Enhancing Cardiomyocyte Survival in Drug Induced Cardiac Injury

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Abstract

Cardiotoxicity associated with many cancer drugs is a critical issue facing physicians these days and a huge hurdle that must be overcome for a side effects-free cancer therapy. Survival of cardiac myocytes is compromised upon the exposure to certain chemotherapeutic drugs. Unfortunately, the mechanisms implicated in cardiac toxicity and the pathways governing myocyte survival are poorly understood. The following thesis addresses the mechanisms underlying the cardiotoxicity of two anticancer drugs, doxorubicin (DOX) and Imatinib mesylate (Gleevec). Transcription factor GATA-4, has recently emerged as an indispensable factor in the adult heart adaptive response and cardiomyocyte survival. Therefore, the specific aim of this project was to determine the role of GATA-4, its upstream regulators, as well as partners in survival. A combination of cell and molecular techniques done on *in vivo*, and *ex vivo* models were utilized to tackle these issues. In this study, we confirmed the cardiotoxicity of the anticancer drug, Imatinib mesylate and found to be age dependent. GATA-4, already known to be implicated in DOX-induced toxicity, was confirmed as an Imatinib target. At the molecular level, we identified IGF-1 and AKT as upstream regulators of GATA-4. Moreover, we confirmed ZFP260 (PEX-1), a key regulator of the cardiac hypertrophic response, as a GATA-4 collaborator in common prosurvival pathways. Collectively, these results provide new insights on the mechanisms underlying drug-induced cardiotoxicity and raise the exciting possibility that cancer drugs are negatively affecting the same prosurvival pathway(s), in which GATA-4 is a critical component. Therapeutic interventions aimed at enhancing GATA-4 activity may be interesting to consider in the context of treatments with anticancer drugs.

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ACE	Angiotensin converting enzyme
AHF	Anterior/secondary heart fields
AIF	Apoptosis inducing factor
ANF	Atrial natriuretic factor
ANP	Atrial natriuretic peptide
ANT	Adenine nucleotide translocator
AO	Aorta
Ao-Root	Aortic Root
Ao-VTI	Aortic outflow velocity-time integral
ARVC	Arrhythmogenic right ventricular cardiomyopathy
ASD	Atrial septal defect
ASK	Apoptosis signal regulating kinase
At	Atria
ATF	Activating transcription factor
ATP	Adenosine triphosphate
AV	Atrioventricular
AVJ	Atrioventricular junction
AVS	Atrioventricular septum
BAV	Bicuspid aortic valve
BH	Bcl-2 homology
BiP/GRP	BiP/glucose regulated protein
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
BW	Body weight
Ca^{+2}	Calcium ion
CAD	Coronary artery disease
Casp	Caspase
CDK	Cyclin D kinase
cDNA	complimentary Deoxyribonucleic acid
CHD	Congenital heart disease
CHF	Congestive heart failure
CML	Chronic myelogenous leukemia
c-KIT	Receptor for stem cell factor
CO	Cardiac output
CREB	cAMP responsive transcription factor
CYP-D	Cyclophilin D
CYT-C	Cytochrome C
DCM	Dilated cardiomyopathy
DD	Diastolic deplorization
DHPR	Dihydropyridine receptor
DISC	Death inducing signaling complex
DMEM	Dulbecco.s Modified Eagles Medium
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECG	Electrocardiogram

EF	Ejection Fraction
eIF	eukaryotic Initiation factor
EMF	Endomyocardial fibrosis
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERK	Extracellular signal-regulated kinase
FDA	Federal drug agency
FGF	Fibroblast growth factor
FHF	First Heart Field
FOG	Friend of GATA
FS	Fractional Shortening
GATA	GATA binding protein
GIST	Gastrointestinal stromal tumor
GPCR	G protein coupled receptor
GSK	Glycogen synthase kinase
hr	Hour
HA-tag	Haemagglutinin tag
HAT	Histone acetyltransferase
HCM	Hypertrophic cardiomyopathy
HCN	non-specific cation funny channel
HDAC	Histone deacetylase
HER	Human epidermal growth factor receptor
HoS	Holt Oram syndrome
HR	Heart rate
HW	heart weight
IAP	Inhibitor of apoptosis
IAS	Interatrial septum
I_f	Funny current
IFT	Inflow tract
IL	Interleukin
I_k	Potassium current
IRE	Inositol-requiring kinase
IVC	Inferior vena cava
IVS	Interventricular septum
IVSd	Interventricular septum thickness at diastole
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K+	Potassium ion
kb	Kilobase
kDa	Kilodaltons
KI	Kinase inhibitor
KO	Knockout
LA	Left atrium
LAD	Left anterior descending artery
LCA	Left coronary artery
LCX	Left circumflex artery
LV	Left ventricle

