hours but returned back to control levels at 6 and 24 hours with doxorubicin treatment (**Figure 12D**). Although an increasing trend was also seen in the *in vivo* rat heart, it did not reach statistical significance (**Figure 11**).

Figure 12. Doxorubicin decreases FGF-16 mRNA levels in neonatal rat cardiac myocytes



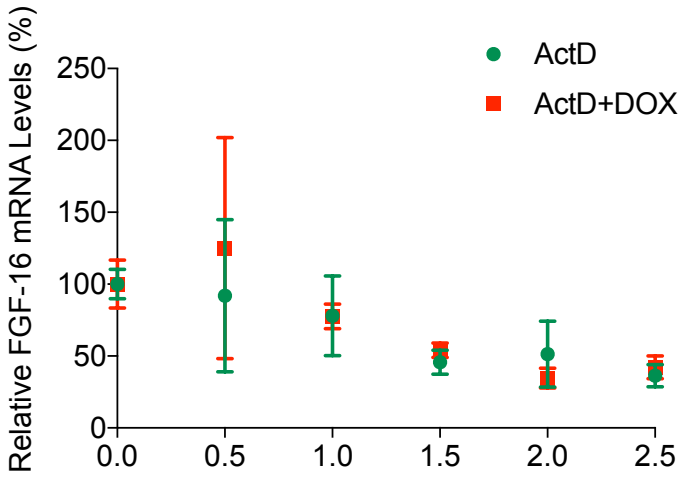
FGF-16 (A), ANP (B), FGF-2 (C), and FGF-9 (D) mRNA levels were assessed by qPCR in neonatal rat cardiac myocyte cultures treated with 1 μM doxorubicin (DOX) or vehicle (Control) for 2, 6 and 24 hours (n=9, triplicate samples from 3 individual experiments). All mRNA levels were normalized to control RNA pol II transcripts. Results at each time point are compared to Contol values, which are arbitrarily set to 100% (A) or 1 (B, C and D). Two-way ANOVA was used for multiple time point comparisons. Mean values for treatment groups were considered significantly different compared to Control alone if $p<0.05$; $p<0.05$*, $p<0.01$**, and $p<0.001$***. (* compared to Control group). *(Figure 12A was reproduced and modified with permission from DNA and Cell Biology [138])*

4.2.2 The effect of 1 μM doxorubicin on FGF-16 mRNA stability using actinomycin D to inhibit general RNA transcription (in vitro)

Total RNA levels are determined by the rate of mRNA synthesis and degradation [218]. Thus, doxorubicin could target either FGF-16 mRNA synthesis or degradation or both to exert early and rapid downregulation of FGF-16 mRNA levels. To assess a possible effect of doxorubicin on FGF-16 RNA stability, neonatal rat cardiac myocytes were treated with 2 μg/ml of the transcription inhibitor actinomycin D for 15 minutes, before 1 μM doxorubicin treatment, and assessment of FGF-16 mRNA levels by qPCR at 0.5, 1, 1.5, 2 and 2.5 hours [138].

As expected, FGF-16 mRNA levels were decreased in response to actinomycin D treatment and time. The time for FGF-16 mRNA to reach 50% of its starting amount in the actinomycin D with and without DOX treated group is ~1.75 hours (**Figure 13**) [138]. However, there was no significant effect of doxorubicin treatment on the rate or pattern of FGF-16 mRNA degradation [138]. This indicates that the decrease in FGF-16 mRNA levels with doxorubicin treatment is exerted on mRNA synthesis at the transcriptional level [138]. Thus, inhibition of RNA synthesis by doxorubicin combined with the relative short half-life of FGF-16 transcripts, contributes to the rapid decrease in FGF-16 mRNA levels detected as early as 2 hours post treatment (**Figure 13**) [138].

Figure 13. Doxorubicin treatment has no significant effect on FGF-16 mRNA degradation



To assess FGF-16 mRNA stability, neonatal rat cardiac myocyte cultures were treated with 2 µg/mL actinomycin D (ActD) for 15 min before 1 µM DOX treatment, and then FGF-16 mRNA levels were assessed by qPCR at the time points 0.5, 1, 1.5, 2 and 2.5 hours (n=9, triplicate samples from 3 individual experiments). The measured values are FGF-16 mRNA levels that were normalized relative to RNA Pol II mRNA levels. RNA Pol II mRNA levels were unchanged with the treatment. A vertical arrow indicates the estimated FGF-16 mRNA half-life. Results at each time point are expressed as a percentage of zero time point values, which are arbitrarily set to 100%. Two-way ANOVA was used for multiple time point comparisons. Mean values for treatment groups were not significantly different compared to ActD ($p>0.05$). *(Reproduced with permission from DNA and Cell Biology* [138]*)*

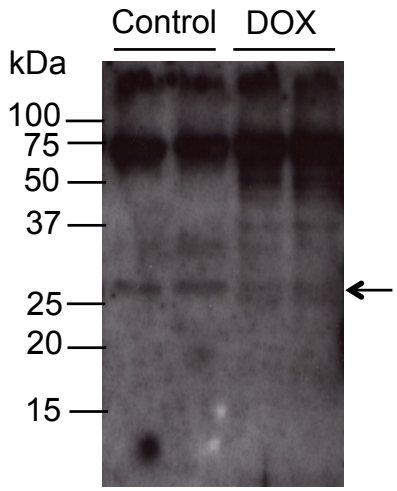
4.2.3 Doxorubicin treatment decreases FGF-16 protein levels in the neonatal rat cardiac myocyte culture medium within 24 hours (*in vitro*)

FGF-16 is secreted into the medium by cardiac myocytes in culture [79, 138]. Thus, the effect of 1 μM doxorubicin on FGF-16 protein levels in the medium was also assessed by heparin extraction, gel electrophoresis and immunoblotting using antibodies to FGF-16 (Abcam). These antibodies were characterized for their ability to detect human recombinant FGF-16 versus highly related (~73%) and fellow FGF subgroup member FGF-9 by immunoblotting, as well as FGF-16 overexpressed in cardiac myocytes, as described previously for three independent FGF-16 antibody preparations (Ab60, Ab61 and AbPeprotech) [79, 137, 138].

The ~26.5 kDa band is consistent with the size and mobility reported for glycosylated and secreted FGF-16 [79, 105, 137, 138]. In contrast to the low mobility bands detected, a decrease in the intensity of the 26.5 kDa FGF-16 band was seen after treatment with 1 μM doxorubicin for 24 hours (arrow) [138], and thus would be consistent with the decrease in FGF-16 mRNA levels observed with doxorubicin treatment (**Figure 12**). However, multiple low mobility FGF-16-related bands were also observed, including the ~45 kDa dimer. The 60-90 kDa bands are presumably due to non-specific binding resulting from the heparin extraction and no significant change was seen with the 60-90 kDa protein bands. However, an increase in intensity of the ~45 kDa band was seen with doxorubicin treatment. Regulated protein dimerization could be important for protein signaling and degradation due to doxorubicin-induced cell death and cell lysis, and leaked to the cell culture medium [219]. Both FGF-1 and FGF-2 were shown to form non-covalent dimers and

oligomers *in vitro* [220]. In addition, heparin modulates dimerization and signaling of FGFs, including FGF-9 and FGF-20 [139, 221]. Human recombinant FGF-16 also appears to include both monomer and dimer isoforms as well [137]. Doxorubicin induces cardiac myocyte death and thus, protein degradation during injury [222]. Thus, FGF-16 protein dimerization could also be due to protein aggreation that is leading to degradation by doxorubicin treatement. This pattern of protein detection by immunoblotting is consistent throughout the thesis.

Figure 14. Doxorubicin decreases FGF-16 protein levels in the neonatal rat cardiac myocyte culture medium



Secreted FGF-16 protein signal intensity in the neonatal rat cardiac myocyte culture medium after 1 μM doxorubicin (DOX) or vehicle (Control) for 24 hours was assessed by heparin sepharose extraction and then immunoblotting using specific FGF-16 antibodies [137] (n=4, duplicate samples from 2 individual experiments). Horizontal arrow indicates the protein band of ~26.5 kDa that is consistent with the reported size of glycosylated and secreted FGF-16 [79, 105, 137, 138]. *(Reproduced with permission from DNA and Cell Biology [138])*

4.3 The negative effect of doxorubicin on *Fgf-16* expression is mediated by the cardiac transcription factor Csx/Nkx2.5

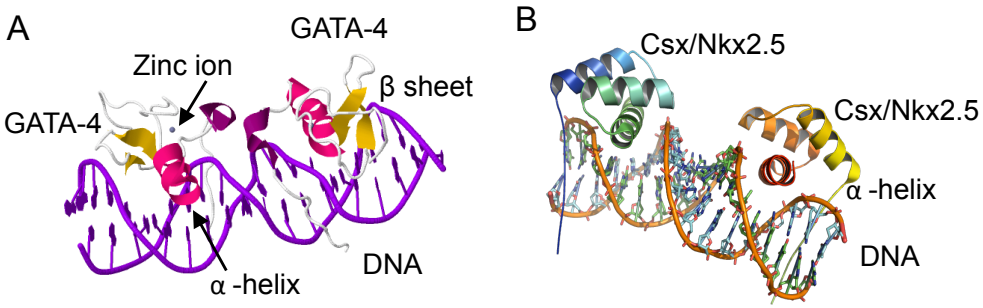
Rationale: The selective effect of doxorubicin on FGF-16 transcripts suggests specificity and raises the possibility that the mechanism for postnatal cardiac-specific *Fgf-16* expression is targeted [79, 123]. It is reported that doxorubicin treatment selectively decreases the level of muscle-specific genes, including cardiac alpha-actin, troponin I, myosin light chain 2, but not the non-muscle genes such as beta-actin and pyruvate kinase [25]. In addition, cardiac transcription factors GATA4, Csx/Nkx2.5, and MEF2C are all rapidly depleted by doxorubicin treatment in cultured cardiac myocytes [150]. Assessment of FGF-16 gene promoter sequences suggests a conservation of putative responsive DNA elements for GATA4 in an intron and Csx/Nkx2.5 in the promoter region of human, rat and mouse *Fgf-16* sequences (**Figures 6** and **7**). GATA4 and Csx/Nkx2.5 are known to act independently but also synergistically by binding to the major groove on DNA to regulate gene expression (**Figure 15**) [223]. FGF-16 mRNA levels are decreased in a GATA4-ablated mouse model and Csx/Nkx2.5 is known to regulate GATA4 gene expression via a Csx/Nkx2.5 site in the proximal promoter region in the early stages of cardiomyogenesis[224]. Like the GATA4 gene, there is a conserved putative Csx/Nkx2.5 site adjacent to the TATA1 sequence in the proximal promoter region of *Fgf-16* [105, 224]. A specific role for Csx/Nkx2.5 in the efficient expression of *Fgf-16* is also supported by the decrease in FOXP1 levels in the postnatal heart [225]. FOXP1 is a known repressor of Csx/Nkx2.5, and the associated increase in Csx/Nkx2.5 levels in the postnatal heart correlates with the increase in FGF-16 levels in the postnatal myocardium [105, 133]. Thus, Csx/Nkx2.5 may act

directly or indirectly (via GATA4) in the postnatal regulation of *Fgf-16*. By extension, if Csx/Nkx2.5 is required for efficient FGF-16 expression, then decreased Csx/Nkx2.5 availability, for example through doxorubicin treatment [181], would be expected to decrease *Fgf-16* expression. However, no direct involvement of Csx/Nkx2.5 in *Fgf-16* gene expression had been reported and was, thus, pursued. It was ***predicted*** that GATA4 and/or Csx/Nkx2.5 regulates *Fgf-16* expression and that a reduction in GATA4 and Csx/Nkx2.5 levels, as in response to doxorubicin, will result in less binding to the proximal *Fgf-16* regulatory region (promoter or intron) and decrease FGF-16 mRNA levels. *Thus, the* ***objective*** *was to identify candidate cardiac transcription factors in the regulation of Fgf-16 expression by doxorubicin.*

Approaches**:** Regulation of FGF-16 by cardiac transcription factors GATA4 and Csx/Nkx2.5 was investigated using adenoviral overexpression. The luciferase reporter gene assay was used to assess the effect of doxorubicin on *Fgf-16* promoter activity [138]. Four different lengths of the FGF-16 promoter-hybrid luciferase constructs (6, 3.6, 1.2 and 0.75kb FGF-16 p.Luc) were described previously and used to identify the shortest length that was responsive to doxorubicin treatment in transfected neonatal rat cardiac myocyte cultures (**Figure 17A**) [119]. Putative responsive elements were predicted using MatInspector software from Genomatix. The level of candidate transcription factors as well as evidence of DNA binding (protein-DNA interaction) with or without doxorubicin treatment *in vitro* and *in situ*, was assessed using electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay, respectively. Finally, siRNA "knockdown" and adenoviral-mediated overexpression was done to validate the effect of a change in levels of a candidate transcription factor, identified through these studies. Unless stated otherwise in

the figure legend, the experimental design, generation, analysis and interpretation of experimental data were done by Jie Wang.

Figure 15. GATA4 and Csx/Nkx2.5 protein-DNA binding structure



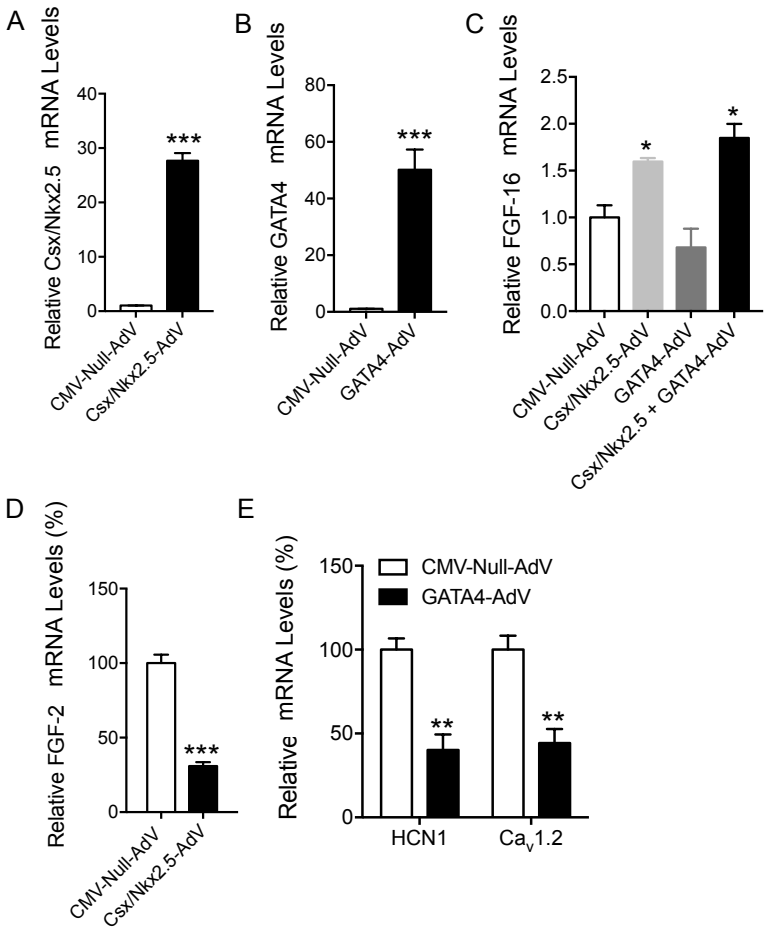
Crystallographic structure of GATA4 [226] (A) and Csx/Nkx2.5[227] (B) protein dimer binding to DNA double helix were obtained from Protein Data Bank (ID: 3RKQ and 3DFV) . (A) Zinc finger transcription factor GATA4 has a small protein structural motif consisting of an α helix and an antiparallel β sheet structure that is characterized by the coordination of one or more zinc ions in order to stabilize the protein folding and binding to the DNA major groove [226]. (B) Homeobox transcription factor Csx/Nkx2.5 has a small protein structural motif consisting of a helix-turn-helix structure with two α helices joined by a short strand of amino acids (turn) that binds to the DNA major groove [228].

Results:

4.3.1 FGF-16 mRNA levels are upregulated by adenoviral overexpression of Csx/Nkx2.5 but not GATA4

Neonatal rat cardiac myocytes were transfected with adenovirus (10 MOI) expressing human Csx/Nkx2.5 (Csx/Nkx2.5-AdV), rat GATA4 (GATA-AdV) or a null adenovirus under the control of the cytomegalovirus (CMV-Null-AdV) promoter for 1 hour [138]. The culture medium was changed and cells were maintained for 48 hours. Increased levels of Csx/Nkx2.5 and GATA4 gene expression were confirmed (**Figures 16A** and **B**) [138]. Overexpression of Csx/Nkx2.5 significantly and specifically increased FGF-16 mRNA levels as, by comparison, FGF-2 transcripts were decreased (**Figures 16C** and **D**) [138]. There was also no effect of GATA4 overexpression alone or when combined with Csx/Nkx2.5 overexpression as, in the case of the latter, FGF-16 mRNA levels were not significantly different compared to Csx/Nkx2.5 overexpression alone (**Figure 16C**) [138]. By contrast, overexpression of GATA4 did significantly down regulate two known GATA4 target mRNAs as reported by others [229]; specifically, potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1) and calcium channel, voltage–dependent, L type, alpha 1C subunit (Ca_v 1.2) mRNA levels (**Figure 16E**) [138].

Figure 16. Overexpression of Csx/Nkx2.5 but not GATA4 upregulates FGF-16 mRNA levels in neonatal rat cardiac myocytes

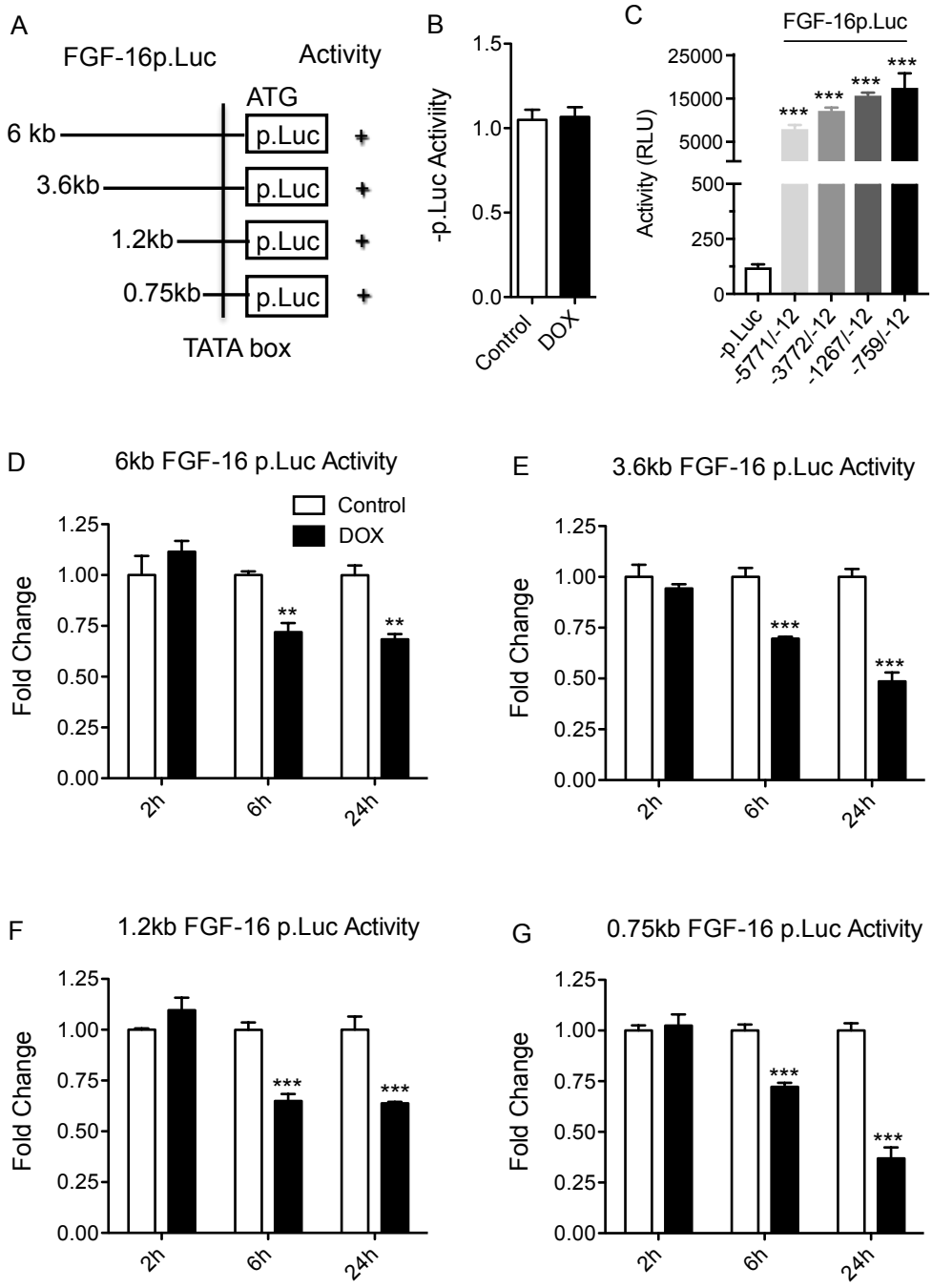


Neonatal rat cardiac myocytes were transduced with 10 MOI CMV-Null-AdV, Csx/Nkx2.5-AdV and GATA4-AdV for 48 hours. The mRNA level of (A) Csx/Nkx2.5 and (B) GATA4 was assessed by qPCR to validate or confirm overexpression. The effects of Csx/Nkx2.5-AdV and/or GATA4 on FGF-16 mRNA levels were then assessed (C). The effect of Csx/Nkx2.5-AdV on (D) FGF-2, and GATA4-AdV overexpression on (E) HCN1 and $Ca_V1.2$ mRNA levels were all also assessed by qPCR. All mRNA levels were normalized to control RNA pol II transcript levels. Results are expressed as fold change (A-C) or percentage (D and E) of CMV-Null-AdV values, which are arbitrarily set to 1 or 100%. Unpaired t-test was applied for (A, B, and D); one-way ANOVA was used for (C); and two-way ANOVA was used for (D and E). Mean values were considered significantly different if $p<0.05$; $p<0.05$*, $p<0.01$**, and $p<0.001$***. * compared to CMV-Null-AdV. Triplicate samples from 3 individual experiments (n=9) were used. *(Reproduced with permission from DNA and Cell Biology [138]).*

4.3.2 Doxorubicin treatment decreases FGF-16 promoter activity

Hybrid firefly luciferase (Luc) genes driven by ~6 to 0.75 kb of mouse *Fgf-16* promoter sequences were used to assess the effect of doxorubicin on promoter (p) activity in transfected neonatal rat cardiac myocytes [138]. A promoterless (-p) Luc gene was also assessed, and all Luc genes were co-transfected with a *Renilla* (R)-Luc gene as a control for DNA uptake (**Figure 17B**) [138]. Transfections were done for 18 hours, and then cells were exposed to defined medium containing 1 μM doxorubicin for 2, 6 or 24 hours, before luciferase activity (Luc/R-Luc.) was measured [138]. No significant effect of 1 μM doxorubicin on -p.Luc activity was observed within 24 hours (**Figure 17B**) [138]. All FGF-16p.Luc genes generated activity that was significantly greater than -p.Luc activity after 18 hours transfection (**Figure 17C**) [138].Similarly, no effect of doxorubicin on all four FGF-16 p.Luc gene expression were observed at 2 hours [138]. However, luciferase activity for all four FGF-16 p.Luc genes was decreased significantly after 6 hours doxorubicin treatment, and a continued decrease was seen at 24 hours compared to controls (**Figures 17D, E, F** and **G**) [138]. The responsiveness of FGF-16 p.Luc to doxorubicin further indicates a potential regulatory region on the FGF-16 promoter that is linked to *Fgf-16* expression by doxorubicin [138].

Figure 17. Doxorubicin treatment decreases hybrid *Fgf-16* promoter/firefly luciferase (Luc) reporter gene activity



(A) Comparison of -5771/-12, -3772/-12, -1267/-12 and -759/-12 mouse (m) FGF-16p.Luc gene activity with a (B and C) promoterless Luc (-p.Luc) gene after transient transfection of neonatal rat cardiac myocytes (n=3, triplicate samples). (C) All p.Luc activity is measured as relative light units (RLU; firefly Luc/Renilla Luc). In addition, the effect of 1 μM doxorubicin (DOX) or vehicle (Control) on Luc gene activity with: (B) -p.Luc or (D) -5771/-12 mFGF-16p.Luc, (E) -3772/-12 mFGF-16p.Luc, (F) -1267/-12 mFGF-16p.Luc and (G) -759/-12 FGF-16p.Luc that all include the TATA1 box region was assessed at 2, 6 or 24 hours (h) in transfected neonatal cardiac myocytes. Results are expressed as a percentage of Control values for each Luc gene, which are arbitrarily set to 1. One-way ANOVA was used for multiple treatment comparisons to –p.Luc (A), and Unpaired t-test was applied for single comparisons (B and C). Two-way ANOVA was used for multiple (treatment/time) comparisons (D, E, F and G). DOX values were considered significantly different compared to control if $p<0.05$; $p<0.05$*, $p<0.01$**, and $p<0.001$***. (* compared to –p.Luc (B and C) or Control (D, E, F and G). Triplicate samples from 3 individual experiments (n=9) were used for (D, E, F, and G). *(Reproduced with permission from DNA and Cell Biology* [138]*).* Contribution: Experimental procedure for Figure B and C were performed with technical support from Ms. Yan Jin. The experimental design, generation, analysis and interpretation of experimental data were done by Jie Wang.

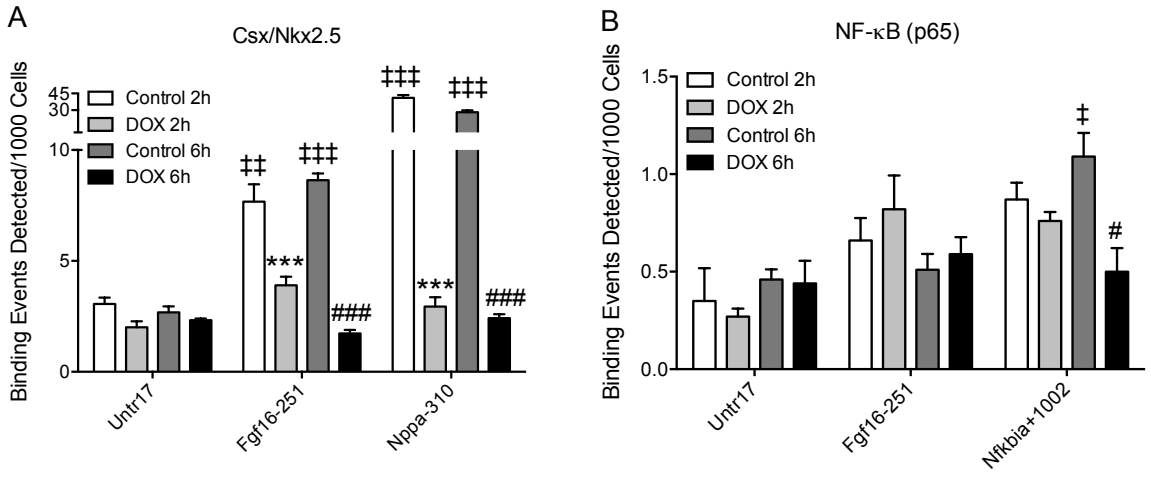
4.3.3 The rapid reduction in FGF-16 mRNA levels is associated with a doxorubicin-induced decrease in Csx/Nkx2.5 but not NF- κB binding at the *Fgf-16* proximal promoter region

The 0.75 kb mouse *Fgf-16* promoter region containing a single TATA box (TATA1) was sufficient to confer responsiveness (decreased activity) to doxorubicin treatment [105] (**Figure 17**). These sequences include a site for Csx/Nkx2.5 at nucleotide position -305/-299 (5′-TAAAGTG-3′), relative to the ATG start codon for rat and mouse FGF-16, where A is designated as nucleotide position +1 (**Figures 6** and **7**) [138]. This putative Csx/Nkx2.5 DNA element overlaps with TATA1 in the human, rat and mouse *Fgf-16* promoter (5′-taTaAAGTG-3′) (**Figure 8**) [138]. In addition, a conserved NF-κB binding site is located at nucleotide position -377/-368 (5′-AGGGGATGCC-3′) in rat, as well as equivalent human and mouse sequences [105, 119, 138].

To assess the effect of doxorubicin treatment on Csx/Nkx2.5 and NF-κB (p65) association with the FGF-16 TATA1 box region *in situ,* a ChIP assay was performed (**Figures 18A** and **B**) [138]. Chromatin from neonatal rat cardiac myocytes treated without or with 1 μM doxorubicin for 2 and 6 hours were crosslinked, fragmented (~500 bp) and immunoprecipitated with specific antibodies to Csx/Nkx2.5 and NF-κB (p65) [138]. Specific PCR primers spanning nucleotides -396/-315 of *Fgf-16* were used to detect fragments associated with Csx/Nkx2.5 and NF-κB (p65) binding in the proximal promoter (TATA1 at nucleotide -307) region by qPCR (**Table 2**) [138]. An untranslated (Untr17) region was used as the non-specific binding negative control. In addition, primers for Nppa-310 and Nfkbia +1002 gene sequences containing known-binding sites for Csx/Nkx2.5 and NF-κB (p65),

respectively, were also used as positive controls (**Table 2**). Significant Csx/Nkx2.5 binding above background (Untr17) levels was seen with both Nppa-310 and FGF-16 sequences at both 2 and 6 hours (**Figure 18A**) [138]. A significant decrease in Csx/Nkx2.5 binding to Nppa-310 and FGF-16 sequences was observed at 2 hours with doxorubicin treatment, and this was still reduced at 6 hours (**Figure 18A**) [138]. By contrast, no significant binding of NF-κB (p65) to *Fgf-16* sequences was detected in either control or doxorubicin-treated samples at 2 and 6 hours (**Figure 18B**) [138]. Significant NF-κB (p65) binding to Nfkbia+1002 sequences was seen in the control at 6 hours but was decreased with doxorubicin treatment (**Figure 18B**) [138]. No significant change was observed at 2 hours (**Figure 18B**) [138].

Figure 18. Doxorubicin decreases Csx/Nkx2.5 protein association with the *Fgf-16* promoter assessed by chromatin precipitation (ChIP) assay

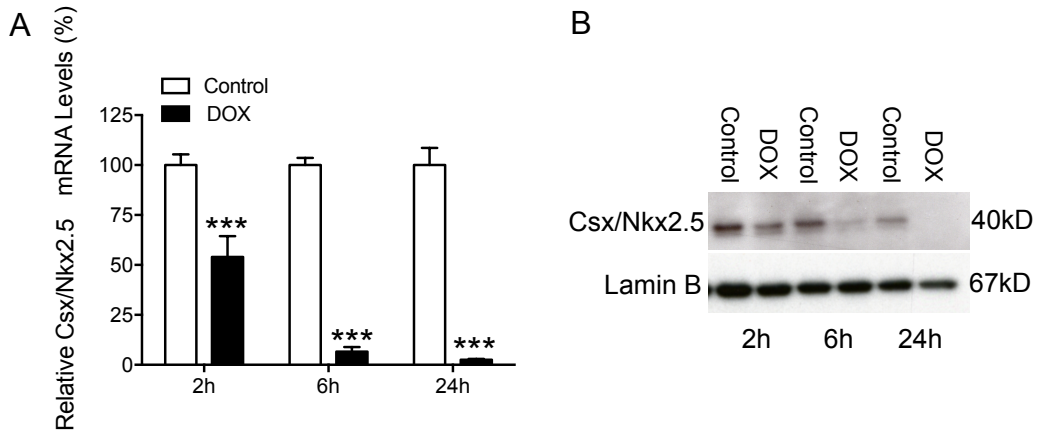


Association of Csx/Nkx2.5 and NF-κB (p65) with *Fgf-16* promoter sequences in neonatal rat cardiac myocytes treated with or without 1 μM DOX or Vehicle (Control) was assessed by ChIP *in situ*, using specific antibodies to (A) Csx/Nkx2.5 and (B) NF-κB, p65 [138] (n=3, triplicate samples). Untr17 was used as negative control for non-specific binding [138]. Nppa-310 and Nfkbia+1002 sequences containing Csx/Nkx2.5 and NF-κB binding sites, respectively, were targeted as positive controls for specific binding. Mean and standard error of the mean were determined, and results are compared to Control 2 hours (h) values [138]. Two-way ANOVA was used for multiple group/treatment comparison. Mean values were considered significantly different if $p<0.05$: $p<0.05$ */#/‡, $p<0.01$ **/‡‡, and $p<0.001$ ***/###/‡‡‡ (* compared to Control 2h; # compared to Control 6h, and ‡ compared to untr17) [138]. *(Reproduced with permission from DNA and Cell Biology [138]).* Contribution: Experimental procedure was performed by Active Motif (Carlsbad, CA, US). The experiment was designed by Jie Wang with technical support from Ms. Yan Jin. Data analysis and interpretation were done by Jie Wang.

4.3.4 Doxorubicin decreases Csx/Nkx2.5 mRNA and protein levels

To assess whether Csx/Nkx2.5 levels are affected under conditions where doxorubicin decreases *Fgf-16* gene expression, neonatal rat cardiac myocytes were treated with 1 μM doxorubicin for 2, 6 or 24 hours [138]. Csx/Nkx2.5 mRNA was then assessed by qPCR, and the effect on protein levels was assessed by protein immunoblotting using specific antibodies to Csx/Nkx2.5 (**Figure 19A and B**) [138]. Csx/Nkx2.5 mRNA levels were significantly decreased at 2 hours, and further decreases were seen until 24 hours (**Figure 19A**) [138]. A corresponding decrease in Csx/Nkx2.5 protein is also shown in response to doxorubicin treatment (**Figure 19B**) [138]. This indicates both Csx/Nkx2.5 levels and its binding to FGF-16 promoter *in situ* are affected by doxorubicin treatment and correlates with endogenous FGF-16 mRNA levels with doxorubicin treatment [138].

Figure 19. Doxorubicin decreases cardiac transcription factor Csx/Nkx2.5 levels

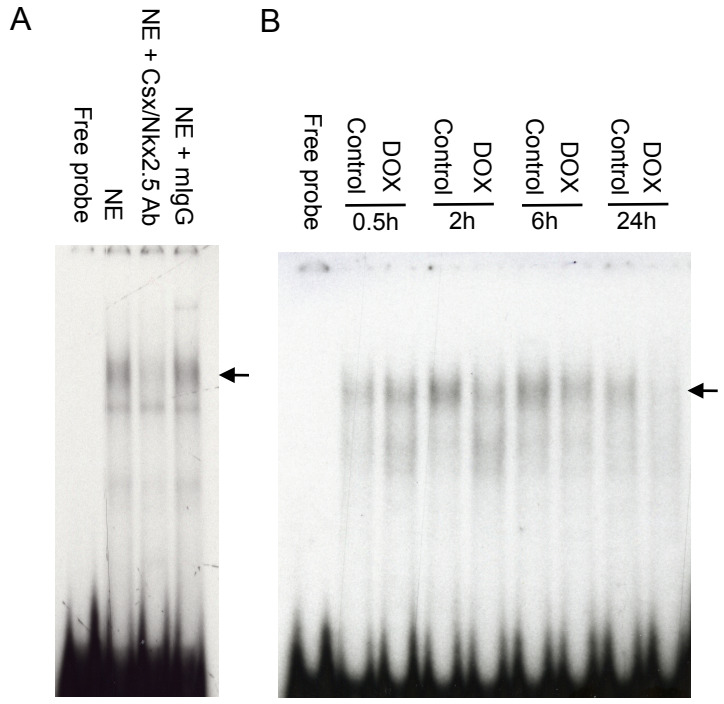


To assess the effect of 1μM doxorubicin (DOX) or vehicle (Control) on Csx/Nkx2.5 levels, neonatal rat cardiac myocytes were treated with DOX for 2, 6 and 24 hours (h), then Csx/Nkx2.5 RNA and nuclear protein were assessed by (A) qPCR and (B) immunoblotting, respectively [138]. All mRNA levels were normalized to control gene RNA pol II transcript levels detected. Mean and standard error of the mean were determined, and results are expressed relative to Control values, which are arbitrarily set to 100% (A) [138]. Two-way ANOVA was used for multiple group/treatment comparison [138]. Mean values were considered significantly different if $p<0.05$; $p<0.05$*, $p<0.01$**, and $p<0.001$***. Triplicate samples from 3 individual experiments (n=9) were used for (A), and triplicate samples (n=3) was used for (B). *(Reproduced with permission from DNA and Cell Biology [138])*

4.3.5 The rapid reduction in FGF-16 mRNA levels is associated with a doxorubicin-induced decrease in Csx/Nkx2.5 protein binding to *Fgf-16* proximal promoter sequences

The DNA sequence containing the putative Csx/Nkx2.5 protein binding site at the *Fgf-16* promoter was also assessed by EMSA using nuclear proteins and specific antibodies to Csx/Nkx2.5 (**Figures 20A** and **B**) [138]. A radiolabeled 20 bp oligonucleotide (-311/-292) containing the *Fgf-16* TATA1 box and Csx/Nkx2.5 DNA element was incubated with neonatal rat cardiac myocyte nuclear extract. A pattern of low mobility complexes was detected [138]. The lowest mobility complex was competed with a specific antibody to Csx/Nkx2.5, but not mouse immunoglobulins (**Figure 20A**) [138]. To assess the effect of doxorubicin on this binding, the same 20 bp oligonucleotide was combined with nuclear protein from neonatal rat cardiac myocytes treated without or with 1 μM doxorubicin for 0.5, 2, 6 or 24 hours [138]. Although no decrease was seen at 30 minutes, a reduction in low mobility complexes was observed after 2 hours doxorubicin treatment, and at all subsequent time points relative to untreated controls (**Figure 20B**) [138].

Figure 20. Doxorubicin decreases Csx/Nkx2.5 protein association with the *Fgf-16* promoter as assessed by electrophoretic mobility shift assay (EMSA)

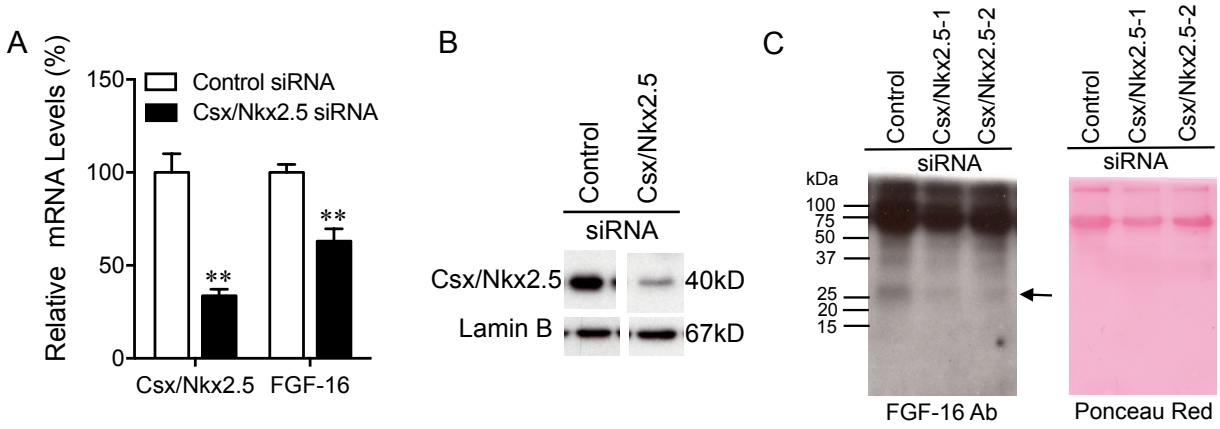


(A) Double-stranded oligonucleotide primers corresponding to nucleotides -311/-292, and containing the (mouse, rat, and human) *Fgf-16* TATA1 box and Csx/Nkx2.5 DNA element, were incubated with neonatal rat cardiac myocyte nuclear extract (NE) [138]. Specific competition for the protein-DNA complex (arrow) was included using Csx/Nkx2.5 antibodies and control mouse immunoglobulins (mIgG) [138]. (B) The effect of 1 μM doxorubicin (DOX) and Vehicle (Control) was also assessed using nuclear extract (NE) from cells treated with or without DOX for 0.5, 2, 6 or 24 hours (h) [138] (Representative blot using pooled samples from triplicates, experiments were done twice in total) . Experiments were reproduced with pooled samples from triplicates. *(Reproduced with permission from DNA and Cell Biology [138]).* Contribution: Experimental design and procedure was performed with the technical support from Ms. Yan Jin. Data analysis and interpretation were done by Jie Wang.

4.3.6 FGF-16 levels are reduced in response to Csx/Nkx2.5 small interfering (si) RNA knockdown

For knockdown, neonatal rat cardiac myocyte cultures were transfected with 50 nM of rat Csx/Nkx2.5 siRNA or a scrambled control siRNA for 72 hours [138]. A significant decrease in Csx/Nkx2.5 mRNA and protein levels was detected with Csx/Nkx2.5 siRNA relative to control siRNA (**Figures 21A** and **B**) [138]. A corresponding and significant 36.9% decrease in FGF-16 transcripts was also seen specifically with Csx/Nkx2.5 siRNA knockdown (**Figure 21A**). In addition, and consistent with a decrease in FGF-16 RNA levels, a decrease in FGF-16 protein in the culture medium is suggested after immunoblotting, including the ~26.5 kDa band associated with glycosylated and secreted FGF-16 protein (**Figure 21C**) [79, 105, 137, 138].

Figure 21. Csx/Nkx2.5 "knockdown" decreases FGF-16 levels

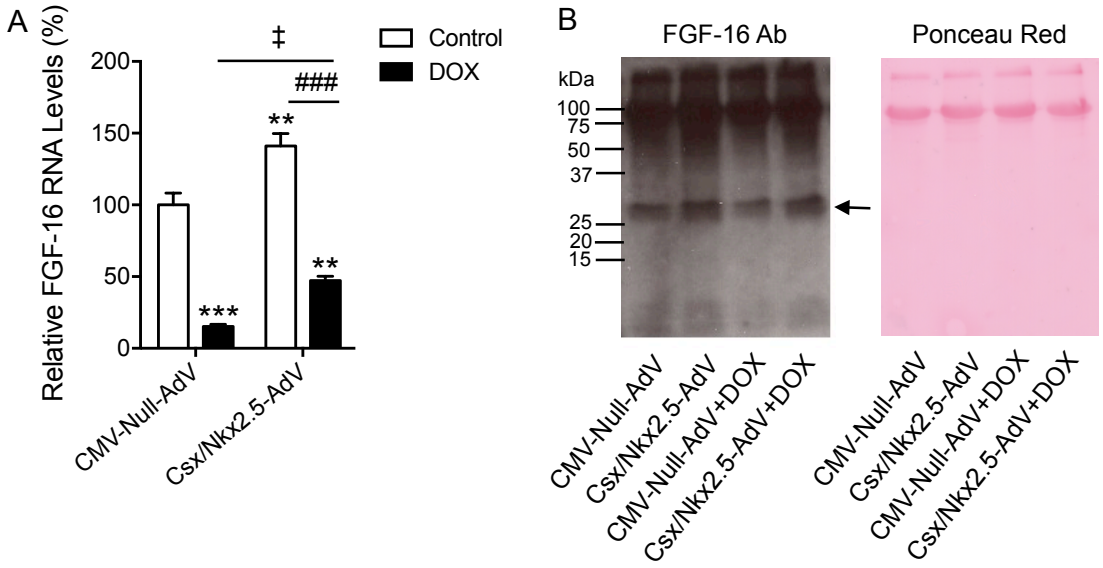


To assess the effect of endogenous Csx/Nkx2.5 on *Fgf-16* gene expression, cells were treated with 50 nM of rat Csx/Nkx2.5 siRNA or "scrambled" control siRNA for 72 hours. (A) Csx/Nkx2.5 and FGF-16 RNAs were assessed by qPCR (n=9, triplicate samples from 3 individual experiments). Csx/Nkx2.5 and control Lamin B protein levels in the nuclear extract (B), and FGF-16 protein levels (arrow) in the culture medium [137] (C) were assessed by electrophoresis and immunoblotting using FGF-16 antibodies [137] (n=3, triplicate samples). Ponceau Red staining of the transfer membrane was used as an indication of sample loading. The mean and standard error of the mean were determined, and results are expressed as the percentage (A) of Control values, which are arbitrarily set to 100%, as appropriate. Two-way ANOVA was used for multiple group/treatment comparison (A). Mean values were considered significantly different if * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. (* compared to control siRNA). *(Reproduced with permission from DNA and Cell Biology [138])*

4.3.7 Overexpression of Csx/Nkx2.5 partially rescues the negative effect of doxorubicin on FGF-16 mRNA levels

Rapid depletion of FGF-16 transcripts by doxorubicin is associated with decreased Csx/Nkx2.5 levels and evidence of reduced binding to the FGF-16 promoter [138]. Thus, the ability of increased Csx/Nkx2.5 expression to prevent the downregulation of endogenous FGF-16 levels in response to doxorubicin treatment was assessed [138]. Neonatal rat cardiac myocytes were transduced with 10 MOI adenovirus expressing Csx/Nkx2.5 or CMV-Null-AdV for 1 hour. The culture medium was changed and after 48 hours cells were treated with 1 μM doxorubicin for 12 hours, before isolation and assessment of FGF-16 mRNA by qPCR and protein by immunoblotting. Csx/Nkx2.5-AdV overexpression alone induced FGF-16 mRNA and protein levels significantly (**Figures 22A** and **B**) [138]. In addition, a significant and greater than 80% decrease in FGF-16 mRNA levels was seen with doxorubicin treatment of cells transduced with CMV-Null-AdV (**Figure 22A**) [138]. By contrast, the doxorubicin-induced decrease in FGF-16 transcripts in neonatal rat cardiac myocytes transfected with Csx/Nkx2.5-AdV were protected significantly by 20% (**Figure 22A**) [138]. Csx/Nkx2.5-AdV transfection before doxorubicin treatment also limited a doxorubicin-induced reduction in the glycosylated and secreted ~26.5 kDa FGF-16 protein band detected in the culture medium by immunoblotting [79, 105, 137, 138].

Figure 22. Overexpression of Csx/Nkx2.5 limits the negative effect of doxorubicin on FGF-16 levels



Neonatal rat cardiac myocytes were transfected with 10 MOI CMV-Null-AdV or Csx/Nkx2.5-AdV for 48 hours cells before treatment with 1 µM doxorubicin (DOX) or vehicle (Control) for 12 hours. (A) FGF-16 mRNA levels were assessed by qPCR (n=9, triplicate samples from 3 individual experiments). All mRNA expression levels were normalized to control gene RNA pol II. The mean and standard error of the mean were determined, and results are expressed as a percentage of Control values, which are arbitrarily set to 100%. Two-way ANOVA was used for multiple group/treatment comparison. Mean values were considered significantly different if $p<0.05$; ‡ $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (* compared to CMV-Null-AdV). (B) FGF-16-related protein in the culture medium was assessed by heparin extraction, electrophoresis, and immunoblotting using specific FGF-16 antibodies [137] (n=3, triplicate samples). The 26.5 kDa band linked to the glycosylated and secreted FGF-16 protein is indicated (arrow) [79, 105, 137, 138]. Ponceau Red staining of the transfer membrane was used as an indication of sample loading. *(Reproduced with permission from DNA and Cell Biology [138])*

Discussion:

The data show for the first time that FGF-16 mRNA levels are rapidly and negatively targeted by doxorubicin in the heart *in vivo* (Chapter 4.1) and neonatal cardiac myocyte cultures *in vitro* (Chapter 4.2) [138]. This is related, at least in part, to a decrease in availability and binding of cardiac transcription factor Csx/Nkx2.5 to the *Fgf-16* promoter, and specifically, TATA1-related sequences that are conserved between human and murine species (Chapter 4.3). Together, these observations suggest that FGF-16 is an early target of doxorubicin-induced cardiotoxicity

The significant decrease in endogenous FGF-16 mRNA levels was seen after 6 hours doxorubicin (i.p.) injection, when cardiac systolic dysfunction is prominent in the rat heart (*in vivo*), as indicated by a decrease in LVEF (**Figure 9)**. However, by 24 hours, both cardiac diastolic and systolic function in the rat heart (*in vivo*) were decreased, as indicated by a further decrease in LVEF and E′/A′, and a significant increase/worsening in both contractility (IVCT) and diastolic function (IVRT), as well as myocardial performance index (LV MPI IV) [230] (**Figure 10**). This may be due to the increased stiffness and impaired relaxation of the left ventricular wall [230]. At this stage, discontinuing chemotherapy and potentially treatment for these cardiovascular side-effects with drugs like beta-blockers, may be effective in preventing or protecting doxorubicin-induced acute cardiotoxicity [231]. However, the symptoms of diastolic dysfunction at this acute stage with a preserved ejection fraction are easily missed under current clinical practice [231]. Thus, by the time LVEF shows any signs of declining, the damage to the heart may have already progressed into heart failure, which would be irreversible [232]. Furthermore, although a significant

decrease in LVEF is observed in the model used here, it is above 59%, which is still within the normal range (55-70%). This suggests the possibility of an early stage of acute diastolic heart failure with preserved ejection fraction (HFpEF) induced by doxorubicin injury [230]. However, the specific diagnosis of heart failure is unclear due to lack of signs or symptoms of heart failure within 24 hours doxorubicin injection.