LVEDD	Left ventricular end-diastolic dimension
LVESD	Left ventricular end-systolic dimension
LVPWD	Left ventricular posterior wall thickness at diastole
MAPK	Mitogen activated protein kinase
MEF2C	Myocyte Enhancer Factor 2
MEKK	MAPK kinase kinase
MHC	Myosin heavy chain
MI	Myocardial infarction
min	Minute
MKK	MAPK kinase
ml	Millilitre
µl	Microlitre
MLC	Myosin light chain
mM	Millimolar
MPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid
mTORC	mTOR complex
MV	Mitral valve
Na^+	Sodium ion
NCX	Sodium/Calcium exchanger
NFAT	Nuclear factor of activated T-cells
NIH 3T3	Mouse embryonic fibroblast cell line
NKX	Nirenberg and Kim X=Mammalian class
NLS	Nuclear localisation signal
OFT	Outflow tract
PA	Pulmonary artery
p300	300kDa histone acetyltransferase
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDK	Phosphoinositide dependent kinase
Peak E	Early filling wave
Peak A	Atrial contraction wave
PE	Phenylephrine
PERE	Phenylephrine response element
PERK	dsRNA-activated protein kinase-like ER kinase
PEX-1	Phenylephrine-induced Complex-1
PFO	Patent foramen oval
PI3K	Phosphoinositol 3 kinase
PK	Protein kinase
PPN	Peripheral Purkinje network
QPCR	Quantitative-PCR
RA	Right atrium
RCA	Right coronary artery
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
RV	Right ventricle

SA	Sinoatrial
Ser	Serine
SERCA	Sarcoplasmic endoplasmic reticulum Ca^{+2} ATPase
SHH	Sonic hedgehog
SRF	Serum response factor
STAT	Signal transducers and activators of transcription
SV	Sinus venosus
SV	Stroke volume
SVC	Superior vena cava
TAD	transactivation domain
TBX	T-box transcription factor
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
ToF	Tetralogy of Fallot
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TV	Tricuspid valve
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VSD	Ventricular Septal Defect
XBP	X-box binding protein
ZnF	Zinc finger
ZFP	Zinc finger protein

1. Introduction

1.1. Heart Development

1.1.1. The Embryonic Heart

In vertebrates, the circulatory system is the first organ to form in response to the increasing need for a suitable transport of nutrients and waste in the exponentially growing embryo. For instance, in a human embryo, a functioning closed circulatory system driven by a simple cardiac pump is completed just four weeks post fertilization. This simple but efficient cardiac pump will develop into a four chambered, double circuited, autonomously beating heart equipped with valves, septa, and its own circulatory system in the following five weeks, while constantly responding to the growing demands of the developing embryo[1]. In mice, this process is much shorter and in a less than a week, mesodermal cells are transformed into the fully functional four chambered heart. Even at this early stage, where other organs of the mouse embryo are hardly recognizable, the heart is perfectly capable of pumping and supplying blood to the entire embryo [2]. This complex but relatively swift organ morphogenesis has always fascinated scientists who diligently tried to decipher the spatially and temporally controlled cellular and molecular events governing the formation of the most intriguing organ in an organism's body, the heart.

1.1.1.1. Stages of Heart Formation

In mammalian systems, cardiac precursor cells of the primary or first heart field (FHF), originating from endoderm associated splanchnic mesoderm, unite and adopt a crescent shape to form the cardiac crescent. Later, specifically at E8.25 in mice, crescent cells migrate ventrally and converge to form the linear heart tube. This linear tube is composed of an inner endothelial lining surrounded by a single layer of myocardial cells.

The first heart beat is initiated at this stage with blood flowing in a caudal to cranial direction through the linear heart tube. Therefore, the arterial outflow tract (OFT) is originally located cranially, and the venous inflow caudally. The heart tube then elongates and grows through both the division of myocardial cells and the addition of cells from the secondary or second heart field (SHF) originating in the pharynx. The lengthening of the outflow tract, as the secondary heart field cardiac cells are added to it, is crucial to the correct alignment of the aortic trunk with the left ventricle and the pulmonary arterial trunk with the right ventricle [3,4]. The heart tube then loops rightward with the outflow region swinging to the right and the inflow part moving antero-dorsally, in such a way that the outflow and inflow complexes fuse [5,6]. In parallel, morphologically different anterior and posterior regions become progressively defined and ultimately these would develop into ventricles and atria, respectively [7] (**Figure 1.1**).