FGF-16 RNA levels showed the same pattern of response to doxorubicin in both *in vitro* culture and the *in vivo* heart model, however, differences in the level of reduction were observed (**Figure 11** and **12**). A possible explanation is that this is related to the "closed nature" (constant exposure to doxorubicin) of the culture system, versus the *in vivo* adult rat heart model where doxorubicin is expected to be metabolized and eliminated from the body gradually [233]. Use of actinomycin D to inhibit transcription before doxorubicin treatment revealed that FGF-16 has a relatively short “half-life” of ~1.75 hours (105 minutes), which is the earliest time point that the effect of doxorubicin on mRNA levels was assessed (**Figure 13**). Estimates do exist for the median mRNA half-life, including 315 minutes in human B-cells and 274 minutes in murine fibroblasts, among 8000 genes tested [234]. Thus, the ~1.75 hours half life of FGF-16 mRNA combined with the decrease in *Fgf-16* transcription could also contribute to the reduction in secreted FGF-16 by doxorubicin (**Figure 12-14**) [234]. However, the exact consequences of a short mRNA half-life in terms of function are still poorly understood [234].

The lack on an effect of doxorubicin on transcript stability with actinomycin D inhibition also supports an effect of doxorubicin on FGF-16 mRNA synthesis but not degradation (**Figure 13**). The relatively short half-life for FGF-16 transcripts and the

inhibition of FGF-16 mRNA synthesis by doxorubicin detected at 6 hours, make FGF-16 an early target of doxorubicin treatment in the heart. This can be compared to ANP mRNA levels, which were unchanged at 6 hours but significantly increased at 24 hours, consistent with the *in vivo* rat heart study (**Figure 11 and 12)**. ANP is primarily expressed by atrial cardiac myocytes, and increased ANP mRNA levels are an important biomarker of cardiac stress and injury [235]. For example, ANP mRNA levels increase under conditions of myocardial stretching due to pressure and volume overload in heart failure [235]. The apparent earlier response from FGF-16 compared to ANP mRNA at 6 hours, suggests that FGF-16 is perhaps a more sensitive marker to assess doxorubicin-induced acute cardiotoxicity.

Consistent data were obtained for the effect of doxorubicin on FGF-16 RNA levels in adult rat heart versus neonatal rat cardiac myocyte models, but this was not the case for FGF-2 and FGF-9 transcripts. In cardiac myocyte cultures, FGF-2 transcripts were decreased initially with doxorubicin treatment but this was followed by a significant increase (**Figure 12**). However, in adult rat hearts, FGF-2 RNA levels remain unchanged at the early 6-hour time point, although, like the *in vitro* cultures, FGF-2 transcripts were increased at 24 hours (**Figure 11)**. FGF-9 RNA levels were unchanged at 2 hours and increased by 24 hours in response to doxorubicin treatment in cardiac myocyte cultures but not in the rat heart model (**Figure 11** and **12**). FGF-16 is predominantly expressed by postnatal cardiac myocytes and is considered to be relatively "cardiac-specific" [79, 105]. By contrast, FGF-2 is widely expressed in prenatal and postnatal mammalian tissues, including both cardiac myocytes and fibroblasts [79, 236], and FGF-9, which is structurally more highly related to FGF-16, is expressed predominantly in the postnatal kidney and only at low levels in the heart [105, 237, 238]. Thus, the response of FGF-2 might be more complex, and

combine different effects of doxorubicin in different cardiac cell types that are all capable of expressing FGF-2 [239, 240]. Specifically, doxorubicin may have differential effects, even in terms of timing, on FGF-2 production in cardiac myocytes versus non-myocyte fibroblasts in the rat heart. For example, a decrease in FGF-2 transcripts could occur in cardiac myocytes with a corresponding increase in fibroblasts, resulting in no change in heart FGF-2 RNA levels detected at 6 hours in response to doxorubicin treatment. In terms of FGF-9, it is possible that the increase in FGF-9 transcripts was compensatory for the presumed decrease in structurally-related FGF-16 and/or FGF-2 expression during early doxorubicin treatment. FGF-9 transcripts returned to control levels with the increase and recovery of FGF-2 RNA levels.

The differential effect of doxorubicin on FGF-16 mRNA versus ANP, FGF-2 and FGF-9 transcripts, supports the notion that FGF-16 is targeted, perhaps related to its postnatal cardiac specificity, at least at this acute stage of doxorubicin treatment, and that this is not simply general inhibition of all transcription. Doxorubicin can intercalate between the DNA double helix and is usually considered a general inhibitor of DNA transcription and, as result can cause massive cellular damage [28, 214]. However, this decrease in mRNA levels is not seen with all genes in response to doxorubicin treatment within 24 hours. In addition, MDR1, MRP1/2 are part of the multidrug resistance system, and their transcripts are known to be upregulated with doxorubicin in a dose and time-dependent manner [26, 198, 241]. The finding that rat *Fgf-16* is specifically regulated by Csx/Nkx2.5, provides support for the notion that *Fgf-16* transcription is targeted acutely by doxorubicin as a result of its postnatal cardiac specificity (**Figure 16, 19, 21** and **22**). The cardiac homeodomain transcription factor Csx/Nkx2.5 is negatively targeted by doxorubicin [181].

Increasing and decreasing Csx/Nkx2.5 levels resulted in corresponding elevated and reduced FGF-16 mRNA levels, respectively (**Figure 21** and **22**). Activity of a truncated mouse 747 bp *Fgf-16* promoter region that contains a highly conserved Csx/Nkx2.5 site within the TATA1 region, but not GATA4 or MEF2 DNA elements, was reduced significantly in response to doxorubicin treatment (**Figure 17**). Furthermore, doxorubicin decreased Csx/Nkx2.5 availability and binding to *Fgf-16* promoter sequences containing TATA1 (**Figure 18** and **20)**, which is conserved in the human, rat and mouse *Fgf-16* promoters [105, 181, 242]. The result was a rapid decrease in FGF-16 mRNA synthesis within 2 hours and evidence of reduced release of FGF-16 at 24 hours (**Figure 12** and **14**).

These data do not exclude a role for additional transcription factors in the doxorubicin-induced decrease in *Fgf-16* expression, as Csx/Nkx2.5 overexpression was only able to partially prevent the negative effect of doxorubicin on FGF-16 mRNA levels (**Figure 22)**. GATA4 is a candidate, as selective knockdown of GATA4 expression in the neonatal mouse heart was associated with a decrease in FGF-16 production, suggesting that *Fgf-16* might be a direct target for GATA4 [86]. GATA4 is an important regulator of gene expression to maintain normal cardiac homeostatic remodeling in the unstressed adult heart by promoting cell survival and inhibiting programmed cell death [243]. With doxorubicin treatment, GATA4 is rapidly decreased in neonatal rat cardiac myocytes [86, 176]. However, unlike the stimulation seen with Csx/Nkx2.5, no increase in FGF-16 mRNA levels was detected with adenoviral overexpression of GATA4 (10 MOI), although mRNA levels of other reported targets of GATA4 were affected [229] (**Figure 16**). There was also no effect when the MOI was increased to 50 (data not shown). In addition, the 747 bp proximal mouse *Fgf-16* promoter region was responsive to doxorubicin but does not contain a

consensus GATA DNA element, although a GATA4 binding site has been described in the second intron of mouse *Fgf-16* [86]. Although this GATA4 site is conserved in the genome of mouse, rat and human species, it is possible that a DNA binding site is not required for GATA4 in the *Fgf-16* promoter region as GATA4 can also act as a co-activator of Csx/Nkx2.5 [244]. However, this was not supported by the results of combined adenoviral overexpression of GATA4 and Csx/Nkx2.5 in neonatal rat cardiac myocyte cultures (**Figure 16**). Specifically, there was no significant difference in the effect of GATA4 and Csx/Nkx2.5 versus Csx/Nkx2.5 overexpression alone on FGF-16 mRNA levels (**Figure 16**). One possible explanation is that Csx/Nkx2.5 is an upstream regulator of GATA4, based on a known binding site in the proximal GATA4 promoter [224]. Thus, GATA4 is not sufficient to rescue the doxorubicin-induced decrease in FGF-16 RNA levels. In addition, GATA4 was reported to antagonize transcription of Csx/Nkx2.5 [244]. Overexpression of GATA4 might have a negative feedback on Csx/Nkx2.5 transcription by interacting with other negative factors, such as CHF1/Hey2, a basic helix-loop-helix (bHLH) transcriptional repressor [245], and therefore a subsequent inhibitory effects *Fgf-16* expression and mRNA levels. Csx/Nkx2.5 is also expressed in the heart of GATA4 null mice, thus, Csx/Nkx2.5 might also play a role in the regulation of endogenous FGF-16 by GATA4 knockout and cryo-injury [86, 246, 247].

Although NF-κB is not cardiac-specific, the proximal *Fgf-16* promoter region does contain a NF-κB DNA element that is conserved between species and binds NF-κB (p50 and p65) *in situ* [119]. However, while the decrease in FGF-16 mRNA levels with doxorubicin treatment of neonatal rat cardiac myocytes corresponded with a decrease in Csx/Nkx2.5 binding at the promoter *in situ*, no change in NF-κB (p65) association was detected (**Figure**

18). In addition to the untranscribed gene desert region on rat chromosome 17 (Untr17) that was used as the negative control in ChIP assay, no effect on NF-κB (p65) protein binding to the *Fgf-16* promoter with doxorubicin treatment, also served as a negative control for non-specific binding in this study. While these data do not rule out any role for GATA4 or NF-κB, they are consistent with Csx/Nkx2.5 as a significant target to explain the negative effect of doxorubicin on *Fgf-16* promoter activity.

Csx/Nkx2.5 is downregulated by doxorubicin, and transgenic mice overexpressing a dominant negative form of Csx/Nkx2.5, as well as knockdown of Csx/Nkx2.5 in neonatal rat cardiac myocytes, result in reduced resistance to doxorubicin-induced cardiac myocyte apoptosis [181, 242]. Complementary to these observations, increasing Csx/Nkx2.5 availability was shown to protect cardiac myocytes from doxorubicin-induced apoptotic cell death [181, 242]. Doxorubicin affects a number of cardiac genes regulated by Csx/Nkx2.5 (e.g., α-actin, ANP, ankyrin repeat protein, and myocardin [152, 248 178]) as well as other transcription factors (e.g., GATA4 and MEF2C [180, 242]). This is consistent with negative effects of doxorubicin on the expression of multiple cardiac-specific genes [249]. It also helps to explain the cardiotoxicity of doxorubicin, and specifically, the damage done to heart structure and contractile function when compared to other tissues at an early stage. Thus, as a downstream target of Csx/Nkx2.5, this also raises the possibility that the rapid decrease in FGF-16 production contributes to acute injury observed in response to doxorubicin treatment. FGF family members have the potential to act as cell survival and even cardioprotective factors [26, 87, 88, 94, 96]. Certainly, FGF-16 "knockout" (C57BL/6) mouse hearts are less resistant to angiotensin II-induced hypertrophy and fibrosis, suggesting FGF-16 might help to maintain postnatal cardiac health [169]. If so, after an initial benefit from pre-

existing FGF-16, a decrease in endogenous FGF-16 availability in response to doxorubicin treatment is expected to reduce resistance to cardiac injury. This might include early negative effects on cardiac myocyte health and heart function, but also contribute to heart failure and death. Cardiac complications can happen at any time during doxorubicin treatment or even years after the last chemotherapy treatment session [28]. In some cases, injury may occur immediately after a single dose or course of doxorubicin therapy, resulting clinically in transient electrophysiological abnormalities within 24 hours, pericarditis, myocarditis syndrome or acute left ventricular failure [250, 251]. Thus, FGF-16 may act as an endogenous cardiac survival factor regulated by cardiac transcription factors to maintain a healthy myocardium under normal conditions. During doxorubicin-induced cardiac injury, FGF-16 was rapidly depleted and the ability to maintain a healthy myocardium was lost. As a result, maintaining the endogenous FGF-16 levels and/or providing exogenous FGF-16 through protein overexpression or supplementation would be beneficial.

Chapter 5: FGF-16 is an Endogenous and Exogenous Cardiac Survival Factor

5.1 The effect of FGF-16 on neonatal rat cardiac myocyte survival

***Rationale*:** FGF-16 is a cardiac FGF, which increases after birth and is a known target of NF-κB and Csx/Nkx2.5 [119, 138]. NF-κB is linked with ischemia-reperfusion injury, stress and cell death signaling pathways [252, 253]. Csx/Nkx2.5 is important for heart development, maintaining differentiated cardiac myocytes and in protecting heart from doxorubicin-induced cardiac injury [181]. This raises the possibility of a role for FGF-16 in cardiac myocyte homeostasis and a response to cardiac stress or injury in the postnatal heart. FGF-16 is specifically synthesized and secreted by cardiac myocytes after birth [79]. There is evidence from FGF-16 null C57BL/6 mice that FGF-16 offers resistance to hearts from angiotensin II stress-induced hypertrophy and fibrosis [169]. Thus, the rapid decrease in FGF-16 synthesis could compromise cardiac cell, including, cardiac myocyte, viability and/or function. However, there is no report on the effect of endogenous FGF-16 on cardiac myocyte survival and death. Identifying the effects of endogenous FGF-16 on cardiac myocyte maintenance and survival may help increase our understanding of acute doxorubicin-induced cardiotoxicity. It may allow identification of an endogenous factor or related signaling pathway as a target to detect or mitigate against doxorubicin-related damage.

It was ***predicted*** that FGF-16 is an endogenous postnatal cardiac myocyte maintenance and/or survival factor. A decrease in neonatal rat cardiac myocyte FGF-16 mRNA levels will increase cell damage and decrease resistance to doxorubicin treatment.

*Thus, the **objective** was to test the effect of FGF-16 siRNA "knockdown" on markers of cell death or injury in postnatal cardiac myocytes in culture.*

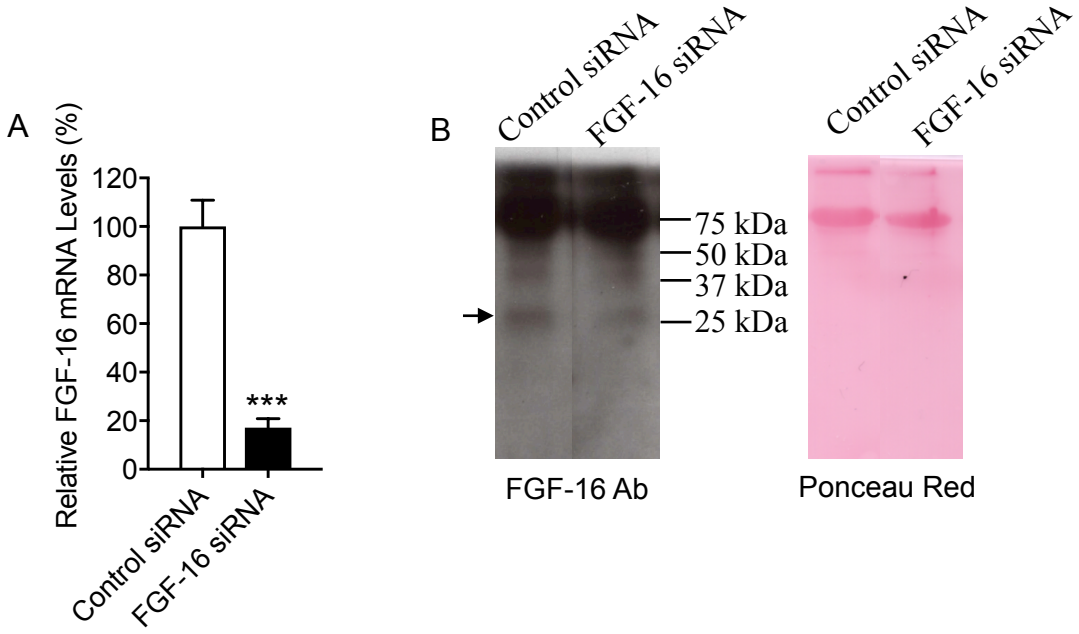
***Approaches*:** Endogenous FGF-16 levels were "knocked down" using siRNA transfection in neonatal rat cardiac myocytes. Apoptotic and necrotic cell death markers, Annexin-V and lactate dehydrogenase (LDH), respectively, were used to assess the outcome in terms of increased potential for programmed cell death and loss of plasma membrane integrity.

***Results*:**

5.1.1 FGF-16 levels were significantly decreased using FGF-16 siRNA transfection in neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes were transfected with 25 nM FGF-16 siRNA for 72 hours. A significant 84% decrease in endogenous FGF-16 mRNA levels was detected by qPCR (**Figure 23A**). The culture medium, following heparin extraction, was also assessed by immunoblotting for FGF-16. As expected, multiple low and high mobility bands were observed [79, 105, 137, 138] (**Figure 23B**). This included the high mobility ~26.5 kDa band linked to glycosylated and secreted FGF-16, and a decrease in this band was suggested with FGF-16 siRNA knockdown.

Figure 23. The effect of FGF-16 knockdown using siRNA on FGF-16 RNA and protein expression in neonatal rat cardiac myocytes

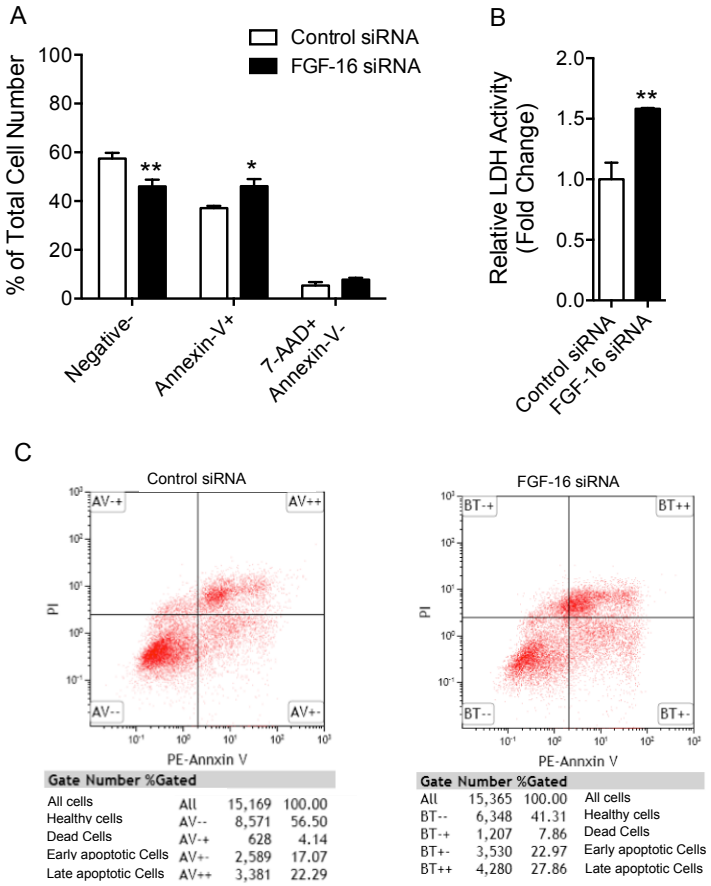


Neonatal rat cardiac myocytes were treated with 25 nM FGF-16 siRNA or control siRNA for 72 hours. (A) Endogenous FGF-16 mRNA was isolated and assessed by qPCR (n=9, triplicate samples from 3 individual experiments). (B) FGF-16 protein was extracted from the culture medium using heparin sepharose beads, and assessed by immunoblotting and antibodies to FGF-16 [137] (n=3, triplicate samples). Ponceau Red staining of the transfer membrane was used as an indication of sample loading. The 26.5 kDa band linked to the glycosylated and secreted FGF-16 protein is indicated (arrow) [79, 105, 137, 138]. Unpaired t-test was applied for single comparison (A). Mean values were considered significantly different if $p<0.05$; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (* compared to control siRNA).

5.1.2 "Knockdown" of FGF-16 using siRNA increases detection of markers for apoptosis and necrosis is neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes were transfected with 25 nM FGF-16 siRNA for 72 hours and then cells were assessed by flow cytometry within 1 hour of harvesting. Flow cytometry combined with markers for apoptosis (Annexin-V) and dead cells (7-AAD), revealed a significant decrease in "healthy" cardiac myocytes (7-AAD$^-$/Annexin-V$^-$), and a corresponding 24% increase in injured (Annexin-V$^+$) cells (**Figure 24A** and **C**). Although a significant increase in 7-AAD$^+$/Annexin-V$^-$ cells was not detected, a significant 1.6-fold increase in LDH release was observed (**Figure 24B**). This is consistent with increased cardiac myocyte membrane damage, and a positive effect of endogenous FGF-16 on maintaining cell viability.

Figure 24. FGF-16 helps maintain neonatal rat cardiac myocyte viability



Neonatal rat cardiac myocytes were transfected with FGF-16 siRNA and Control siRNA for 72 hours. (A) Flow cytometry was performed using apoptosis (Annexin-V) and dead cell (7-AAD) markers. Cells that are double negative for both Annexin-V and 7-AAD (unstained) were considered healthy, Annexin-V^+ as apoptotic (early apoptosis and late apoptosis combined), and 7-AAD^+/Annexin-V^- as dead cells. (B) LDH activity in the cardiac myocyte culture medium was assessed by spectrophotometry. Total cell number was set to 100% (A) and control siRNA group was set to 1 (B). Results were analyzed by two-way ANOVA with a *post-hoc* Bonferroni test in (A) and unpaired t-test in (B). Mean values were considered significantly different if $p<0.05$; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (* compared to control siRNA). (C) Representative flow cytometry data used in the preparation of (A). Cells that are double negative for both Annexin-V and 7-AAD negative (AV--and BT--) were considered healthy, Annexin-V^+ as apoptotic (early apoptosis, AV+- and BT+-; late apoptosis AV++ and BT++), and 7-AAD^+/Annexin-V^- (AV-+ and BT-+) as dead cells. Triplicate samples from 2 individual experiments were used (n=6).

5.2 The effect of FGF-16 overexpression on the resistance of neonatal rat cardiac myocytes to doxorubicin-induced cell death

Rationale*:** There is evidence from FGF-16 null (C57B/6) mice [114] and siRNA "knockdown" (**Figure 24**) of FGF-16 in neonatal rat cardiac myocytes that endogenous FGF-16 is important for cardiac myocyte maintenance and survival. *Fgf-16* expression and mRNA levels were rapidly and negatively targeted by doxorubicin within a few hours, and during a time when cardiac dysfunction was already detectable (**Figures 9, 11** and **12**). Doxorubicin appears to weaken the heart's own "self-defense" system, FGF-16. Thus, efforts to maintain or supplement FGF-16 levels might be beneficial [80, 86, 105]. FGF-16 protein is secreted and can act as an autocrine or paracrine signal by binding to FGFRs. Thus, FGF-16 supplementation or adenoviral expression are expected to have similar protective effects to endogenous FGF-16 [79]. It was ***predicted that FGF-16 is a cardiac myocyte survival factor that can also offer protection against doxorubicin-induced cardiac injury. Overexpression of FGF-16 in neonatal rat cardiac myocytes will increase resistance to doxorubicin-induced cell death. *Thus, the **objective** was to test whether increasing FGF-16 levels in cardiac myocytes before doxorubicin treatment can maintain or protect cardiac myocytes against doxorubicin-induced damage*

***Approaches*:** FGF-16 levels were increased in neonatal rat cardiac myocytes using an adenoviral vector with a cytomegalovirus promoter to drive FGF-16 cDNA expression [254]. Adenoviral gene delivery has been widely used in cardiac myocyte primary cultures with the infection efficiency as high as 100% for both neonatal and adult rat cardiac myocytes [255, 256]. FGF-16 was expected to be secreted and free to bind and signal via cell

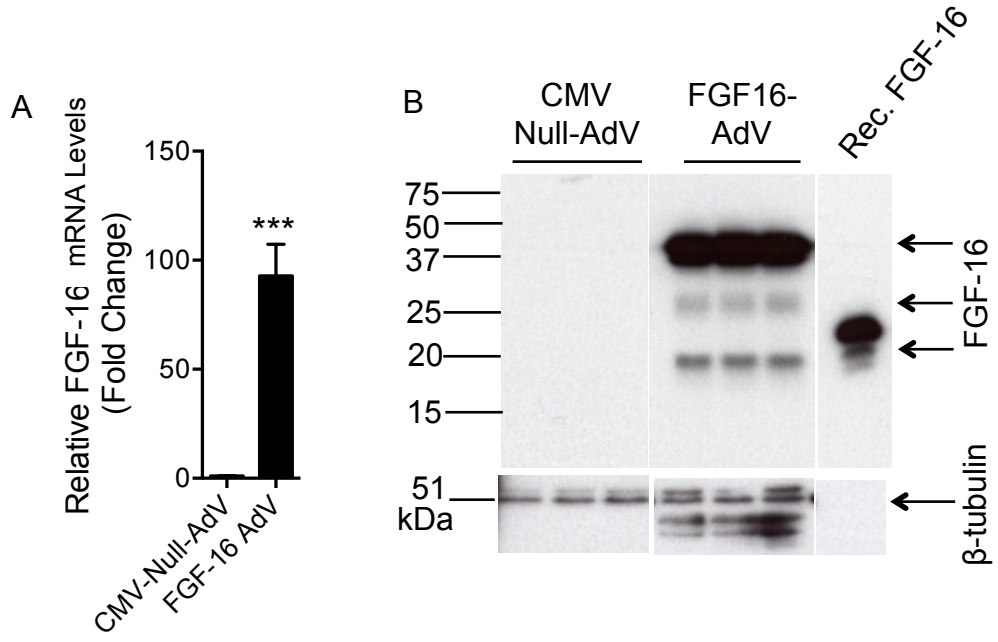
surface FGFRs. Apoptotic and necrotic cell death markers were used as before to assess the effect of FGF-16 overexpression on cell survival following doxorubicin treatment.

***Results*:**

5.2.1 FGF-16 levels are significantly increased following FGF-16 adenoviral transduction of neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes were transduced with 10 MOI FGF-16 adenovirus (FGF-16 AdV) or CMV-Null AdV (Control AdV) for 48 hours. A significant increase in FGF-16 mRNA was detected by qPCR in the FGF-16 AdV transduced group compared to CMV-Null-AdV (**Figure 25A**). Intracellular FGF-16 protein levels were also significantly increased following FGF-16 AdV versus CMV-Null-AdV transduction of neonatal rat cardiac myocytes, assessed by immunoblotting using FGF-16 antibodies (**Figure 25B**). Due to overexpression, no enrichment through heparin binding was required. The two most prominent bands detected in the intracellular protein sample were ~19.5 and ~45 kDa, consistent with non-glycosylated intracellular monomers and dimers (**Figure 25)** [79]. A minor ~26.5 kDa band was also seen and is consistent with the presence of the intracellular N-glycosylated and releasable FGF-16 isoform (**Figure 25B**) [79, 105, 137, 138]. No bands were detected in the CMV-Null-AdV transduced cardiac myocytes under identical immunoblotting conditions due to: 1) the relatively high intracellular concentration of FGF-16 protein in FGF-16 AdV overexpressed neonatal cardiac myocytes; and 2) FGF-16 is normally secreted and not stored by cardiac myocytes based on immunoblotting and immunohistochemistry data, and thus thus endogenous intracellular concentrations are low and enrichment of heparin binding proteins is normally required for detection [79, 137].

Figure 25. Overexpression of FGF-16 using adenoviral delivery in neonatal rat cardiac myocytes



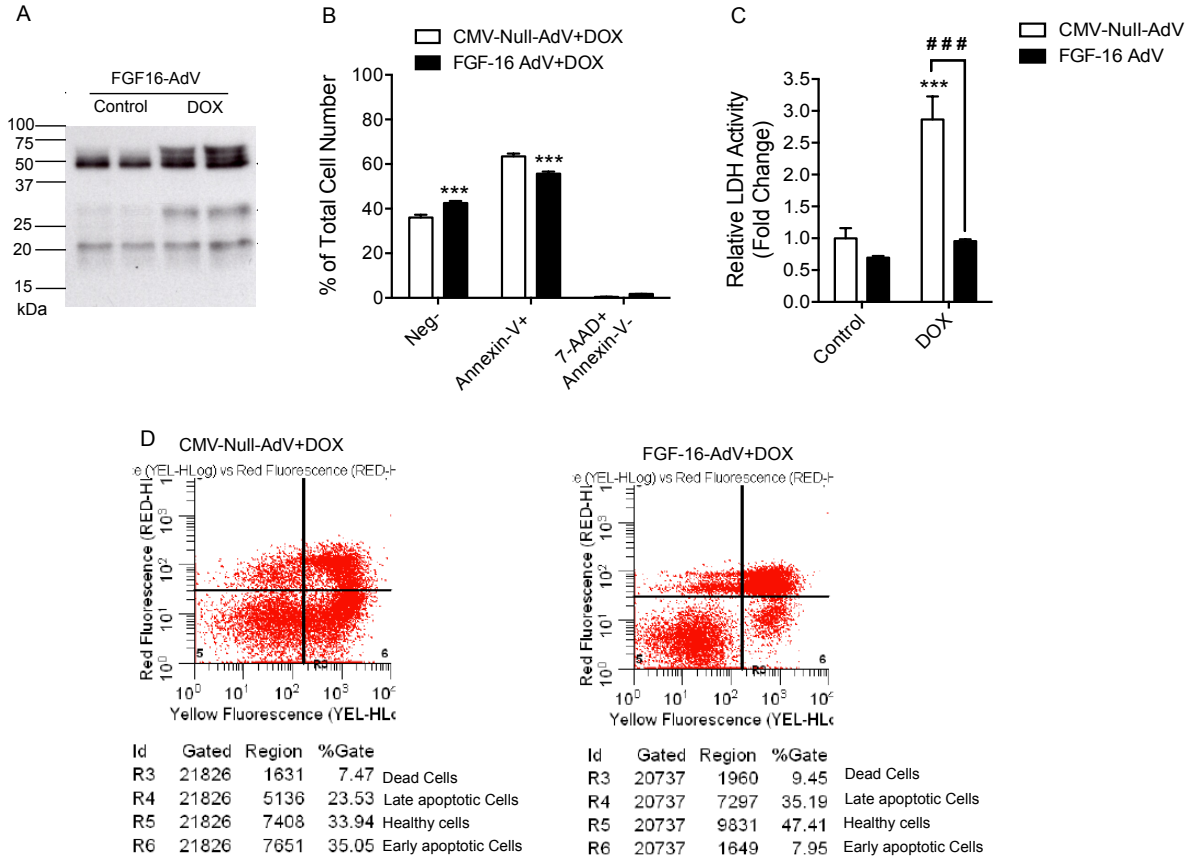
Neonatal rat cardiac myocytes were transduced with FGF-16-AdV or CMV-Null-AdV for 48 hours. (A) FGF-16 relative to RNA pol II transcripts were assessed by qPCR (n=9, triplicate samples from 3 individual experiments). The value for the CMV-Null-AdV transduced group was arbitrarily set to 1. An unpaired t-test was used for this comparison (n=3, triplicate samples). Mean values were considered significantly different if $p<0.05$; *** $p<0.001$. (* compared to CMV-Null-AdV). (B) Detection of FGF-16 in intracellular protein from transduced cardiac myocytes, as well as a recombinant human FGF-16 protein (non-glycolylated) sample by immunoblotting [137] (n=3, triplicate samples). Three bands were detected and the 26.5 kDa band linked to the glycosylated and secreted FGF-16 protein is indicated (arrow) [79, 105, 137, 138]. The 19.5 kDa (non-secreted, non-glycosylated and amino-terminal truncated) and 45 kDa (dimer) FGF-16 protein bands were also observed in the FGF-16-AdV overexpressed sample. Level of β-tubulin (~51 kDa) was assessed as a control for loading. Human recombinant FGF-16 protein showed two proteolytic FGF-16 products possibly due to protein degradation at sizes of 21 kDa and 18 kDa.

5.2.2 Overexpression of FGF-16 using adenovirus increases resistance to doxorubicin-induced apoptosis and necrosis in transduced neonatal rat cardiac myocytes

The effect of FGF-16 overexpression on doxorubicin-induced neonatal rat cardiac myocyte death was assessed using FGF-16-AdV transduction (**Figure 26)**. FGF-16 protein was extracted from the culture medium using heparin and assessed by immunoblotting using FGF-16 antibodies. As expected, both low (>45 kDa) and high (19.5 and 26.5 kDa) mobility bands were detected in the culture medium. The non-glycosylated 19.5 and possible dimer ~45kDa FGF-16 protein levels were unchanged with doxorubicin treatment in FGF-16-AdV overexpressed cardiac myocyte cultures (**Figure 26A**). However, levels of ~26.5 kDa FGF-16 (glycosylated and secreted FGF-16) as well as a band >50 kDa, presumably reflecting a glycosylated dimer, were increased with doxorubicin treatment compared to non-doxorubicin treated cells [79, 105, 137, 138] (**Figure 26A**).

The effect of FGF-16-AdV (10 MOI) overexpression on doxorubicin-induced neonatal cardiac myocyte damage was also assessed by flow cytometry using Annexin-V, a marker of apoptosis, and 7-AAD staining as a marker of dead cells, (**Figure 26B** and **D**), as well as LDH release/activity in the culture medium as an indication of plasma membrane damage (**Figure 26C**). FGF-16 overexpression before doxorubicin treatment resulted in a significant 12.2% reduction in Annexin-V^+ cells (**Figure 26B and D**) and 1.9-fold decrease in LDH activity compared to CMV-Null-AdV treated group (**Figure 26C**). These observations are consistent with the ability of FGF-16 to increase resistance of cardiac myocytes to doxorubicin-induced damage.

Figure 26. FGF-16 overexpression increases neonatal rat cardiac myocyte viability



Neonatal rat cardiac myocytes were treated with 10 MOI FGF-16-AdV and CMV-Null-Ad for 48 hours before being treated with 1 μM doxorubicin for 12 hours. (A) FGF-16 protein was extracted from the culture medium using heparin and assessed by immunoblotting using FGF-16 antibodies [137] (n=3, triplicate samples). All 19.5 kDa (non-glycosylated, non-secreted and amino-terminal truncated), 26.5 kDa (glycosylated and secreted), and the ~45-50 kDa (dimer) FGF-16 protein bands were observed with FGF-16-AdV overexpression. In addition, the 26.5 kDa band increased in combination with DOX treatment. The effects of FGF-16 overexpression on cell death was assessed by: (B) flow cytometry using Annexin-V and 7-AAD staining as previously described in **Figure 24**; and (C) measuring LDH activity in the FGF-16-AdV conditioned culture medium versus CMV-Null-AdV medium (n=9, triplicate samples from 3 individual experiments). Total cell number was set to 100% (B) and CMV-Null-AdV group was set to 1 (C). Results were analyzed by two-way ANOVA with a *post-hoc* Bonferroni test. Mean values were considered significantly different if $p<0.05$; ***/### $p<0.001$. (* compared to CMV-Null-AdV+DOX in (B), and * to CMV-Null-AdVand # to CMV-Null-AdV+DOX in (C)). Neg-: Negative in both Annexin-V and 7-AAD staining. (D) Representative flow cytometry data used in the generation of (B). Cells that are double negative for both Annexin-V and 7-AAD (R5) were considered healthy, Annexin-V^+ as apoptotic (early apoptosis, R6; late apoptosis R4), and 7-AAD^+/Annexin-V^- (R3) as dead cells.

5.3 The effect of FGF-16 supplementation on doxorubicin-induced dysfunction in perfused isolated mouse hearts

***Rationale*:** FGF-16 overexpression is cardioprotective against DOX-induced cardiac myocyte death **(Figure 26).** Cardiac myocytes are the contractile units of the heart [257]. However, the effects of increased FGF-16 levels in cardiac function as well as the underlying mechanisms have not been reported. Using an isolated heart model and pretreatmented with a perfusate containing FGF-16 just prior to doxorubicin treatment, it allows us to assess a direct and immediate effect of FGF-16 supplementation on doxorubicin-induced cardiac dysfunction in a whole organ system.

The retrograde perfused isolated mammalian heart preparation was developed by Oscar Langendorff in 1895 [258]. It provides an *in vitro* isolated organ approach to investigate contractile force and other parameters of the heart under known physiological conditions without the neural and hormonal complications of an *in vivo*, whole animal experiment. In this model, the heart is isolated from the animal, a cannula is inserted into the ascending aorta and the heart is perfused in a retrograde direction with, most commonly, a nutrient-rich Krebs-Henseleit (or modified) solution. The perfusate can be supplemented with drugs or proteins for testing, and is oxygenated and normally delivered from a gravity fed reservoir. The retrograde perfusion causes pressure on the aortic valve, which closes and forces the solution into the coronary circulation, which exits through the coronary sinus into the right atrium. The heart continues to contract and parameters can be measured. In addition, this model was used previously in the laboratory to assess the effects of FGF-2 after transgenic overexpression of recombinant FGF-2 on contractile function for up to two

hours after ischemia-reperfusion injury [173].

Exposure of cardiac myocytes in culture and in hearts (*in vivo)* to chronic low levels of doxorubicin in the range of 0.1-2 μM is associated with injury [26, 259]. By contrast, higher concentrations of doxorubicin in the range of 10 μM to 0.5 mM have been required to see negative effects on cardiac function, including developed pressure, and even damage in isolated murine hearts [260-263]. While some effects have been reported with lower doses (2 μM) in isolated heart studies, this has not included an effect on developed pressure [260, 264]. Thus, in an effort to balance both dose and negative effect, 10 μM doxorubicin was used to induce dysfunction within the acute/two-hour time frame available.

It was ***predicted*** that FGF-16 is cardioprotective and that perfusion of an isolated mouse heart with FGF-16 prior to doxorubicin treatment will increase the maintenance of heart function and resistance to doxorubicin-induced cardiac dysfunction. *Thus, the* ***objective*** *was to test the ability of FGF-16 supplementation to protect against doxorubicin-induced cardiac dysfunction in isolated mouse hearts.*

Approaches**:** Isolated heart studies were done in collaboration with Dr. David Sontag [90]; Langendorff heart preparations were done by Dr. Sontag, while data analysis and graphing were done and the initial complete draft of the resulting manuscript generated by Jie Wang [90]. Briefly, hearts were isolated from adult CD-1 mice (8-9 weeks, 36-46 g) and perfused with a 2-ml bolus of Krebs-Henseleit solution supplemented with or without 10 μg of recombinant FGF-16 for 7 minutes to allow the activation of FGF signaling pathways and the potential cardioprotective signaling pathways [90]. Hearts were then perfused with or

without 10 μM doxorubicin for 2 hours, and the effect of FGF-16 protein supplementation and/or acute doxorubicin-induced cardiotoxicity on the heart function was recorded and analyzed by a Digimed Heart Performance Analyzer [90]. For protein kinase C (PKC) inhibition, 5 μM chelerythrine was added to the Krebs-Henseleit solution before the FGF-16 [90]. In addition, released lactate dehydrogenase levels in the perfusate were also measured as an indication of doxorubicin-induced cardiac cell damage [90].

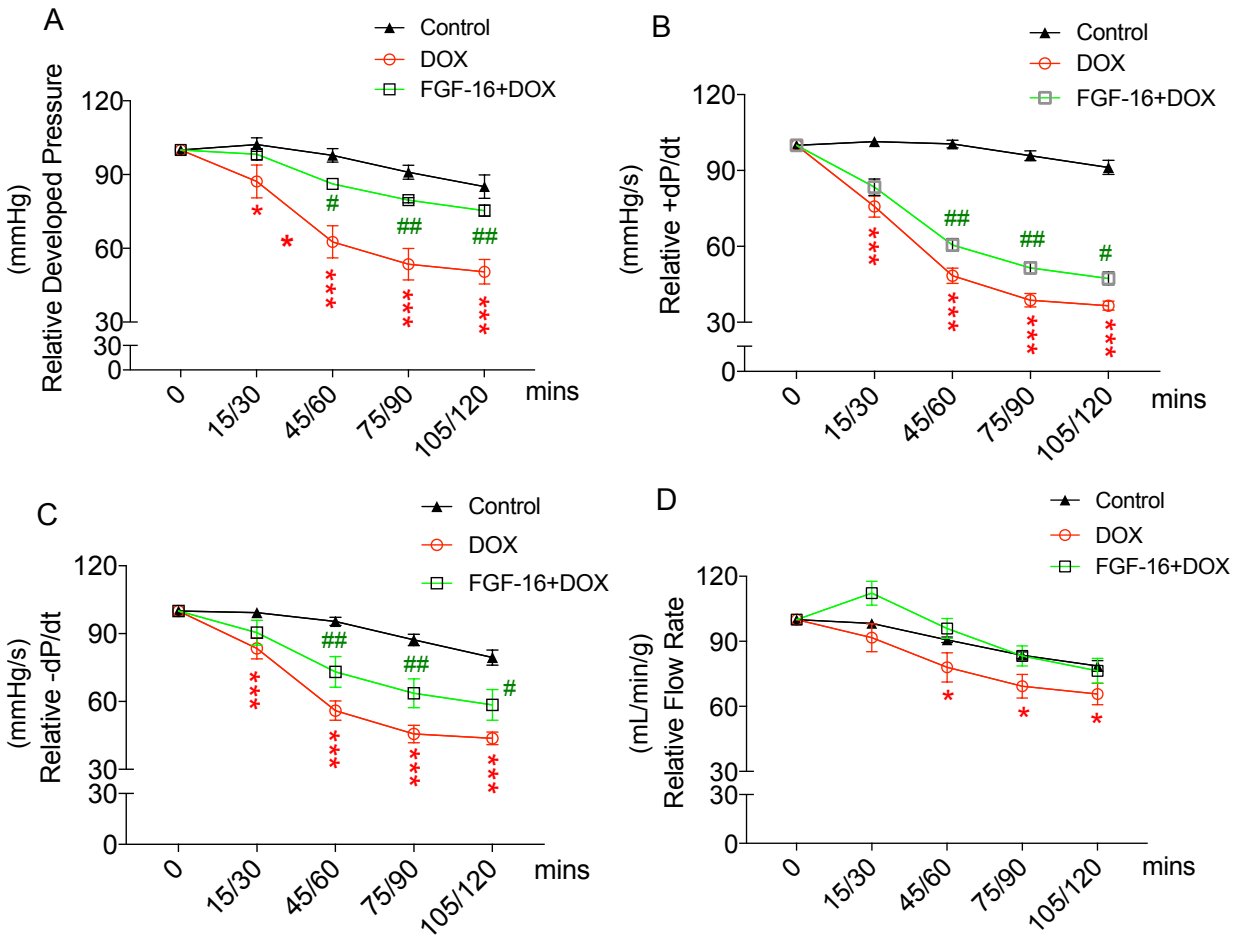
***Results*:**

5.3.1 FGF-16 increases resistance to a doxorubicin-induced decrease in left ventricle contractility

The effect of doxorubicin in Krebs-Henseleit buffer on left ventricle contractility was examined in isolated mouse hearts over a period of 120 minutes [90]. Doxorubicin caused a significant decrease in developed pressure, as well as in the rate of rise (+dP/dt) or decline (-dP/dt) of left ventricular pressure compared to control (vehicle) treatment (**Figures 27A-C**). The decrease in developed pressure detected at 15/30 minutes was significant and persisted until the end point of the study, where a 41% reduction in developed pressure was detected relative to control levels (**Figure 27A**) [90]. In addition, the decrease in +dP/dt and -dP/dt with doxorubicin treatment was significant at 15 and 30 minutes, respectively, with a persistent decrease until the 120-minute end time points (**Figures 27B** and **C**) [90]. The flow rate was also decreased significantly from the 60 minutes time point and persisted throughout the study (**Figure 27D**) [90].

Pre-treatment with FGF-16, given 7 minutes prior to doxorubicin blunted the decrease in developed pressure caused by doxorubicin, starting from 45 to 60 minutes until the end of the study (**Figure 27A**) [90]. At 120 minutes, the developed pressure in the presence of FGF-16 and doxorubicin was 74% of control, and significantly higher compared to 50% seen with doxorubicin alone [90]. This response was also reflected in the significant changes detected in measurements of +dP/dt and -dP/dt, the rate of rise and decline of left ventricular pressure, respectively (**Figures 27B** and **C**) [90]. No significant change in flow rate was observed with the FGF-16 pre-treatment group with doxorubicin at any time points measured (**Figure 27D**) [90].

Figure 27. The effect of FGF-16 supplementation on doxorubicin-induced dysfunction in the isolated mouse hearts

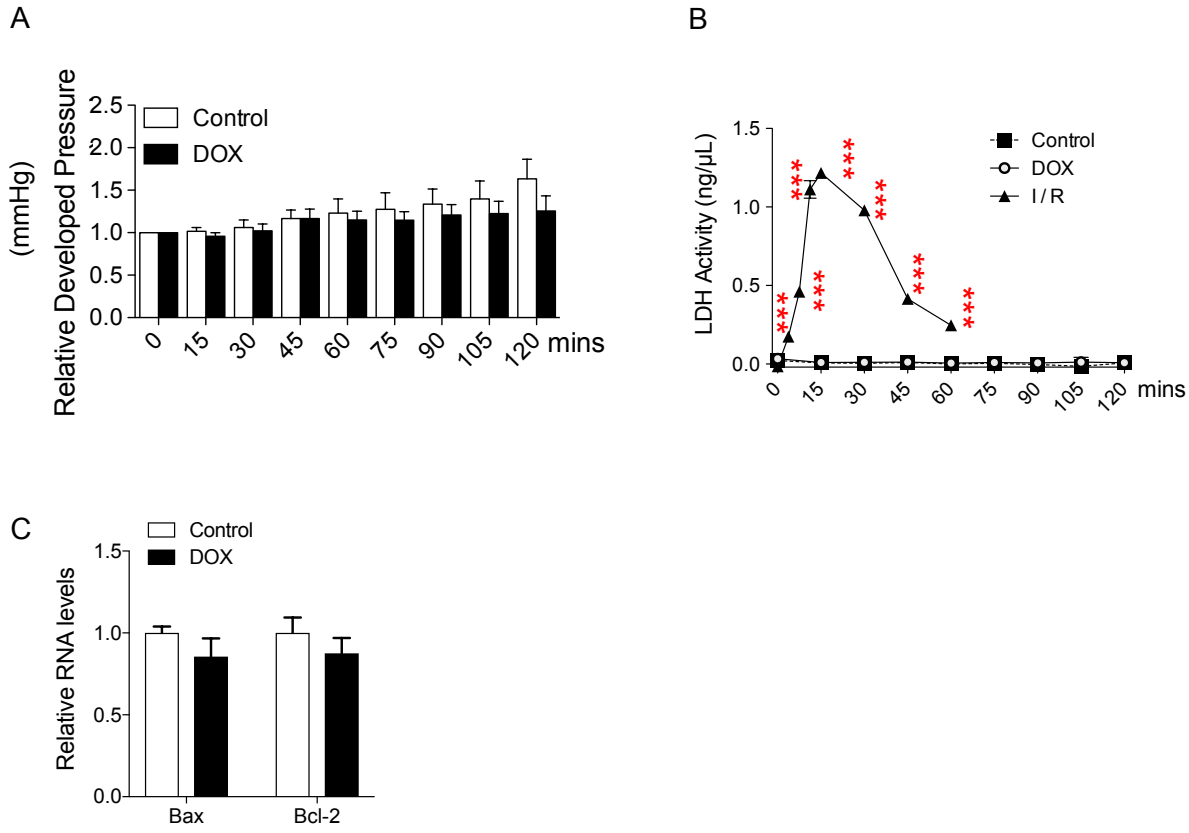


Effect of FGF-16 pre-treatment on (A) developed pressure (mmHg), (B) rate of pressure increase (+dP/dt) (mmHg/s) and (C) decline (-dP/dt), and (D) flow rate (mL/mm/g) detected and analyzed in isolated mouse hearts perfused with DOX. Hearts were perfused with or without a single bolus of FGF-16 (7 minutes) and/or DOX in Krebs-Henseleit buffer for 120 minutes. The vehicle (Control) value is arbitrarily set to 100. Data were assessed by two-way ANOVA, with n=5 mouse hearts per treatment group. A value of $p<0.05$ is considered statistically significant; $p<0.05$, */#; $p<0.01$, **/##, $P<0.001$ ***/###. (* compared to control and # compared to doxorubicin). *(Reproduced with permission from Springer, Cardiovascular Toxicology* [90]*)*

5.3.2 Doxorubicin treatment has no significant effect on heart compliance and cardiac myocyte damage

End diastolic pressure was also assessed as an indication of heart compliance, a side effect of doxorubicin-induced acute damage [90]. An increasing trend was observed, however, significance was not reached (**Figure 28A**) [90]. The perfusates from control and doxorubicin-treated hearts were also examined for the presence of lactate dehydrogenase, as an indicator of plasma membrane disruption and cell damage [90]. No significant difference was observed between the control and doxorubicin-treated groups (**Figure 28B**) [90]. There was also no significant effect of doxorubicin treatment on the pro-apoptotic Bax and anti-apoptotic Bcl-2 mRNA levels ratio from the heart at the end of the study by qPCR (**Figure 28C**) [90]. However, an increase in lactate dehydrogenase release was detected during reperfusion after a 20 minutes of 'no-flow' global ischemia, and used as a positive control for the induction of injury (**Figure 28B**) [90].