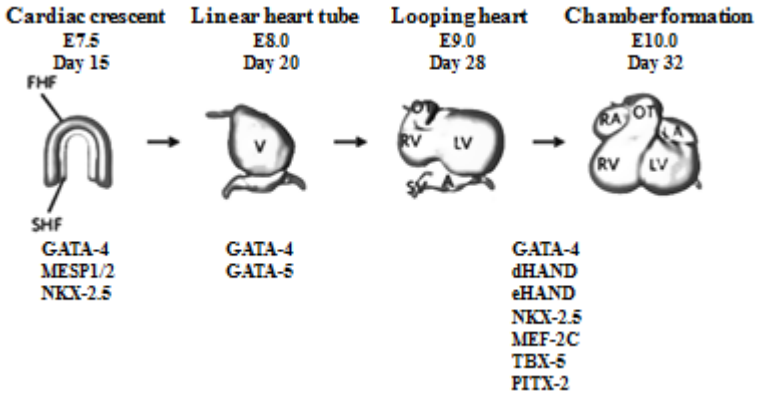


Figure 1.1. Mammalian Heart Development. Heart formation starts with the cardiac crescent and involves two pools of cardiac precursors, the first heart field (FHF) and the second heart field (SHF). The FHF contributes cells to the left ventricle (LV), while the SHF contributes cells to the right ventricle (RV) and later to the outflow tract (OT), sinus venosus (SV), and left and right atria (LA and RA, respectively). Outflow tract (OFT) septation separates the common outflow tract (OT) into the aorta (AO) and the pulmonary artery (PA). A Modified figure from [31,32].

A Complex series of remodeling events are necessary to obtain the structure that contains the four distinct chambers characteristic of a mammalian heart. The formation of right and left atria is established early on and is reflected through the differences in the

lateral mesoderm right and left progenitor pools. On the other hand, ventricles are originally specified along the antero-posterior axis and afterwards will be oriented along the left-right axis due to looping of the heart tube [7]. The formation of ventricles starts early when progenitor cells from both the primary and secondary heart fields migrate to form the linear heart tube. The spatial expression of different cardiogenic genes along the antero-posterior axis of the heart tube is evident at this early stage of development. Once the heart tube has looped and realigned, it differentially grows to form outer and inner curvatures [8,9]. The growing chamber myocardium is phenotypically different from the primary myocardium, such that cells now have gap junctions for rapid conduction and a faster rate of proliferation [10]. Trabeculae, the morphological markers of chamber formation, develop along the ventricular luminal surface from a sponge-like layer of cardiomyocytes. These cells are the power generators of this early heart and are also believed to enhance oxygen uptake by increasing the surface area [5]. The formation and correct positioning of the ventricular myocardium is crucial and likely involves complex signaling along both the cranio-caudal and dorso-ventral axes [8-10].

On the other hand, the formation of the primary atrial septum (*septum primum*) divides the atrial chambers at first, and then a secondary atrial septum (*septum secundum*) formed by an atrial myocardium ingrowth completes the separation. Finally, the interventricular septum (IVS), a single structure, separates the left and the right ventricles. The muscular component of IVS is formed early in development where myocytes of the ventricular wall proliferate at the interventricular groove. Conversely, its mesenchymal component is formed by the fusion of the conotruncal endocardial and atrio-ventricular cushions [11].

The autonomous cardiac conduction system develops around this same period as well. Whereas the slow conducting tissue of the inflow tract (IFT) is at the origin of the cardiac pacemaker (sinoatrial (SA) node), the atrioventricular (AV) node arises inside tissue proximal to the atrioventricular canal. Later on, the bundle of His forms in the IVS and connects to the Purkinje fibre network formed by cells of the ventricular trabeculae. Eventually the heart rate-regulating autonomic nervous system innervates both the SA and AV node [12,13]. Meanwhile, cardiac chamber mass and volume are built through the thickening of the highly proliferative ventricular outer layers. Additionally, the pulmonary and systemic circuits are directed by the endocardial cushions associated with myocardial non chamber areas [13]. Mitral and Tricuspid valves are formed by endocardial cushions within the atrioventricular canal, while the aortic and pulmonary valves are formed by OFT endocardial cushions. These OFT cushions are also at the origin of the aortic-pulmonary septum dividing the OFT into the pulmonary artery and aorta [5]. On the other hand, proepicardial cells migrate from the dorsal body wall to form the epicardium, which is at the origin of the coronary vasculature. This proepicardium originates from mesothelial cells situated close to the liver primordium [14,15]. As the ventricle thickens, O_2 diffusion distance from the ventricular lumen increases triggering the formation of the coronary circulation. This growth dictates the migration and differentiation of epicardial precursor cells. Later, vascular capillary tubes formed by endothelial cells start fusing and growing in a branching pattern. A venous connection is formed by the fusion of a capillary plexus just as it makes contact with the coronary sinus. Afterwards, this venous system acquires its smooth muscle component. The capillary plexus encircling the aortic base fuses and some of its endothelial cells penetrate the aorta at the left and right coronary sinuses [16].