Figure 28. The effect of doxorubicin on end diastolic pressure (EDP) and cardiac myocyte damage in isolated mouse heart

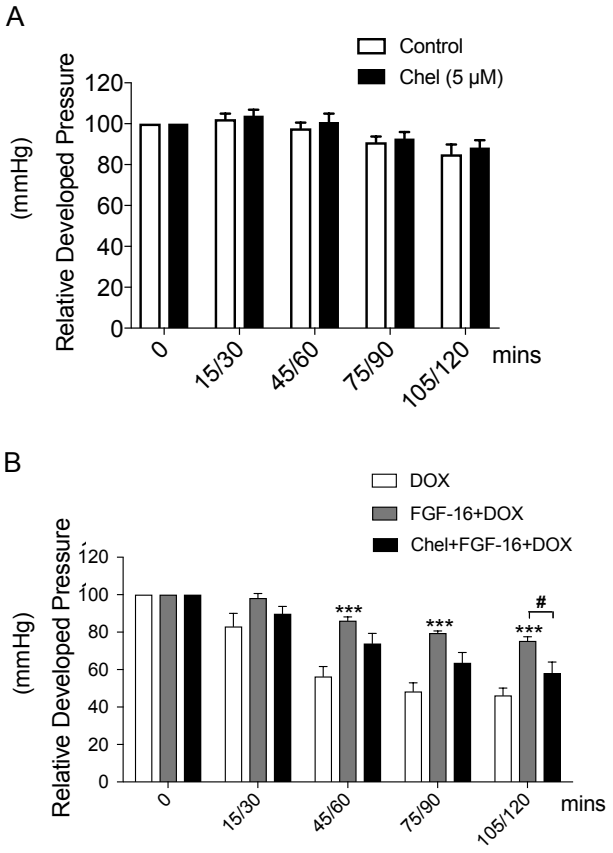


Effect of doxorubicin (DOX) perfusion on (A) end diastolic pressure (EDP), n=5 (B) lactate dehydrogenase (LDH) release (n=3), and (C) Bax and Bcl-2 mRNA levels were also assessed (n=3) to measure the effects on the base line cardiac cell or function changes. Global ischemia-reperfusion (I/R) was used as positive control (B) for LDH release and hearts were re-perfused after 20 minutes of global ischemia. LDH activity in the perfusate (ng/µL) was measured. All mRNA expression levels were normalized to control gene RNA Pol II. Vehicle (Control) value was arbitrarily set to 1. Data were assessed by two-way ANOVA (A and B), and unpaired t-test for (C). A value of $p<0.05$ is considered statistically significant; $p<0.001$ ***. (* compared to Control). *(Reproduced with permission from Springer, Cardiovascular Toxicology* [90]*)*

5.3.3 Protection by FGF-16 against a doxorubicin-induced decrease in left ventricle contractility is sensitive to chelerytherine treatment

Chelerythrine has been used to inhibit PKC signaling [265-268]. Hearts were pre-perfused with chelerythrine, at 5 μM, followed by perfusion with FGF-16 and subsequently doxorubicin [90]. Chelerythrine alone had no significant effect on developed pressure compared to the control (**Figure 29A**) [90]. By contrast, chelerythrine blunted the protective effect of FGF-16 against the doxorubicin-induced decrease in developed pressure, in the 105 to 120-minute treatment range (**Figure 29B**) [90].

Figure 29. Protection by FGF-16 against a doxorubicin-induced decrease in left ventricle contractility is sensitive to chelerytherine treatment

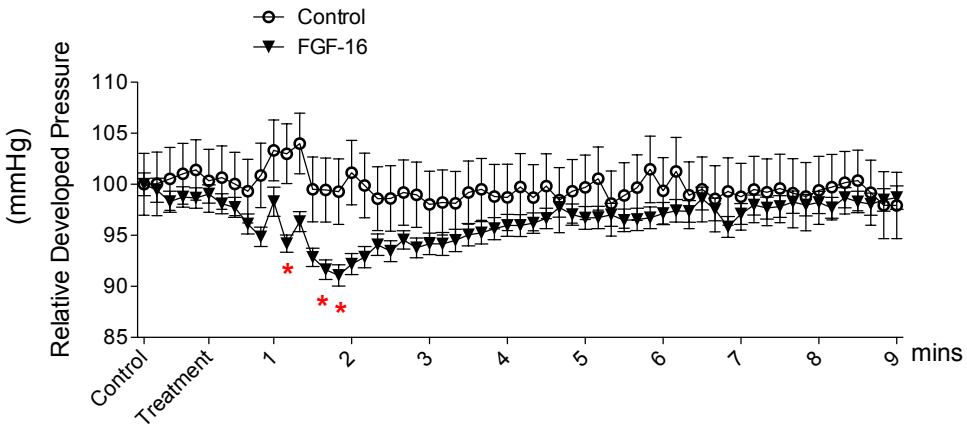


Developed pressure (mmHg) was compared between isolated mouse hearts perfused with: (A) the protein kinase C inhibitor chelerythrine (Chel) alone (n=3); (B) doxorubicin (DOX), FGF-16 and DOX with and without prior treatment with Chel (n=3). The DOX value was arbitrarily set to 100. Developed pressure values were assessed by two-way ANOVA for multiple comparisons for the times as indicated. A value of $p<0.05$ is considered statistically significant: $p<0.05$, #; $p<0.001$, ***. * compared to DOX and # compared to FGF-16 +DOX. *(Reproduced with permission from Springer, Cardiovascular Toxicology* [90]*)*

5.3.4 Evidence that FGF-16 exerts negative inotropism

FGF-2 (low molecular weight, 18 kDa) was reported to confer cardioprotection that was also sensitive to protein kinase C activation [90], and cause a negative inotropic effect in the heart that was seen as a significant decrease in developed pressure after FGF-2 perfusion [97, 269]. To assess the possibility of a similar effect with FGF-16, developed pressure was measured in isolated perfused mouse hearts during and after the FGF-16 bolus infusion period at 10-second intervals on average [90]. A transient, but significant, reduction in developed pressure was observed starting at 1 minute of perfusion with a peak reduction of about 10% at 2 minutes of perfusion (**Figure 30**) [90]. Developed pressure returned to pre-drug values after 2 minutes of FGF-16 pre-treatment [90].

Figure 30. Detection of a pattern of transient negative inotropism with FGF-16 perfusion



Isolated mouse hearts were perfused without (n=8) or with FGF-16 (n=9) in Krebs-Henseleit buffer, and the developed pressure was recorded and analyzed for the first 9 minutes (mins). The vehicle (Control) value was arbitrarily set to 100. Data were assessed by two-way ANOVA. Significant differences were only seen at points assessed between 1 and 3 minutes. A value of $p<0.05$ is considered statistically significant; $p<0.05$, *. *(Reproduced with permission from Springer, Cardiovascular Toxicology* [90]*)*

Discussion:

"Knockdown" of FGF-16 in neonatal rat cardiac myocyte suggests that cells with low endogenous FGF-16 levels are at an increased risk of apoptotic and necrotic cell death (**Figure 23** and **24**). Furthermore, increased levels of FGF-16 protected neonatal rat cardiac myocytes from doxorubicin-induced cardiac cell death and dysfunction, whether by adenoviral FGF-16 overexpression (**Figure 25** and **26**) or protein supplementation (**Figure 27** and **28**). These data suggest that FGF-16 is an endogenous and exogenous cardiac survival factor.

Accumulation of secreted 26.5 kDa FGF-16 protein was also reduced with FGF-16 siRNA transfection, which is consistent with the decrease in FGF-16 RNA levels [79, 105, 137]. Although multiple bands were detected, the 26.5 kDa FGF-16 protein is the expected size of the N-glycosylated and secreted form, and thus presumably binds to heparan sulfate proteoglycan (HSPG) and the extracellular matrix, as described previously [79, 105, 137, 138]. This further supports a role for endogenous FGF-16 in the maintenance of postnatal cardiac myocyte viability and function [166, 169]. Thus, rapid depletion of FGF-16 in response to doxorubicin treatment as a result of a negative effect on Csx/Nkx2.5 availability and *Fgf-16* transcription, is expected to contribute to the reduced resistance of cardiac myocytes to damage.

The data from FGF-16 null C57BL/6 mice suggest their hearts are able to function within normal parameters until they are subjected to stress (by angiotensin II) [169]. By contrast, the results from FGF-16 "knockdown" in neonatal rat cardiac myocytes suggest

there is an increased risk of cell death or damage. This might be explained by seeing isolated cardiac myocytes in culture after disaggregation of a heart as being stressed. Alternatively, the increased risk of cell death may reflect the greater potential for additional factors, perhaps produced by non-cardiac myocytes, that are able to compensate for the loss of FGF-16 in cardiac function until a significant stress (e.g., angiotensin II treatment-induced hypertrophy and fibrosis [270]) or injury (e.g. ischemia-reperfusion [271]) are applied . For example, FGF-2 from cardiac fibroblasts can also bind to FGFR on cardiac myocytes, thus, offering protection to cardiac myocyte death [26]. FGF-16 levels were also significantly decreased in the GATA4-ablated mice with severe cardiac dysfunction and an increased level of cell death, while FGF-16 overexpression partially rescues the phenotype [86].

Using FGF-16 adenoviral overexpression in neonatal rat cardiac myocytes, the levels of apoptosis and necrosis were reduced (**Figure 25** and **26**). This is consistent with FGF-16 as a cardioprotective factor. Doxorubicin rapidly decreases endogenous FGF-16 synthesis (**Figure 11** and **12**). When cardiac myocytes are pre-exposed to increased FGF-16 before doxorubicin treatment, secreted FGF-16 presumably acts as a local cardiac autocrine and/or paracrine factor by binding to heparan sulphate proteoglycan and/or FGFRs and triggers intracellular cardioprotective pathways [79, 124].

Intracellular levels of FGF-16 are relatively low in the murine heart based on immunofluorescence microscopy, postnatal production and secretion of glycosylated FGF-16 by cardiac myocytes and not fibroblasts, and the short FGF-16 RNA half-life (**Figure 13**) [79]. In addition, FGF-16 recombinant protein added into the rat chondrosarcoma culture medium was decreased significantly within the first 2 hours without heparin addition [272].

Thus, heparin extraction both stabilizes and enriches secreted FGF-16 protein through binding. As a result, in the absence of overexpression, detection of intracellular or secreted FGF-16 from cardiac cells and tissues, has benefited from combined heparin extraction and immunoblotting (**Figures 14, 22 and 23)** [79, 137, 273]**.** However, even with heparin extraction, the levels of FGF-16 protein in neonatal cardiac myocytes are still relatively low compared to adults (**Figure 5**), and multiple bands are detected by immunoblotting with available FGF-16 antibodies [105, 137]. Independent FGF-16 antibody preparations (Abcam, PeproTech, A61 and A60) have been tested with or without heparin extraction and showed consistent pattern of detection of multiple bands [137]. Their specificity is suggested by the higher affinity of all four antibody preparations for FGF-16 versus FGF-9 based on serial dilution and immunodetection [137], as these two FGFs share ~73% amino acid sequence similarity[105, 110, 126]. The 26.5 kDa FGF-16 isoform was shown to be glycosylated and the predominant FGF-16 protein secreted by neonatal rat cardiac myocytes in culture [79, 105, 137, 138]. The 19.5kDa was shown to be a non-glycosylated isoform of FGF-16 [79, 105, 137, 138]. In addition, all of these FGF-16 antibody preparations detect recombinant human FGF-16 at sized consistent with both monomers and dimers [137]. Thus, the ~45 kDa band (**Figure 14, 22, 23 and 26**) as well as the 50-60 kDa band seen after FGF-16-AdV overexpression and doxorubicin treatment (**Figure 26**), are consistent with dimers of the 19.5 kDa and 26.5 kDa FGF-16 isoforms, respectively. Although a low mobility band above 60 kDa was detected in some immunoblots, this is presumed to be due to a non-specific signal related to excess transferred protein, as suggested by Ponceau Red staining of the membrane (**Figure 21** and **22**). In addition, doxorubicin treatment increased the ~26.5 glycosylated FGF-16 protein form in the FGF-16-AdV overexpressed cardiac myocyte culture medium. This could be contributing to the increased resistance or protection seen in the FGF-16-AdV

overexpression group compared to the Null-AdV group. Increased necrotic cell death from doxorubicin -induced injury could release more FGF-16 protein into the culture medium, while unfolded protein response due to endoplastic reticulum (ER) stress with FGF-16-AdV overexpression may also contribute to increased or facilitated protein secretion [274].

Doxorubicin decreased contractile function (developed pressure) in the absence of any detectable damage to cell membrane integrity (lactate dehydrogenase) within 2 hours in an isolated mouse heart model (**Figure 27** and **28**). FGF-16 protein supplementation increased resistance to doxorubicin-induced heart contractile dysfunction (**Figure 27**). These observations support a cardioprotective role for FGF-16, when taken together with increased resistance to doxorubicin-induced cardiac myocyte damage due to FGF-16 overexpression in cardiac myocytes (**Figure 26**). This is also consistent with the proposed role of postnatal FGF-16 in the heart as a maintenance factor [80, 86, 105, 169].

Doxorubicin is reported to induce dilated cardiomyopathy characterized by a decrease in developed pressure and rate of rise of left ventricular pressure (+/-dP/dt), which can lead to heart failure at the end stage of treatment in both the animal model and when used clinically [90, 275, 276]. Doxorubicin-induced damage includes swelling of the cytoplasm and mitochondria, loss of membrane integrity and cell lysis resulting in lactate dehydrogenase release [90, 277]. Doxorubicin has been reported to increase lactate dehydrogenase release in primary mouse and rat cardiac myocyte cultures, as well as in mouse models *in vivo* within 8-14 days of treatment [90, 278-280], but there was no detectable increase in lactate dehydrogenase release, or change in Bax and Bcl-2 mRNA levels, and thus Bax/Bcl-2 ratio in doxorubicin-treated hearts levels during the 120-minute study

compared to the control group [90] (**Figure 28**). An increase in lactate dehydrogenase activity was clearly detected, however, after subjecting the same isolated heart model to ischemia and reperfusion [90]. This suggests that in the isolated mouse heart model, if the insult is sufficient and the cell plasma membrane is compromised, then lactate dehydrogenase will be released and be detectable. Thus, the data suggest that the decrease in contractility observed with doxorubicin in the isolated mouse heart model is not caused by disruption of the plasma membrane and cell damage, at least not sufficient to be detected within the 2-hour time frame of the study. Acute effects of doxorubicin include impairment of mitochondrial metabolism and dysregulation of cellular energy homeostasis; the latter has been reported to occur before major cardiac dysfunction and, therefore, may contribute to the decrease in developed pressure observed [260, 281].

Pre-perfusion with FGF-16 increased resistance to the negative effect of doxorubicin on developed pressure and was evident by 45 to 60 minutes of perfusion (**Figure 27**). Similar results were observed with the rate of rise (+dP/dt) and decline (-dP/dt) of left ventricular pressure [90] (**Figure 25**). Other than a brief and transient negative inotropic effect during the pre-perfusion period, there was no effect of FGF-16 alone on contractile function [90] (**Figure 30**). Like FGF-2, the negative inotropy effect from FGF-16 may also directly contribute to cardioprotection [240]. FGFR1 and PKC signaling have been implicated in protection of the myocardium, including against ischemia-reperfusion injury in a mouse model [282]. However, the effects of FGF-16 on cardioprotection and related signaling pathways were unknown [79]. Although two or more structurally unrelated inhibitors should preferably be used in order to claim the direct effect on PKC signaling, the ability of chelerythrine to blunt the developed pressure improvement observed with

FGF-16 and doxorubicin perfusion raised the posiblity that PKC signaling either directly or indirectly is involved in the increased resistance to doxorubicin-induced damage seen with FGF-16 (**Figure 29**). The PKC family of kinases, and in particular PKCε, is strongly implicated as mediators of cardioprotection and cytoprotection [283]. Many cardioprotective manipulations activate PKCε, which translocates to various subcellular compartments, including mitochondria. Mitochondrial targets of PKCε include the K^+ATP channel and putative components of the mitochondrial permeability transition pore [284]; PKCε-mediated phosphorylation of the mitochondrial permeability transition pore results in enhanced mitochondrial resistance to oxidative stress and calcium-overload induced damage, and promotes cell survival [285]. However, FGF-16 was reported to inhibit the activation of PKCα and ε by FGF-2 [79]. Thus, it is also possible that other PKC signaling pathways are involved that were also sensitive to chelerythrine treatment (**Figure 29**). PKC-dependent protection would raise the possibility that FGF-16 may increase resistance to other cardiac insults including hypoxic injury [90, 105]. In addition, the PKC inhibiton effect from chelerythrine is controversial. Although there are a large number of reports using chelerythrine as a PKC inhibitor with observed biological effects consistent with PKC signaling [265-268], chelerythrine was also reported to have no effect on inhibiton of PKC or any protein kinase[286]. It was proposed that these effects were probably from unknown targets from this plant extracted alkaloid [286]. Thus, there is also the possibility that additional protective FGF signaling pathways, which have been implicated in protection against doxorubicin damage, are involved including PI3K-Akt-mTOR [92, 287], extracellular signal-regulated kinases (ERK), and protein kinase B (Akt) that are all reported as downstream targets of FGF-2 and FGFR interaction, and have been linked with multiple processes including cell proliferation and survival [85]. Further investigation on potential FGF-16 downstream

signaling pathways will help us to better understand the underlying molecular mechanisms of FGF-16 induced cardioprotection.

FGF-16 shares another characteristic with FGF-2, in addition to FGFR1 binding and implied PKC signaling. Specifically, FGF-16 showed evidence of biological activity by exerting a transient negative inotropic effect on cardiac muscle albeit to a lesser degree than FGF-2 [97, 269] (**Figure 30**). Other cardioprotective agents, such as volatile anesthetics, are known to elicit a degree of negative inotropism when given pre-ischemically in models of ischemia-reperfusion injury [288]. Mechanisms for the transient, negative inotropic effect of FGF-2 and now FGF-16 have not been investigated; it is possible that similar downstream signaling activation to FGF-2 may also be involved [97]. It is noted, however, that the negative inotropic effect of FGF-16 is small, brief, and transient; the hearts had resumed normal contractile function before the administration of doxorubicin. However, the downstream signaling pathways responsible for the negative inotropic effect versus the cardioprotective effect were unknown. Although it appears to be a correlation between a negative inotropic effect and protection, this is not necessarily directly related.

Several strategies have been employed to counteract the adverse cardiotoxic effects of doxorubicin [28]. Examples include: administration of erythropoietin, which protected from doxorubicin-induced oxidative stress and cell death via the Akt and glycogen synthase kinase-3 (GSK-3) beta pathway [289]; modulation and benefit in terms of the cardiac response to oxidative stress by anti-oxidant flavonoid compounds such as resveratrol [290]; and the observation that phosphodiesterase-5 inhibitors can reduce doxorubicin-induced oxidative stress and damage to the heart, while increasing oxidative stress in cancer (prostate) cells [291].

A common denominator in many of these strategies is an increased resistance of cardiac mitochondria to doxorubicin-induced oxidative stress and calcium-induced permeability transition. Additional studies are needed to determine the extent and sustainability of FGF-16 cardioprotection from doxorubicin-induced cardiac injury, and the underlying mechanisms of the FGF-16 induced cardioprotection, which was pursued.

Chapter 6: FGF-16 Regulates Multidrug Resistance Protein 1a (MDR1a): An Underlying Mechanism of FGF-16 Cardioprotection Against Acute Doxorubicin-Induced Cardiotoxicity

6.1 The effect of FGF-16 overexpression on efflux drug transporters in neonatal rat cardiac myocytes

***Rationale*:** Unlike FGF-16 that is expressed preferentially by postnatal cardiac myocytes, FGF-2 is widely expressed including in the heart and mainly by cardiac fibroblasts [87, 88, 292], including the well-described cardioprotective 18 kDa isoform [26, 90]. However, FGF-16 does share a number of properties with FGF-2, including an affinity for the tyrosine kinase FGFR1 binding, protein kinase C activation, and an inotropic effect on heart function [79, 90]. This suggests the presence of some shared downstream signaling as a result of receptor activation [79, 97, 269], which presumably may be triggered with FGF supplementation or when in excess. Previous work in our laboratory implicated the ability of 18 kDa FGF-2 to positively affect "pumping out" or efflux of doxorubicin, without it being metabolized, as a novel mechanism for FGF-mediated cardioprotection [26]. This effect was blunted by chelerythrine treatment [26, 90], a known downstream target of tyrosine kinase FGF receptor binding by FGF-2 [26]. In addition, tyrosine kinase inhibitors such as PD173074 were reported to inhibit efflux drug transporter ABCB1 but not ABCC1 or ABCG2 or MRP1 activity, increase the intracellular concentration of the substrate chemotherapy drugs, and thus reverse the multidrug resistance of the cancer cells [293]. This suggests a potential role of FGF16-FGFR signaling on efflux drug transporters. Thus, a potential effect of FGF-16 on efflux drug transport in cardiac myocytes expression and activity was investigated.

As introduced in section 1.7.2, efflux drug transporters or multidrug resistance proteins (MDRs) were first described in cancer cells, but are also found in many other normal tissues including the heart [241, 294-297]. MDR1a and the multidrug resistance-associated protein 1 (MRP1) are abundantly expressed in cardiac myocytes compared to MDR2 and MRP2 [188]. The MDR1a$^{-/-}$ knockout mouse model showed increased accumulation of chemotherapy drug doxorubicin in the cardiac tissue, as well as brain and liver [298, 299]. Although increased MDR1 levels have a negative effect for cancer, it is beneficial to the heart since the removal of doxorubicin protects against cardiac myocyte death and heart dysfunction [186, 188, 192]. FGF-16 is predominantly expressed in cardiac myocytes, and thus overexpression of FGF-16 in neonatal cardiac myocyte cultures allows efflux drug transport to be assessed without systematic complications [79]. Using the fluorescent properties of MDR substrates like calcein and even doxorubicin itself, the effect of FGF-16 and doxorubicin on efflux drug transport function can be assessed and quantified in real time in cardiac myocyte cultures by flow cytometry [26, 300].

It was ***predicted*** that FGF-16 signals through FGFR and specifically upregulates efflux drug transporter function to decrease doxorubicin entry in neonatal rat cardiac myocytes. *Thus, the* ***objective*** *was to assess the effect of FGF-16 overexpression on efflux drug transport.*

Approaches**:** The underlying mechanism related to FGF-16 overexpression and cardioprotection against doxorubicin was investigated. Neonatal rat cardiac myocytes were transduced with FGF-16 adenovirus, and the effect of FGF-16 overexpression on both efflux drug transporter RNA levels and efflux transport function were assessed. Calcein-

AM is a substrate for MDR, and is converted to green-fluorescent calcein that can be measured by its intensity using flow cytometry. As a result, if there is an increase in the levels of MDR expression with FGF-16 overexpression in cardiac myocytes, efflux of calcein-AM will be elevated resulting in a reduction of the fluorescent signal. As neither calcein-AM nor calcein affect MDR levels, this is a direct measurement of efflux pump levels and function in real time. Doxorubicin itself is autofluorescent, and as a result can also be measured by its intensity using flow cytometry [26]. However, unlike FGF-16, doxorubicin is not only a substrate but also an inducer of MDRs [301, 302]. As such, the intracellular doxorubicin concentration is dynamic and constantly changing, depending on the balance of influx and efflux pump levels in response to doxorubicin treatment [26, 302, 303]. Thus, the final measurement of doxorubicin is an indication of the overall or cumulative effects on efflux transport with doxorubicin treatment. Doxorubicin-induced cardiotoxicity is also dose-dependent and cumulative [26]. The lower the intracellular doxorubicin concentration at the end of the treatment, the less damage there is to the cardiac myocyte [28].

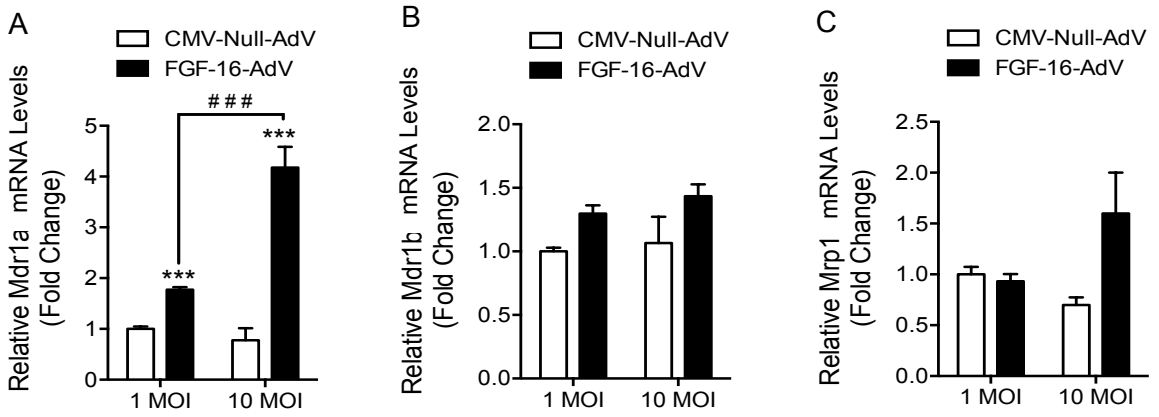
Results:

6.1.1 FGF-16 overexpression specifically increases efflux drug transporter MDR1a but not MDR1b mRNA levels in neonatal rat cardiac myocytes

To assess the effect of FGF-16 on efflux drug transporter gene expression, FGF-16-AdV (1 or 10 MOI) was used to overexpress FGF-16 in neonatal rat cardiac myocytes. The levels of efflux drug transporter mRNA levels were then assessed by qPCR after 48 hours. Efflux drug transporter MDR1a but not MDR1b or MRP1 transcripts were increased significantly in a dose-dependent manner with FGF-16 overexpression (**Figures 31A-C**),

and MRP2 mRNA was not detected under the conditions used. These data suggest specific regulation of MDR1a mRNA levels by FGF-16.

Figure 31. FGF-16 overexpression increases efflux drug transporter MDR1a RNA levels

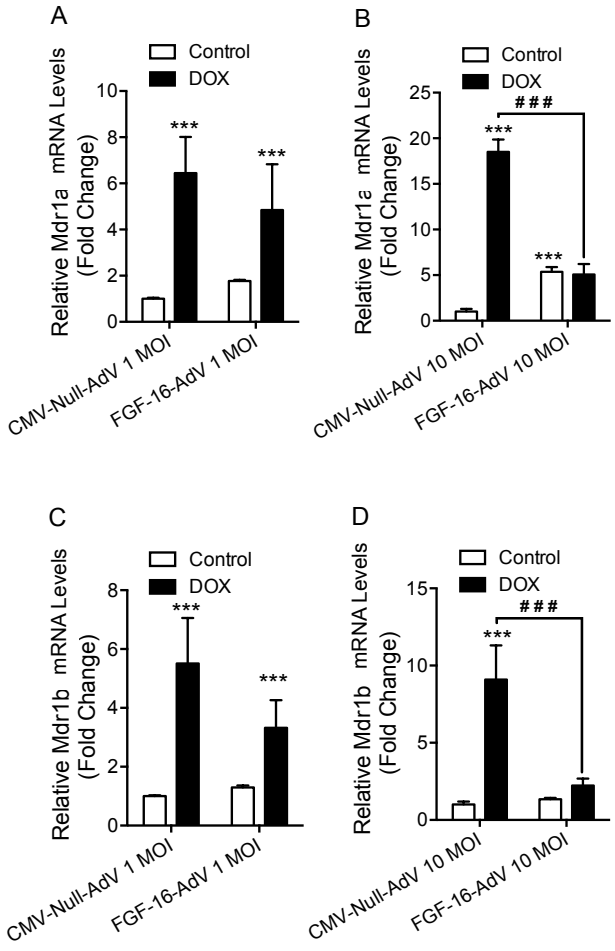


(A) MDR1a, (B) MDR1b, (C) MRP1, and MRP2 mRNA levels were assessed relative to RNA pol II transcripts by qPCR in neonatal rat cardiac myocytes transduced with FGF-16-AdV versus control CMV-Null-AdV (1 or 10 MOI) after 48 hours (n=9, triplicate samples from 3 individual experiments). MRP2 was not detected as tested. CMV-Null-AdV 1 MOI was arbitrarily set to 1.0. Results were analyzed by unpaired t-test with single comparison to CMV-Null-AdV 1 MOI (*) and FGF-16-AdV 1 MOI (#) treatment groups. A value of $p<0.05$ is considered statistically significant; $p<0.001$, ***/###.

6.1.2 The effect of FGF-16 overexpression and doxorubicin on efflux drug transporter mRNA levels in neonatal rat cardiac myocytes

MDR1a/b mRNA levels were assessed in neonatal rat cardiac myocytes transduced with FGF-16-AdV (1 or 10 MOI) before treatment with 1 μM doxorubicin for 12 hours (**Figures 32A-D**). FGF-16-AdV and doxorubicin alone both significantly induced MDR1a mRNA levels in the neonatal rat cardiac myocytes as expected (**Figures 32A** and **B**). However, the effect of the doxorubicin-induced increase in MDR1a levels was decreased with FGF-16 AdV at 1 MOI treatment, and was abolished by FGF-16 AdV 10 MOI transduction (**Figures 32A** and **B**). In addition, MDR1b mRNA levels were increased with doxorubicin alone, but FGF-16-AdV overexpression before doxorubicin limits this effect. (**Figures 32C** and **D**).

Figure 32. Effect of FGF-16 with or without doxorubicin on MDR1a/b mRNA levels



Neonatal rat cardiac myocytes were transfected with FGF-16-AdV or CMV-Null-AdV (1 or 10 MOI) for 48 hours before treatment with or without 1 μM doxorubicin (DOX) or Vehicle (Control) for 12 hours. MDR1a (A and B), MDR1b (C and D) mRNA levels relative to RNA pol II transcripts were assessed by qPCR (n=9, triplicate samples from 3 individual experiments). CMV-Null-AdV 1 MOI is arbitrarily set to 1.0 for (A and C) and CMV-Null-AdV 10 MOI is arbitrarily set to 1.0 for (B and D). Results were analyzed by two-way ANOVA with a *post-hoc* Bonferroni test, and comparisons were made to (*) CMV-Null-AdV (1 or 10 MOI, respectively) and (#) CMV-Null-AdV+DOX 10 MOI treatment groups. A value of $p<0.05$ is considered statistically significant; $p<0.001$, ***/###.

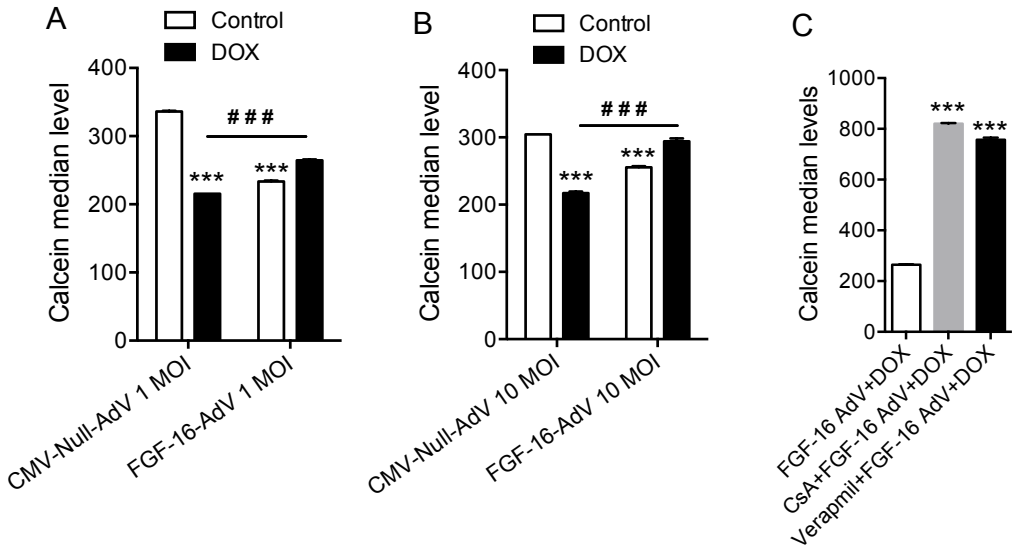
6.1.3 FGF-16 overexpression increases efflux of calcein-AM in neonatal rat cardiac myocytes

Neonatal rat cardiac myocyte cultures were transduced with FGF-16-AdV (1 or 10 MOI) for 48 hours, calcein-AM (1:1000) was added 30 minutes before harvesting for flow cytometry. The level of intracellular calcein fluorescence was significantly decreased with FGF-16-AdV (1 or 10 MOI) overexpression compared to transfection with control CMV-Null-AdV (**Figures 33A** and **B**). This is consistent with increased MDR1a mRNA levels with FGF-16 overexpression, and presumably increased transporters resulting in increased (calcein) efflux activity. As a positive control, transport inhibitors verapamil or cyclosporine A significantly blocked the increased efflux of intracellular calcein observed with FGF-16 overexpression (**Figure 33C**).

Neonatal rat cardiac myocyte cultures were then treated with doxorubicin for 12 hours after FGF-16-AdV (1 or 10 MOI) transduction for 48 hours, and calcein-AM (1:1000) was then added 30 minutes before harvesting for flow cytometry. The level of calcein fluorescence was significantly decreased in CMV-Null-AdV (1 or 10 MOI) transduced cells (**Figure 33A**). This decrease was not seen, however, in cardiac myocytes treated with doxorubicin that had been transduced with FGF-16-AdV (compare **Figures 33A** and **B**). In addition, FGF-16-AdV plus doxorubicin treatment was associated with a higher signal for calcein fluorescence than in cardiac myocytes treated with CMV-Null-AdV plus doxorubicin (compare **Figures 33A** and **B**). This is consistent with: (i) increased MDR1a/1b levels and efflux transport with doxorubicin treatment alone; and (ii) decreased

MDR1a and 1b mRNA levels and efflux transport with FGF-16 overexpression before doxorubicin treatment compared to doxorubicin treatment alone.

Figure 33. FGF-16 overexpression stimulates calcein-AM efflux

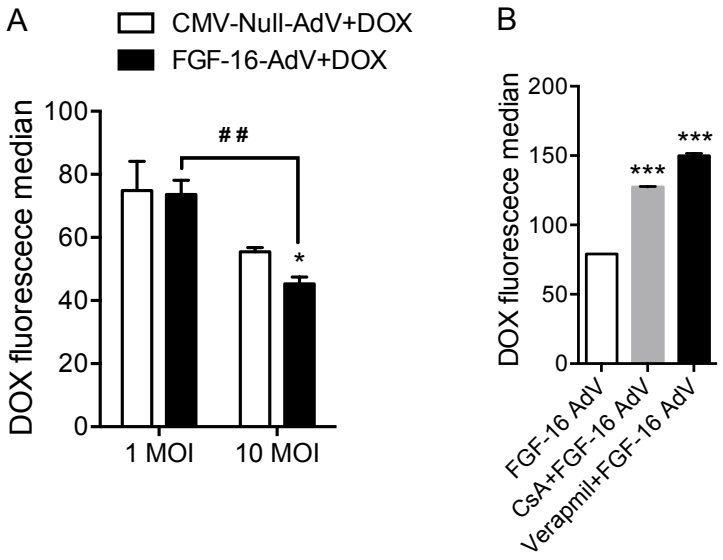


(A) and (B) Neonatal rat cardiac myocytes were transduced with FGF-16-Ad or CMV-Null-AdV (1 or 10 MOI) for 48 hours before treatment with or without 1 μM doxorubicin (DOX) or vehicle (Control) for 12 hours (n=9, triplicate samples from 3 individual experiments). Calcein-AM was added 30 minutes before preparation for flow cytometry. For (C), efflux drug transport inhibitors cyclosporine A (CsA, 20 μM) or verapamil (2 μM) were added for 30 minutes before calcein-AM (n=3, triplicate samples). The medians for calcein fluorescence were assessed relative to cells without calcein-AM treatments by flow cytometry. Results for (A) and (B) were analyzed by two-way ANOVA with a *post-hoc* Bonferroni test, and comparisons were made to (*) CMV-Null-AdV (1 or 10 MOI) and to (#) FGF-16 AdV+DOX (1 or 10 MOI). For (C), results were analyzed by one-way ANOVA with a *post-hoc* Tukey test and * compared to FGF-16-AdV+DOX treatment groups. A value of $p<0.05$ is considered statistically significant; $p<0.001$, ***/###.

6.1.4 FGF-16 overexpression increases removal of doxorubicin in neonatal rat cardiac myocytes

Unlike calcein-AM, doxorubicin is not only a substrate but also a regulator of efflux drug transporters [302]. The effect of FGF-16 overexpression on doxorubicin removal was assessed in neonatal rat cardiac myocyte cultures with FGF-16-AdV transduction (1 or 10 MOI) for 48 hours before 12 hours of doxorubicin treatment. Cells were then harvested for doxorubicin autofluorescence flow cytometry analysis. Doxorubicin autofluorescence intensity showed a significant decrease in FGF-16-AdV transfected cells with 10 but not 1 MOI, compared to CMV-Null-AdV (**Figure 34A**). As a positive control, transport inhibitors verapamil and cyclosporine A (CsA) significantly blocked the increased efflux of intracellular doxorubicin observed with FGF-16 overexpression (**Figure 34B**). No significant effect was observed with CMV-AdV-Null transduction alone on doxorubicin retention. Thus, these observations are consistent with FGF-16 decreasing intracellular doxorubicin, which is consistent with increased efflux drug transporter MDR1a mRNA levels and activity.

Figure 34. FGF-16 overexpression increases removal of doxorubicin in neonatal rat cardiac myocytes

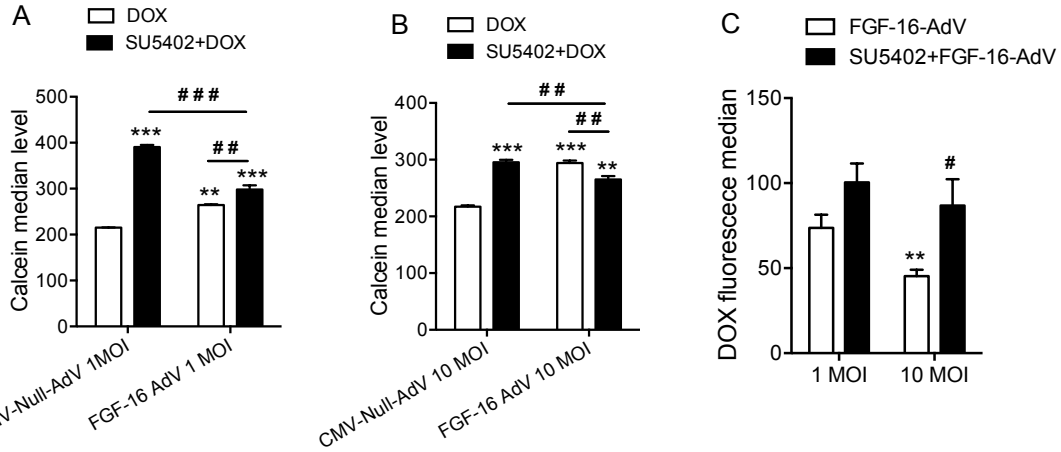


Neonatal rat cardiac myocytes were transfected with (A) FGF-16-AdV or CMV-Null-AdV (1 or 10 MOI) for 48 hours before treatment with or without 1 μM doxorubicin (DOX) for 12 hours (n=9, triplicates samples from 3 individual experiments). (B) Efflux drug transport inhibitors cyclosporine A (CsA, 20 μM) or verapamil (2 μM) were added for 30 minutes before DOX (n=3, triplicate samples). The medians for DOX autofluorescence were assessed relative to cells without DOX treatment by flow cytometry. Results for (A) were analyzed by two-way ANOVA with a *post-hoc* Bonferroni test, and comparisons were made to * CMV-Null-AdV 10 MOI and to # FGF-16 AdV+DOX (1 MOI). For (B), results were analyzed by one-way ANOVA with a *post-hoc* Tukey test. * compared to FGF-16-AdV+DOX treatment groups. A value of $p<0.05$ is considered statistically significant; $p<0.05$ *, $p<0.01$ ##, $p<0.001$, ***.

6.1.5 FGFR inhibition reverses the increased efflux transport of calcein-AM and doxorubicin in neonatal rat cardiac myocytes overexpressing FGF-16

To assess if the increased efflux transport by FGF-16 in cardiac myocytes is signaled through FGFR binding, cells were treated with the FGFR inhibitor, 20 μM SU5402, before FGF-16-AdV transduction and doxorubicin treatment. SU5402 interfered significantly with the positive effects of FGF-16-AdV overexpression on efflux of calcein (both 1 and 10 MOI) and doxorubicin (10 but not 1 MOI) in neonatal rat cardiac myocytes (**Figures 35A-C**). SU5402 treatment also increased the calcein fluorescence median in CMV-Null-AdV transfected cells treated with doxorubicin (**Figures 35A** and **B**). This presumably reflects SU5402 inhibition of endogenous background FGF signaling, resulting in a further decrease in efflux drug transport and increase in calcein fluorescence in cardiac myocytes.

Figure 35. FGFR inhibition limits the positive effect of FGF-16 overexpression on efflux transport



Neonatal rat cardiac myocytes were treated with FGFR inhibitor SU5402 (20 μM) before (1 or 10 MOI) FGF-16 overexpression and 1 μM doxorubicin (DOX) treatment. (A) and (B) Calcein-AM and (C) DOX fluorescence were assessed relative to cells without calcein or DOX treatment by flow cytometry (n=6, triplicate samples from 2 individual experiments). Results for (A) and (B) were analyzed by two-way ANOVA with a *post-hoc* Bonferroni test, and comparisons were made to (*) CMV-Null-AdV (1 or 10 MOI) and (#) SU5402+FGF-16 AdV+DOX (1 or 10 MOI) treatment groups. For (C), comparisons are made to (*) FGF-16-AdV+DOX 1 MOI and (#) FGF-16-AdV+DOX 10 MOI treatment groups. A value of $p<0.05$ is considered statistically significant; $p<0.05$ #, $p<0.01$ **/##, $p<0.001$, ***/###.

6.2 The effect of decreased endogenous FGF-16 expression on MDR1a levels

***Rationale*:** FGF-16 overexpression increases efflux drug transport and offers cardioprotection against doxorubicin-induced cardiac myocyte death. However, doxorubicin rapidly depleted endogenous FGF-16 expression levels by 2 hours in isolated neonatal rat cardiac myocytes and by 6 hours in the rat heart *in vivo*. Thus, a negative effect on doxorubicin efflux as a result of a specific decrease in FGF-16 availability is expected to contribute to the severity of doxorubicin. To date, there is no report on the effect of decreased FGF-16 on efflux drug transporters mRNA levels. In addition, doxorubicin alone increases MDR1a levels as part of the multidrug resistance mechanism [304]. Thus, the level of MDR1a, including transcripts, in neonatal rat cardiac myocyte cultures treated with doxorubicin, has potentially multiple regulators depending on the time and duration of the treatment. If MDR1a levels are decreased rapidly in response to the rapid reduction in FGF-16 synthesis, then this would be expected to occur before the development of doxorubicin-induced multidrug resistance. As a result, if drug efflux is decreased due to decreased MDR1a level at this early stage, more doxorubicin is likely to enter the cells and cause more cardiac myocyte damage. Thus, investigating how the endogenous FGF-16 affects multidrug resistance protein levels alone or in combination with the effects of doxorubicin may help us to better understand the role of FGF-16 in doxorubicin-induced cardiotoxicity.

It was ***predicted*** that a decrease in endogenous FGF-16 mRNA levels will be associated with a specific decrease in MDR1a but not 1b transcripts in the neonatal rat cardiac myocytes. In addition, downregulation of endogenous FGF-16 by doxorubicin will decrease MDR1a mRNA levels before multidrug resistance develops in response to

doxorubicin treatment. The initial decrease in MDR1a and efflux transport related to the loss of FGF-16 may contribute to the specific and severe cardiotoxicity induced by doxorubicin. As a result, preventing the initial decline in endogenous FGF-16 levels from doxorubicin may also be cardioprotective. *Thus, the **objective** was to assess the effect of decreased FGF-16 levels on MDR1a mRNA levels*

***Approaches*:** Two models were used to test the effect of decreased endogenous FGF-16 on MDR1a mRNA levels:

1) siRNA "knockdown" of endogenous FGF-16 levels in neonatal rat cardiac myocyte cultures as described in Chapter 5.2. Cultures were transfected with FGF-16 siRNA and endogenous MDR1a and 1b mRNA levels were assessed by qPCR; and

2) doxorubicin-induced rapid depletion of endogenous FGF-16 levels in rat hearts *in vivo*. Eight- week -old male Sprague-Dawley rats were injected with a single 15 mg/kg dose of doxorubicin (i.p.) as described in Chapter 4.1, and endogenous FGF-16 and MDR1a/1b mRNA levels were assessed by qPCR at both 6 and 24 hours.

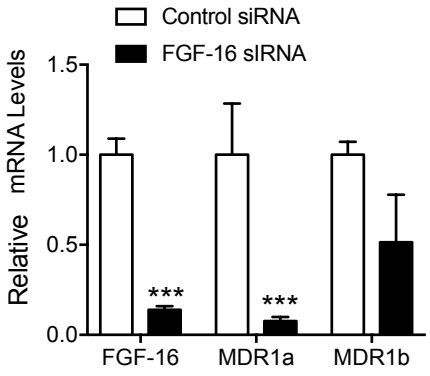
***Results*:**

6.2.1 The effect of FGF-16 siRNA knockdown on efflux drug transporter mRNA levels in neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes were transfected with 25 nM FGF-16 siRNA or control siRNA for 72 hours. Cells were harvested, RNA isolated and then MDR1a, MDR1b, and FGF-16 transcripts were assessed by qPCR normalized to endogenous control RNA Pol

II. As expected, a significant decrease in endogenous FGF-16 mRNA was detected. A significant >90% decrease in Mdr1a mRNA levels was observed but no reduction was seen with MDR1b transcripts (**Figure 36**). The regulation of MDR1a but not MDR1b mRNA levels with FGF-16 "knockdown", is consistent with the specific regulation of MDR1a by FGF-16 overexpression. Together, they suggest a direct regulation of efflux drug transporter MDR1a levels by FGF-16.

Figure 36. FGF-16 siRNA knockdown decreases MDR1a mRNA levels

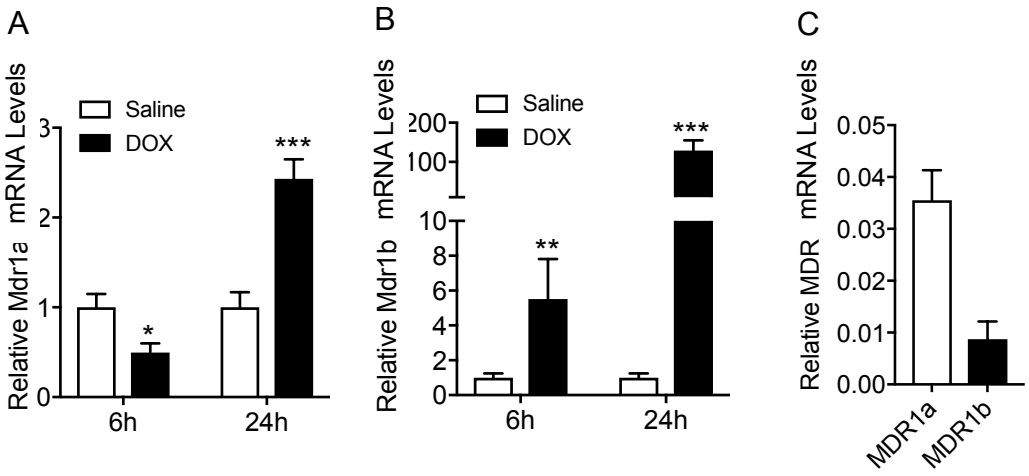


Neonatal rat cardiac myocytes were treated with 25nM FGF-16 or control siRNA for 72 hours, endogenous FGF-16, MDR1a, and MDR1b mRNA levels were then assessed by qPCR normalized to control gene RNA pol II (n=6, triplicate samples from 2 individual experiments). Control siRNA levels were arbitrarily set to 100%. Results were analyzed by unpaired t-test and * compared to Control siRNA. Data is considered significant if $p<0.05$; $p<0.001$***.

6.2.2 The effect of doxorubicin on endogenous FGF-16 and MDR1a mRNA levels in rat hearts

Eight-week-old male Sprague-Dawley rats were injected intraperitoneally with 15 mg/kg doxorubicin, and the endogenous MDR1a and MDR1b mRNA levels were assessed at both 6 and 24 hours by qPCR. The MDR1a levels were decreased significantly ~50 % at 6-hours post-doxorubicin treatment, but then increased ~2.4-fold by 24 hours (**Figure 37A**). By contrast, MDR1b transcripts were already significantly increased at 6 hours by ~5.5-fold, and increased further to ~129-fold at 24-hours (**Figure 37B**). However, relative to RNA pol II mRNA, MDR1a transcripts are ~4-fold more abundant than MDR1b transcripts, suggesting that MDR1a is the predominant form of multidrug resistance protein expressed in postnatal cardiac myocytes (**Figure 37C**). FGF-16 appears to target MDR1a over MDR1b mRNA levels (**Figure 36**), while there is a much greater effect of doxorubicin on MDR1b transcripts (**Figures 37A** and **B**). Thus, a combination of FGF-16 and doxorubicin effects will need to be taken into consideration when analyzing multidrug resistance in cardiac myocytes.

Figure 37. The effect of doxorubicin on FGF-16 and MDR1a /1b mRNA levels in rat hearts



Eight week old male Sprague-Dawley rats were injected with 15 mg/kg DOX, and endogenous MDR1a and MDR1b RNA levels were then assessed by qPCR at both 6 (A) and 24 (B) hours (n=6 rats for each group). All mRNA expression levels were normalized to control gene RNA Pol II. Saline group were arbitrarily set to 1. Results were analyzed by unpaired t-test and * compared to the saline group. Data is considered significant if $p<0.05$: $p<0.05$ *; $p<0.01$ **; and $p<0.001$***. (C) The relative MDR1a to MDR1b mRNA levels in rat hearts.

Discussion:

The observations made suggest that the protection from doxorubicin-induced injury with FGF-16 overexpression is related, at least in part, to an increase in efflux drug transport. FGF-16 overexpression upregulated MDR1a mRNA levels in neonatal rat cardiac myocytes (**Figure 31**). This increase was associated with reduced intracellular doxorubicin (and calcein) concentration, which is consistent with lowering doxorubicin-induced damage, as seen by decreases in Annexin-V staining and lactate dehydrogenase levels (**Figure 31-34**). In addition, decreased endogenous FGF-16 levels are associated with reduced MDR1a mRNA levels.

Both FGF-16 and doxorubicin alone increases MDR1a mRNA levels in cardiac myocytes (**Figure 32**). However, when combined through pretreatment with FGF-16 before doxorubicin addition (FGF-16-AdV+DOX), the effect on MDR1a mRNA levels is antagonistic (**Figure 32**). In fact, little or no increase in MDR1a mRNA levels was seen in cardiac myocytes treated with FGF-16-AdV+DOX (**Figure 32**). A similar effect was also seen in the MDR1b mRNA data. FGF-16 treatment alone has no effect on MDR1b, but pretreatment with FGF-16 antagonized the doxorubicin-induced increase in MDR1b mRNA levels (**Figure 32**). The antagonistic effect between FGF-16 and doxorubicin presumably reflects the increased efflux of doxorubicin as a result of increased pump activity due to FGF-16 overexpression (**Figure 38**). This increased efflux would reduce the intracellular doxorubicin concentration and thus mute a stimulus of MDR1a as well as 1b production, as there is less doxorubicin to be removed (**Figure 38**). This is further supported by measurement of efflux drug transporter function as a surrogate for its expression levels

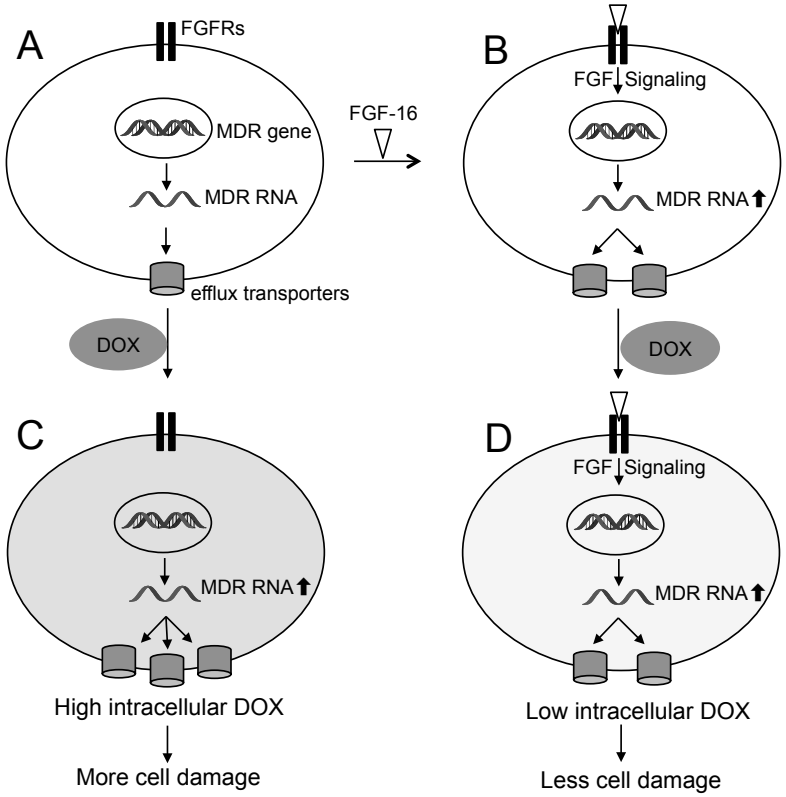
(**Figure 33** and **34**). Decreased intracellular doxorubicin levels were observed with FGF-16 pretreatment before doxorubicin (**Figure 34**). The toxic effect of doxorubicin is cumulative [28], thus, by increasing efflux transport, FGF-16 increases cardiac myocytes resistance to doxorubicin-induced injury and is cardioprotective. This is mainly due to prevention or reduction of the overall exposure of cardiac myocytes to doxorubicin, resulting from a FGF-16-induced increase in MDR levels.

In addition, FGFR inhibition reversed the FGF-16 induced increased efflux in cardiac myocytes (**Figure 35**). This suggests FGFR signaling is involved in the regulation of efflux drug transport by FGF-16. FGFR inhibition has been used in clinical trials to increase cancer sensitivity to chemotherapy drugs [305]. However, the observation made here suggests that FGFR inhibition also increases the sensitivity of cardiac myocytes to doxorubicin. Similar to FGFR inhibition, MDR inhibition is also beneficial to cancer treatment but detrimental to the heart [306]. Thus, increased cancer sensitivity and decreased cardiotoxicity from doxorubicin must be balanced if FGFR or MDR inhibitors are to be considered for therapeutic development and applied systematically. In addition, SU5402 can also inhibit Abelson murine leukemia viral oncogene homolog 1, Insulin-like growth factor receptor 1, and Janus kinase 3 signaling [307], thus, more specific FGFR inhibition could be used to determine a more specific effect of FGF-16 on FGFRs.

Numerous stimuli are reported to evoke a stress response and alter MDR1 gene expression. This includes chemotherapy, heat shock, and inflammation [182]. In addition, human P-glycoprotein is inactive when its maturation is inhibited during biogenesis, such that the transporter itself loses the ability to undergo ATP-induced conformational change

and cannot reach the cell surface to become functional [308]. As a consequence, assessment of functional efflux pump activity by calcein AM was pursued as a more accurate indication over protein levels at the end (12 hours) time point [308] (Figure 33).

Figure 38. FGF-16 pretreatment increases efflux transport of doxorubicin (DOX) and decreases DOX-induced efflux transporter expression in cardiac myocytes



Schematic representation of cardiac myocytes that are (A) untreated, or (B) treated with adenoviral FGF-16 overexpression, or (C) DOX supplementation, or (D) pretreated with FGF-16 overexpression and then treated with DOX supplementation. This graph represents two aspects of FGF-16 overexpression, specifically (1) removal of DOX and (2) efflux drug transporter expression levels. (1) Removal of DOX: Overexpression of FGF-16 (A➔B) results in an increase in efflux transporters and thus increased capacity for efflux DOX transport before DOX treatment. Thus, subsequent treatment with DOX (B➔D) fails, at least initially, to reach the same intracellular DOX concentration (depicted by grey shading) seen in cardiac myocytes that are treated with DOX directly (A➔C). While DOX treatment itself will eventually increase efflux drug transporters, the lower intracellular DOX concentration seen with FGF-16 pretreatment is expected to increase resistance to cardiac myocyte injury in (D) versus (C). (2) Efflux transporter expression levels: FGF-16 pre-treatment with DOX has lower efflux transporter expression levels (A➔B➔D) compared to DOX treatment alone (C). This is presumably due to the antagonistic effects from FGF-16 against DOX on efflux transporter levels (B) by reducing intracellular DOX concentration and there is a reduced stimulation of efflux drug transporter expression in (D) versus (C).

FGF-16 siRNA "knockdown" induced a decrease in MDR1a but not MDR11b mRNA levels in isolated neonatal rat cardiac myocytes (**Figure 36**), and is expected to result in reduced efflux drug transport function. Certainly, an increase in efflux substrate transport was observed with an increase in MDR1a transcripts following FGF-16 overexpression (**Figure 33** and **34**). These efflux pumps not only mediate drug resistance in human and disease-causing pathogens, but are also required for normal physiology and removal of body metabolites [183, 193, 194]. Although MDR1 knockout mice are viable, they do exhibit an increased risk of injury as well as an accumulation of xenobiotics [183, 309]. Efflux drug transport in the heart is relatively understudied when compared to cancer cells [188]. FGF-16 may also play a significant role in modulating absorption, accumulation, distribution and/or excretion of certain metabolites, as well as influence the efficacy/toxicity of cardiovascular drugs, given its ability to influence efflux activity and the potential as a cardiac-specific maintenance/survival factor in the postnatal heart.

Doxorubicin injection also induced "knockdown" (reduced expression) of endogenous FGF-16, and thus, MDR1a mRNA levels, at 6 hours in the Sprague-Dawley rats (**Figure 37**). This initial decline in endogenous FGF-16 and MDR1a mRNA levels with doxorubicin treatment potentially weakens the heart in two aspects: (1) a reduction in FGF-16 levels, and its cardiac maintenance/cardioprotective properties, would mean less resistance to doxorubicin-induced damage; and (2) a decrease in MDR1a levels, would mean less efflux doxorubicin transport out of the cardiac myocyte resulting in an increased probability of more damage. As a result, negatively targeting endogenous cardiac FGF-16 and MDR1a-related efflux transport could be one of the mechanisms contributing to

doxorubicin-induced acute cardiotoxicity, by increasing intracellular doxorubicin and causing more severe heart damage.

After the initial decline of both FGF-16 and MDR1a transcripts at 6 hours, FGF-16 mRNA synthesis in the cardiac myocytes remain suppressed by doxorubicin treatment until 24 hours (**Figure 37)**. However, doxorubicin also induces MDR1a and MDR1b in primary rat cardiac myocytes and mouse hearts *in vivo* [182, 198, 302, 310]. The level of efflux drug transporters including MDR1a, MDR1b, MRP1, and MRP2 increase in order to efflux more doxorubicin as components of the multidrug resistance system, increasing the chance of cardiac myocyte survival. In addition, when other non-specific MDR regulators such as FGF-2 levels increase with doxorubicin treatment at 24 hours, MDR levels remain increased even with a decrease in the endogenous FGF-16 levels. Unlike FGF-16, FGF-2 not only increased MDR1a mRNA levels but also MDR1b and MRP1/2 transcripts [26]. FGF-2 mRNA levels were unchanged at 6 hours but increased at 24 hours in the heart with doxorubicin treatment (**Figure 11**). Thus, the increase in MDR1a RNA levels at 24 hours with doxorubicin and increased FGF-2 transcripts, appears to counteract any decrease as a result of reduced *Fgf-16* expression, resulting in a net increase in MDR1a *in vivo*. However, FGF-2 levels are decreased with doxorubicin treatment in primary cardiac myocyte cultures (**Figure 12**). A possible explanation is the presence of non-myocytes in the intact heart *in vivo*, and specifically the presence of cardiac fibroblasts that are the predominant source of FGF-2 [240]. The released FGF-2 from cardiac fibroblasts are stored in the extracellular matrix and may become mobilized and free to act on cardiac myocytes, including causing protection, in a paracrine manner [240].

Together, observations from the *in vitro* and *in vivo* studies suggest that FGF-16 overexpression will prime cardioac myocytes with increased efflux transporters to reduce early damage by preventing DOX intracellular accumulation. However, once doxorubicin-induced multidrug resistance (> 100 fold increase in MDR1 levels) develops, the compensatory effect of endogenous FGF-16 on increased efflux drug transport (<10 fold increase in MDR1a) might be negligible by comparison (**Figure 37**). Thus, an initial short intervention by FGF-16 may be sufficient to protect DOX-induced damage. The effect of doxorubicin on MDR levels and efflux drug transport in the heart is dynamic depending on the class, time and duration of the drug treatment. Understanding how levels of FGFs and MDRs change at different stages with doxorubicin treatment may provide further insight into how to precisely minimize the cardiac damage from doxorubicin, and maximize the cardioprotection using FGF-16 and/or FGF signaling and the underlying efflux drug transport mechanism.

The MDR1 gene in humans and MDR1a and MDR1b genes in rodents all encode P-glycoprotein, a multidrug resistance protein that plays an important role in disposition and excretion of toxins including chemotherapy drugs and xenobiotics [182]. Although MDR1a and MDR1b-produced P-glycoprotein has a similar function, the tissue distribution pattern of MDR1a and MDR1b transporters are very different in rodents [311]. In mice, MDR1a is predominantly expressed in the gastrointestinal tract and the heart [26, 311], while MDR1b is predominantly expressed in ovary, placenta, kidney, and liver [311]. A previous study in Wistar rats indicated that cardiac expression of the MDR1a gene is more specifically located in the cardiac myocytes compared to other cardiac cells [312]. A similar study suggested that P-glycoprotein is present in cardiac muscle with immunostaining only in the

mid-myocardium but not endocardium and epicardium using specific MDR1 antibodies [313]. In addition, age and gender differences are also an important influence in MDR1a versus 1b expression pattern and/or levels [311]. However, little is known about the endogenous specific regulation of MDR1a and 1b expression in rodents versus MDR1 in human [182]. Evidence also suggests that P-glycoprotein derived from MDR1a and MDR1b is different in terms of resistance to doxorubicin [308, 314]. Here, FGF-16 was shown to specifically upregulate MDR1a but not MDR1b mRNA levels in neonatal rat cardiac myocytes, while FGF-2 and doxorubicin can upregulate both MDR1a and MDR1b [26] (**Figure 31**). Thus, the specific regulation of the human MDR1 homolog rat MDR1a but not MDR1b by FGF-16 is consistent with different regulatory mechanisms for MDR1a and MDR1b, and thus for multidrug resistance.

In addition to the effect of FGF-16 on efflux drug transport, there may also be an independent effect of FGF-16 on influx drug transporters. The net intracellular substrate concentration indicates a balance between drug influx and efflux, and this could be changed due to regulators of drug transporters including doxorubicin and FGF-16. Thus, FGF-16 overexpression could decrease the influx/efflux ratio, while FGFR inhibition may reverse this effect. Copper influx transporter 1 degradation has been linked to alkylating agent Cisplatin resistance in cancer patients [315], and is required for FGF signaling [316]. An organic cation transporter, SLC22A16, has been characterized as a doxorubicin importer [317]. Thus, FGF-16 could also decrease doxorubicin intracellular concentration by limiting doxorubicin influx in addition to increasing efflux. Regardless of the level of influx, the net intracelluar doxorubicin concentration measured here using substrate calcein and doxorubicin autofluorescence, indicates the overall balance of influx and efflux of doxorubicin.

Observations presented in this chapter suggest FGF-16 can both prevent and protect against doxorubicin-induced cardiotoxicity in postnatal cardiac myocytes including through a specific effect on MDR1a efflux transport. This presents three potential targets to consider in terms of intervention:

(1) To supplement or overexpress FGF-16 in the heart and increase MDR1a levels before doxorubicin treatment, thereby preventing or reducing doxorubicin entry and facilitating more drug removal in the heart. Cardiac myocytes would have more efflux transporters and thus pump activity before doxorubicin treatment. Adenoviral overexpression of FGF-16 in neonatal rat cardiac myocytes increased resistance to doxorubicin-induced cell death. However, the effect of increased FGF-16 on cardiac fibroblasts and cancer cell models still needs to be addressed, to assess the potential for systematic side effects, for example, in terms of promoting proliferation and developing multidrug resistance;

(2) To prevent the initial decline of endogenous FGF-16 and MDR1a by doxorubicin treatment, thereby protecting the heart from early and specific doxorubicin-induced cardiac damage. This means maintaining the ability of the cardiac myocyte to defend itself by removing doxorubicin out of the cell effectively at an early stage before doxorubicin-induced multidrug resistance develops. Manipulating mediators such as Csx/Nkx2.5 can rescue decreased FGF-16 levels by doxorubicin, and thus, can offer potential cardioprotection by maintaining FGF-16 levels and efflux transport ability;

(3) MDR1 is also present in the endothelial cells of capillaries and arterioles which is similar to the P-glycoprotein expression pattern in the blood-brain barrier; thus, MDR1 could also serve as a functional barrier between the blood and cardiac muscles [318]. In addition, single nucleotide polymorphisms in the MDR1 gene can affect the expression and function level of P-glycoprotein [318]. Therefore, the expression of P-glycoprotein is genotype dependent and could be used as an important modulator of the action of its substrate drugs such as cardiac and chemotherapy drugs [318].

Using FGF-16 to "condition" cardiac myocytes with increased efflux drug transport in the heart is beneficial by reducing intracellular doxorubicin levels and thus doxorubicin-induced cardiotoxicity [26]. However, efflux drug transporters are also involved in multidrug resistance in cancer cells, and an increased efflux drug transport means cancer cell survive and chemotherapy treatment failure [302]. Although the cardiac-specific expression pattern and limited proliferative effects of FGF-16 are favorable, the effect of FGF-16 in cancer growth, survival and multidrug resistance development requires further study.

Chapter 7: Conclusions

The contribution of FGF-16 to neonatal rat cardiac myocyte viability as well as the cardiac function was studied here under normal and injury conditions *in vitro*, specifically as a result of exposure to the chemotherapy drug doxorubicin. Observations made, combined with existing data, suggest that FGF-16 is preferentially expressed by cardiac myocytes after birth [79], and that FGF-16 has both cardiac myocyte maintenance and cardioprotective properties, including against doxorubicin cardiotoxicity (**Figures 24** and **26**) [80, 86, 90, 138]. FGF-16 synthesis is decreased rapidly and specifically within 6 hours in response to doxorubicin, largely through a negative effect on gene expression and RNA levels (**Figures 11** and **12**). As a consequence, cardiac myocyte viability is compromised, thereby decreasing resistance to damage, which is expected to contribute to doxorubicin-induced cardiotoxicity *in vivo*. Overexpression of or supplementation with FGF-16 increased resistance to doxorubicin-induced injury in neonatal rat cardiac myocytes and an isolated rat heart model *in vitro* (**Figures 26** and **27**). As a result, maintaining endogenous FGF-16 levels or increasing FGF-16 availability or signaling are all expected to offer some protection of the heart against doxorubicin-induced injury *in vivo*.

Insights were also obtained into the mechanisms involved in the rapid reduction in FGF-16 synthesis, and potential contribution to the cardiotoxic effects associated with doxorubicin treatment. A highly conserved Csx/Nkx2.5 site was identified in the human, rat and mouse *Fgf-16* promoter region (**Figures 6** and **7**), and Csx/Nkx2.5 and this region were shown to regulate *Fgf-16* promoter activity (**Figures 17**). This site, which includes TATA sequences, was shown to support Csx/Nkx2.5 binding *in vitro* and in neonatal cardiac

myocytes *in situ* (**Figures 18**). Csx/Nkx2.5 availability and association with the *Fgf-16* promoter region were decreased in response to doxorubicin treatment (**Figure 19** and **20**). This identified increasing Csx/Nkx2.5 levels as a target to maintain FGF-16 availability, and protect against doxorubicin-induced damage (**Figure 16**). In support, FGF-16 mRNA levels and doxorubicin-induced damage was partially rescued with Csx/Nkx2.5 overexpression (**Figure 22**). However, while consistent with the protective effects of FGF-16 observed, this cannot be considered established as additional downstream target genes of Csx/Nkx2.5 may also be rescued and provide protection against doxorubicin-induced cardiac damage.

In terms of possible mechanisms related to maintenance or protective function, FGF-16 specifically regulated MDR1a mRNA levels, and efflux transport, including of doxorubicin, in neonatal rat cardiac myocytes, and thus potentially in the heart (**Figures 31-34**). Efflux transporters are required for normal physiology and removal of body metabolites, but also mediate removal of disease-causing pathogens and drug resistance, like doxorubicin [183, 193, 194]. A consequence of the rapid decrease in FGF-16 availability in response to doxorubicin treatment is an increase in intracellular doxorubicin concentration, thereby presumably contributing to its cardiotoxicity (**Figures 12** and **24**). Thus, as expected, FGF-16 overexpression offered protection to neonatal rat cardiac myocytes against doxorubicin treatment, which was associated with an increase in efflux transport and a reduction in intracellular doxorubicin concentration (**Figure 32** and **34**).

Chapter 8: Future Directions

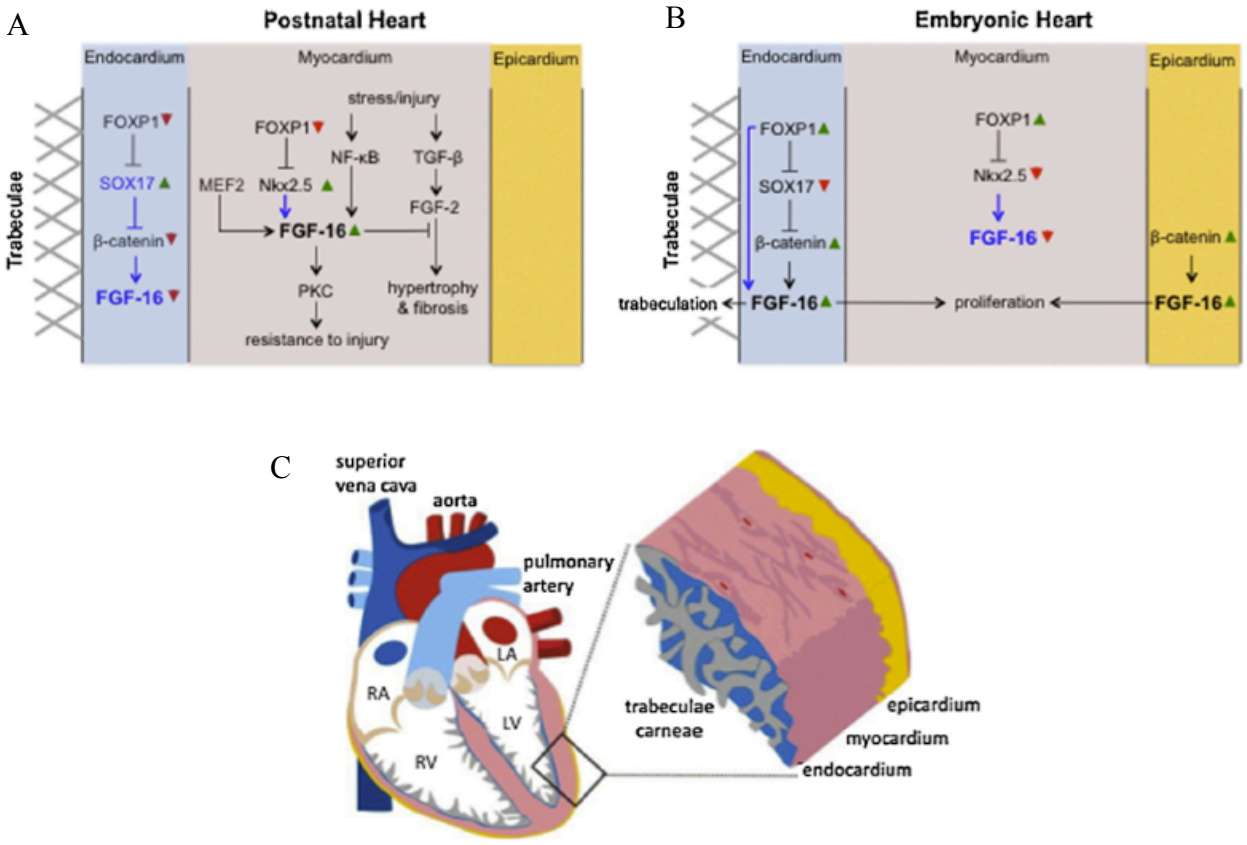
Based on the observations in this thesis, the opportunity is taken to further discuss additional aspects of FGF-16 biology that have been touched through observations made during these tudies, related to: (i) embryonic versus postnatal heart expression of FGF-16; (ii) potential FGF-2 and FGF-16 relationship; (iii) the identification of a pre-multidrug resistance stage; (iv) the unconventional release of FGF-16; (v) FGF-16 and cancer cells; and (vi) human FGF-16.

(i) The regulation of *Fgf-16* gene expression in the embryonic and postnatal heart

FGF-16 has a different cellular site of expression in the heart before and after birth [79, 133, 319]. Before birth, FGF-16 is mainly expressed in the epicardium and endocardium under the regulation of the FOXP1-Sox17-Wnt/β-catenin signaling pathway (**Figure 39**) [133, 319]. It has been suggested that FGF-16 is released from the epicardium and endocardium and acts on the myocardium, where it plays an important role in embryonic heart development by promoting cardiac myocyte proliferation [79, 105]. In support, evidence of decreased myocardial formation has been described in FGF-16 null Black Swiss mice [114]. FOXP1 regulates the balance of cardiac myocyte proliferation by targeting different pathways in the endocardium and myocardium [133, 320]. In the myocardium, FOXP1 represses Csx/Nkx2.5 expression, thus based on the observations made here, presumably inhibiting FGF-16 expression in cardiac myocytes (**Figures 18** and **20**) [133]. FOXP1 protein is also expressed at higher levels during embryogenesis than postnatally [225]. Thus, decreased FOXP1 levels after birth may facilitate an increase in Csx/Nkx2.5 availability in

the myocardium leading to increased FGF-16 levels in the postnatal heart (**Figure 39**). The regulation of two different FOXP1 signaling pathways in the endocardium and myocardium may explain why there is a "shift" and increased expression of FGF-16 after birth [79, 105]. An initial study to explore this relationship would be to compare the association of Csx/Nkx2.5 with the proximal *Fgf-16* promoter binding site by ChIP assay in embryonic cardiac myocytes during mid and late gestation, versus neonatal and adult stages. It is predicted that binding will be seen postnatally but will be absent or at low levels in the embryonic cardiac myocytes. If the results are as expected, embryonic cardiac myocytes could be isolated and FOXP1 siRNA knockdown could be attempted to see if Csx/Nkx2.5 availability increases, binding to the FGF-16 promoter occurs and FGF-16 mRNA levels increase.

Figure 39. Schematic representation summarizing the expression and suggested role of FGF-16 in the embryonic and postnatal heart



The schematic includes a section through the mammalian heart and identifies the epicardial, myocardial and endocardial layers of the heart wall. Signaling pathways (Wnt/β-catenin, PKC signaling, TGF-β and high molecular weight FGF-2) and transcription factor (FOXP1, MEF2, Csx/Nkx2.5, NF-κB) linked to expression and suggested functions (myocardial development, trabeculation, response to stress/injury) are also shown. Pathways for which there is evidence are indicated with black arrow/type and those suggested but not demonstrated are shown in blue. Relative (embryonic versus postnatal) levels are indicated with green (higher) and red (lower/not detectable) arrowheads. *(Reproduced with permission from Elsevier, Cytokine & Growth Factor Review* [105]*)*

(ii) A potential inter-relationship between FGF-16 and FGF-2

The principal cellular sites of FGF-2 and FGF-16 production and release in the postnatal murine heart are the cardiac fibroblasts and myocytes, respectively [79, 85]. This raises the potential for crosstalking between these factors from different cell types. FGFR1c is predominantly expressed in cardiac myocytes [88]. Previous studies suggest that FGF-16 can compete with FGF-2 for binding to FGFR1, and can also inhibit FGF-2 activation of PKC α and PKC ε signaling pathways [79]. Observations made here, however, indicate that exogenous addition of FGF-16 offers protection against doxorubicin-induced cardiotoxicity and is sensitive to chelerythrine treatment (**Figures 27** and **29**) [90]. This may reflect a differential activation of downstream PKC signaling from FGF-16 compared to FGF-2. PKC isoforms other than α and ε have been linked to cardioprotection, such as PKC delta (δ) [321]. For example, FGF-16 may activate other PKC isoforms signaling while inhibiting PKC α and ε signaling, or FGF-16 could compete with FGF-2 binding to FGF receptors and thus inhibit downstream PKC signaling activation. Another possible explanation is that this may reflect a non-specific effect due to the high level of recombinant FGF-16 protein; FGF-16 is subject to posttranslational modification when secreted [79], which could affect stability [322]. Binding in this manner, even if it provides a weaker signal than FGF-2, might activate FGFRs like FGF-2 and offer cardioprotection because it is in excess. When not in excess and added at lower amounts, FGF-16 might still presumably competes with FGF-2 for receptor binding, but the weaker signal results in reduced activation compared to FGF-2 alone. In addition, binding to the same FGFR might have differential effects from the FGF ligands. Both FGF-7 and FGF-10 can activate FGFR2b, however, binding to the same

receptor led to different downstream signaling patterns due to differences in internalization and recycling [323]. FGF-2 and FGF-16 may also have very different downstream effects other than PKC signaling by binding to the same FGFR1 [79, 90]. In addition, FGF-16 has been reported to have hight affinity to FGFR3c in BAF3 (mouse pro-B cell line) [103]. Thus, a diversion of signaling by FGF-16 binding to FGFR3c could also lead to differential effects seen with PKC signaling and other downstream pathways related to FGFR signaling.

The best evidence for a reciprocal relationship between FGF-2 and FGF-16 comes from models of cardiac injury. When challenged with angiotensin II and a prolonged increase in blood pressure, the C57BL/6 FGF-16 null mouse showed enhanced cardiac hypertrophy and fibrosis that was linked to FGF-2-induced TGF-β signaling [169]. Similar angiotensin II treatment of FGF-2 null mice (lacking both 18 kDa and high molecular weight isoforms), resulted in dilated cardiomyopathy but failed to increase cardiac mass in response to the elevated blood pressure compared to control mice [324]. These data are consistent with the ability of FGF-16 to interfere with the FGF-2-induced hypertrophic and fibrotic process [169]. There is also evidence that FGF-2 can stimulate its own and FGF-16 synthesis in cardiac myocytes [169, 292]. However, FGF-16 did not affect FGF-2 expression in both cardiac myocyte and fibroblast cultures in the absence of cardiac stress or injury [169].

FGF-16 pretreatment resulted in a reduced infarct size in a diabetic myocardial infarcted mouse heart model [80]. In addition, pretreatment was also associated with significantly decreased infiltration of monocytes [80], suggesting that FGF-16 plays a role in post-myocardial infarction inflammation [80]. Chronic overexpression of FGF-2, resulting in increased 18 kDa but also high molecular weight isoforms, was shown to exacerbate the

cardiac inflammatory response and increase T-cell infiltration [240]. This raises the possibility that FGF-16 might limit an inflammatory response related to FGF-2. Based on the above, the relationship between FGF-2 and FGF-16 may depend on dosage, bioavailability and tissue distribution. Thus, further studies may be warranted to titrate FGF-2 and FGF-16, for example, through viral overexpression to optimize for cardioprotection during cardiac remodeling and using inflammation, for example, cellular infiltration, as a measure.

Although both protective during doxorubicin-induced injury [90], FGF-16 appears to have opposite expression pattern or effect compared to FGF-2 signaling [79, 169]. Thus, further investigation of the mechanisms of FGF-2 and FGF-16 crosstalking or interaction may provide further insight in maximize the benefits of using FGFs as cardioprotective agents.

(iii) FGF-16 and the pre-multidrug resistance stage

The identification here that FGF-16 increases MDR1a mRNA levels and efflux transport activity in cardiac myocytes before multidrug resistance occurs, provides another potential stage to offer cardioprotection. A decrease in MDR1a mRNA levels was observed with siRNA "knockdown" of FGF-16, while doxorubicin inhibited FGF-16 transcription (**Figures 11, 12** and **37**). MDR1a mRNA levels in the heart after one bolus of doxorubicin injection *in vivo* first decreased rapidly at 6 hours before significantly increasing at 24 hours. Increased multidrug resistance is intrinsic to the heart during doxorubicin treatment. The upregulation of MDR1a with FGF-16 addition appears less significant when compared to doxorubicin-induced multidrug resistance. Thus, the use of FGF-16 to offer

cardioprotection at least by increasing multidrug resistant protein levels at the stage when doxorubicin-induced multidrug resistance has already developed, is not predicted to be efficient or beneficial. By contrast, the decrease in MDR1a RNA levels due to a reduction in FGF-16 with doxorubicin treatment is specific and acute. This allows more doxorubicin to get into the cardiac myocytes and cause more damage. Inhibition of this decrease in FGF-16 and MDR1a in response to doxorubicin treatment by using FGF-16 supplementation, is expected to rescue and potentially enhance endogenous cardioprotection at this early stage. Doxorubicin-induced cardiac injury is dose and time-dependent, so less drug entering the cell is expected to result in lower intracellular concentrations and less initial damage. Cardiac myocytes, unlike cancer cells, are post-mitotic [325], thus, the damage induced by doxorubicin is permanent and irreversible [27]. Protection from doxorubicin-related cardiac injury could be offered through multiple mechanisms including increase drug efflux, decrease drug influx, or activation of doxorubicin-induced cell damage repair mechanisms [326]. However, while reducing initial doxorubicin entry into cardiac myocytes is expected to be beneficial to overall cardiovascular health in the long run, this would not be the opposite for cancer cells, where increased influx and cell death is desired. A higher concentration of doxorubicin may kill more cancer cells but would induce higher endogenous multidrug resistance protein expression in the surviving cell, which upon cell division becomes more resistant to doxorubicin treatment due to the increased drug efflux. Thus, although high endogenous multidrug resistance levels in cancer cells offers challenges for further doxorubicin treatment, it also provides an indication of the cells already being exposed to high levels of doxorubicin. Thus, the overall MDR efflux effects (net intracellular drug concentration) appear to provide a more accurate standard to maximize the effect of chemotherapy in

treating the cancer, while minimizing the cardiotoxicity in the heart, compared to the actual MDR levels at any particular time. As a result, the idea that the pre-multidrug resistance stage may offer an opportunity for protection could begin to be tested by increasing local FGF-16 levels in the heart. Three possible approaches include: (1) An acute *in vivo* model of doxorubicin injury, as described here (**Figures 9** and **10**), could be pretreated with adenovirus expressing FGF-16 under the control of a cardiac-specific promoter to deliver increased FGF-16 to the heart [86]. (2) Alternatively, direct intramyocardial injections of FGF-16 might be attempted [327]. (3) Thirdly, transgenic mice with an inducible and cardiac specific FGF-16 transgene could also be used to assess FGF-16 and cardioprotection in multiple injury models including doxorubicin treatment.

(iv) The unconventional release of FGF-16

The protein immunoblot for FGF-16 following doxorubicin treatment after FGF-16 adenovirus transduction indicates an increased secretion of the glycosylated 26.5 kDa FGF-16 protein and its possible dimer (50-60 kDa) into the cardiac myocyte culture medium, when compared to that detected after FGF-16 adenovirus treatment alone without doxorubicin treatment (**Figure 26**). This does not appear to be due to doxorubicin-induced plasma membrane damage and cell lysis [328], as there is no equivalent increase in the 19.5 kDa and ~45 kDa FGF-16 isoforms (**Figure 26**). This suggests a possible unconventional release of FGF-16 protein via increased efflux drug transport induced by doxorubicin or FGF-16 itself (**Figure 31**). The efflux drug transporter MRP has been linked to the secretion of another FGF family member, FGF-2 [329]. In addition, secretory vesicles as well as endoplasmic reticulum-Golgi-mediated secretion have all been studied as unconventional

secretion pathways for FGF-2 protein [330]. Like FGF-2, FGF-16 is reported to have no N-terminal single peptide for traditional protein secretion [330], however, deletion of the N-terminal of FGF-16 protein sequence abolished its secretion [124]. Furthermore, an uncleaved bipartite signal sequence with both the N-terminal region and central hydrophobic region all appear to be important for successful secretion of the FGF-16 protein [124]. Thus, overexpression of MDR1a, as well as other efflux transporters, by transfecting or virally transducing postnatal cardiac myocytes may reveal an effect on and thus role in FGF-16 protein release. In addition, efflux drug transporters are involved in the removal of a wide variety of endogenous toxins including metabolic waste products [331]. Thus, increased MDR1a levels from FGF-16 overexpression may also increase resistance to higher metabolic stress in cardiac myocytes. Alternatively, an increase in protein secretion may also reflect an ER stress-related effect and an error in protein folding due to the large amount protein of expression in cardiac myocytes induced by adenoviral overexpressionc [274]. Thus, inhibition of Golgi-ER protein transport using Brefeldin A may offer further insight in the regulation of FGF-16 protein secretion [332].

(v) FGF-16 and cancer cells

As a cardiac maintenance and survival factor, FGF-16 increases resistance to doxorubicin-induced cardiac injury through, at least in part, a positive effect on efflux drug transport [90]. Postnatal FGF-16 is a heparin binding protein that is produced and secreted preferentially by cardiac myocytes [79]. Thus, it is expected to bind locally to the extracellular matrix and act on cardiac cells in the heart [79]. However, FGFRs are widely expressed in many different cancer cell types and FGF signaling plays a key role in regulating cancer

cell proliferation, differentiation, migration and survival [84]. Thus, an increased understanding of the effects of FGF-16 on non-cardiac cells is warranted, if FGF-16 were to be supplemented systemically, and not through modifying endogenous production or local delivery. Specifically, in the context of increasing resistance to doxorubicin-induced damage, the effect of FGF-16 on cancer cell proliferation and efflux drug transport is of interest.

FGFs, including FGF-16, may also protect tumor cells as well as promote tumor cell proliferation, and thus compromise their potential therapeutic cardioprotective benefit [90, 333]. ABCB1, ABCC1, and ABCG2 are all expressed in both normal tissue and cancer cells [334]. The types and levels of efflux drug transporters can be critical to cancer cell survival [335-337]. However, the relative potency of FGF as a cardioprotective agent may still permit its beneficial use in chemotherapy, particularly if the protective and proliferative properties of an FGF can be uncoupled [26, 90]. Uncoupling of these activities has been demonstrated for FGF-2, where modification results in an isoform that confers acute protection while lacking mitogenic activity. As a result, this FGF-2 isoform has the potential to protect the heart from doxorubicin-induced injury, without the risk of stimulating cancer cell proliferation and tumor-associated angiogenesis [26, 90]. Although data suggest a proliferative effect of FGF-2/FGFR related signaling in tumor progression in small lung cancer cells [338], overexpression of FGF-2 downregulated Bcl-2 and promoted apoptosis in human breast cancer MCF-7 cells [339]. Also, while FGF-2 protects cardiac myocytes from doxorubicin-induced damage, exogenous addition of 18 kDa FGF-2 was unable to protect MCF-7 tumor cells from doxorubicin treatment [92]. This suggests that cardiac and cancer cells can respond differently to FGF signaling, refleced by different pattern of FGF and FGFR expression,

and different FGFR specificity/affinity for available FGF(s) [92, 103, 340-343]. For example, FGFR1 is present in both human breast cancer MCF-7 and MDA-MB-231 tumor cell lines [340], but FGFR2 is only present in MCF-7 but not in MDA-MB-231 cells [340]. Aberrant FGF signaling has oncogenic potential by promoting tumorigenesis and progression [84]. On the other hand, several studies suggest that FGFR2 is also tumor suppressive [84]. For example, in the bladder cell line T24, FGFR2-IIIb was downregulated possibily due to epithelial to mesenchymal transition and overexpression of FGFR2-IIIb expression blocks cell proliferation [344]. Thus, depending on the tumor cell origin, different effects of FGF ligand-FGFR signaling on cancer cell growth and survival may be observed.

A positive property in this regard could be the relatively low proliferative effect of FGF-16 when compared, for example, to 18 kDa FGF-2 on neonatal rat cardiac myocytes [79]. Although FGF-16 shares some FGFR binding with FGF-2, when tested in a comparative study with FGF-2, FGF-16 did not stimulate the proliferative potential of cardiac cells [79]. FGF-16 appears to have a higher affinity for FGFR1 and blocks FGF-2 activation of the PKC signaling pathway [79]. While the proliferative activity of FGF-16 has been reported during embryonic development and for some cancer cells in culture, specifically, SKOV3 ovarian cancer cells and NCL-H460 lung cancer cells [345-347], an assessment of cancer cells from a wide range of tissue sources has not been reported. FGF-16 was also reported to enhance the survival of Tera-2 cells by counteracting apoptosis at a concentration of 1-10 ng/ml, while an effect on cell motility was observed when given at higher concentrations [167]. This suggests a differential effect of FGF-16 on cancer cell growth or survival, which is perhaps dependent on the specificity and/or affinity of FGFR binding as a result of ligand concentration. There are over 100 different types of cancers (American Cancer Society

Guideline) based on the original site and cell type. Thus, in addition to effects of FGF-16 on cancer cell proliferation and doxorubicin multidrug resistance, further studies could include the presence, type, and levels of FGFRs in cancer cell lines/types to determine whether this might serve as an indicator of FGF-16 responsive versus non-responsive cancer cells.

Testing FGF-16 in a doxorubicin model of cancer chemotherapy and cardiotoxicity *in vivo* would establish a basis to explore targets and methods of delivery that could be applied clinically. Based on the literature, it is anticipated that colorectal carcinoma CT26 cells would meet the requirements for this study [60, 348]. This allows the use of a syngeneic BALB/c mouse model, and retention of an intact immune system, which is preferred [348]. In addition, CT26 cells: (1) will grow after subcutaneous injection in the left or right flank of syngeneic BALB/c (or athymic) mice [60, 349, 350]; (2) have been extensively used in testing cytotoxic agents and treatments in the context of the CT26-BALB/c mouse model *in vivo*; and specifically was used successfully to assess (3) doxorubicin-induced cardiotoxicity, and (4) cardioprotection [60, 348, 349]. If human tumor cells are to be pursued, then athymic mice xenograft model would be used, as a syngeneic model is not possible. Others have previously examined skin A431 and breast MDA-MB-231 cancer cell growth after introduction as a subcutaneous xenograft, in response to doxorubicin [351, 352]. A doxorubicin treatment regimen that produces a "clinically relevant cardiomyopathy" in nude mice is described [353-355]. For these studies, cancer cells will be tagged through stable transfection with firefly luciferase to allow *in vivo* detection and assessment of growth and effectiveness of treatment through bioluminescence imaging[356]. Mice will be treated with FGF-16, prior to treatment with doxorubicin using an attenuated adenoviral expression vector under the

control of the cardiac troponin T promoter, which has been used successfully by others to express FGF-16 in the heart *in vivo* after the introduction by tail vein injection [86]. Sustained expression up to 28 days was reported with recombination-deficient adenoviral delivery via a single tail vein injection [357]. Heart function, hypertrophy, and morphology will be assessed by echocardiography, heart weight/body weight ratio, and microscopy. Damaged related mRNAs and/or proteins (serum creatine kinase, serum cardiac troponin T, and lactate dehydrogenase) will be determined as indicators of myocardial damage [60]. In addition, drug uptake or influx transporters, as well as efflux drug transporters (MDR1a/b, MDR2, MRP1, and MRP2) would be assessed at the end of the study by qPCR and immunoblotting to further investigate the interaction between doxorubicin and drug transporters.

In summary, FGF-16 is expected to be secreted, bind to the extracellular matrix and signal locally in the heart. In terms of satisfying the specific characteristics of a "good candidate" for investigation as an endogenous cardioprotective agent, insight into whether FGF-16 will offer little or no interference with the negative effect of doxorubicin on tumour cell growth and survival (under conditions where protection of cardiac cells is observed) is desirable.

(vi) Human FGF-16

The conservation of the FGF-16 gene and chromosome location, its apparent regulation by developmental and cardiac-specific transcription factors (e.g., Csx/Nkx2.5 and GATA4), as well as results with zebrafish and mice are consistent with a significant

role for FGF-16 in embryonic heart development [105, 108, 114, 138, 166]. Suppression of *Fgf-16* expression in zebrafish affected the pectoral fin bud development in 46% of the 101 embryos examined compared to heart edema in 76%; this phenotype was rescued by FGF-16 mRNA injection [105, 166]. FGF-16 null Black Swiss mice are embryonic lethal at day 11.5, by which time there is evidence of thinning of the atrial and ventricular walls as well as chamber dilation [105, 114, 132]. An equivalent role in human development is less clear. In support, an increased incidence of cardiovascular abnormalities including atrial fibrillation and myocardial infarction was observed in a family carrying a nonsense mutation in the FGF-16 gene [105, 166]. However, more commonly, mutations in exons 1, 2 or 3 of human *Fgf-16* result in X-linked recessive hand malformations, with a fusion between the fourth and the fifth metacarpals (MF4) and hypoplasia of the fifth digit [105, 166, 358, 359]. Paradoxically, the relatively low incidence of reporting of cardiovascular problems could also indicate a major role for FGF-16, which has not been readily detected because of early embryonic fatality [105]. Equally, however, this same observation might indicate little or no effect, perhaps related to compensation by other family members or factors [105]. Based on the phenotypic differences seen between FGF-16 null Black Swiss and C57BL/6 mice, the genetic background within a species, or the presence of modifier genes or redundancy, may result in a range of outcomes from embryonic lethality to some increased risk for cardiovascular abnormality [105, 132]. In addition, while murine *Fgf-16* contains two gene promoter regions (TATA 1 and TATA2), only one of these (TATA1) is conserved in human sequences (**Figure 6**) [105]. Thus, even though a second more distal promoter maybe present in the human sequences, the potential exists for differences in the pattern or site of *Fgf-16* expression and by extension function between human and murine species [105, 119]. It would be of interest to determine the role of TATA2, however, it is noted that lower levels

of FGF-16 mRNA are detected in the human versus rodent heart based on qPCR analysis [119]. Additional studies on FGF-16 production and function, and specifically in cases associated with cardiovascular abnormalities will help resolve the issue of the relative importance of human versus murine FGF-16 [105, 166].

Chapter 9: Final Comments

FGF-16 is preferentially produced and secreted by cardiac myocytes in the postnatal heart [79]. Based on the findings in this thesis, FGF-16 synthesis is negatively and rapidly affected in response to acute doxorubicin treatment *in vitro* and *in vivo*, and is associated with reduced cardiac cell viability and contractile function (**Figures 9-12**). This is due to decreased cardiac transcription factor Csx/Nkx2.5 availability, *Fgf-16* promoter activity and transcription combined with a relatively short FGF-16 RNA half-life (**Figures 13** and **16-21**). Evidence from this thesis also supported a protective role for FGF-16 against doxorubicin-induced cardiac dysfunction and cardiac myocyte death (**Figures 26** and **27**). In addition, for the first time, a direct role for endogenous FGF-16 in the control of efflux transporter MDR1a and removal of toxic metabolic substrates in the postnatal myocardium was reported (**Figures 31-37**). Thus, (1) the rapid decrease in FGF-16, a survival factor for cardiac myocytes, may contribute to doxorubicin-related cardiotoxicity through a negative effect on drug efflux (**Figures 11, 12, 24, 36** and **37**); and (2) preventing this loss, and/or rescuing or increasing FGF-16 levels may increase resistance to cardiac injury (**Figure 22, 26, 27, 32** and **34**). Together, these data support that FGF-16 or FGF-16-related signaling is a promising therapeutic agent and/or target in offering cardioprotection. Further studies in animal models in which the effects of pre-treatment with FGF-16 on cardiotoxicity and cancer cell chemotherapy can be co-assessed are therefore warranted, and will provide greater insight into the potential of FGF-16-related therapy. With the development of translational research in the FGF field (mainly in Japan and China), the hope then is that this thesis and specifically studies on FGF-16 is a beginning and not an end that will see further exploration of fundamental mechanisms of FGF-16 regulation and function, as well as potential benefits of applying FGF-16 therapeutically in the future.

Chapter 10: References

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