1.1.1.2. Complex Combinatorial Regulation by Transcription Factors

The complex morphogenetic events that help form the chambered four vertebrate heart require a tightly controlled spatiotemporal expression of different genes. This is strictly regulated by the combinatorial interactions of various transcription factors as well as their cofactors [17] (**Figure 1.1**).

Signals from the endoderm and neighboring tissues induce mesodermal cardiac precursor cells to become cardiogenic. Some of these positive-acting signals are, bone morphogenetic protein 2 (BMP2), Crescent, fibroblast growth factor 8 (FGF8), and WNT-11 [18-21]. On the other hand, inhibitory signals seem also indispensable, possibly to limit this induction to a specific group of mesodermal cells [22]. These signals include WNT ligands such as, WNTs 3a and 1, and anti-BMPs such as Noggin [5,7]. On the other hand, the secondary heart field induction is controlled by BMP2 and FGF8, expressed in the outflow tract and caudal pharynx, SHH (Sonic Hedgehog), and WNT/β-Catenin signaling. These in turn induce the expression of transcription factors, such as GATA-4, NKX2.5, and TBX-5 [5,7,23].

In vertebrates, NKX-2.5 transcription factor, which portrays the role of a Drosophila transcription factor called *tinman* [24], is known to be the earliest marker of heart field formation, where it is expressed in the lateral plate mesoderm. Emerging data propose a role for NKX2.5 in later stages of myocyte differentiation, such as spatial and asymmetric regionalization. Moreover, NKX2.5 is known to physically interact with GATA-4 and cooperatively activate the expression of target genes. GATA-4 is a key transactivator of many cardiac promoters including promoters of contractile genes, and similar to NKX2.5, it is expressed in the bilateral cardiac primordia. As the heart develops, the specialization of contractile protein machinery ensues with the expression of chamber specific contractile

genes that are often detectable before any morphologic separation. For example, IRX-4, a homeobox protein, was only detected in ventricular cardiomyocytes, and thus seems to impose a ventricular phenotype over a default atrial pathway [25]. The asymmetric heart looping originates from the asymmetric process of egg fertilization, while the establishment of the left and right axis signaling pathways seem to be coordinated by Vg-1 factor, a TGF-β family member. Vg-1 then induces the expression of sonic hedgehog (SHH) particularly on the left side of the developing embryo. Afterwards, SHH binds to its receptor (PATCHED) and induces the expression of two TGF-β family members (NODAL and LEFTY-2) in the caudal region of the heart. Later, NODAL and LEFTY activate PITX-2, which is exclusively expressed along the developing heart left side [26]. On the other hand, heart tube looping is controlled by the HAND and the MEF-2 families of transcription factors. For instance, the expression of dHAND becomes restricted to the future right ventricle during looping, while the expression of eHAND becomes restricted to the anterior and posterior heart tube regions, which will later eventually give rise to the conotruncus and left ventricle, respectively [27].

The endocardium originates from the endocardial progenitors found at the cardiogenic field periphery. Later, the fusion of the cardiac primordia surrounds these progenitors by the two myocardial layers. Endocardial-specific gene expression is preferentially induced by GATA-5, a GATA family member. Furthermore, the expression of the nuclear factor of activated T-cells C (NFAT-c) in endocardial cells seems to play a crucial role in valve and membranous septa formation [28]. Finally, the adhesion of the pericardium to the myocardium requires the expression of both the epicardial α4-Integrin and the myocardial VCAM-1 cell adhesion molecules [29].

1.1.2. Congenital Heart Diseases

1.1.2.1. Definition and Introduction

Heart formation is an extremely complex process, and as mentioned earlier encompasses a large number of molecular pathways. These signaling pathways, which are controlled by a definite set of factors, require exceptionally complicated regulation mechanisms as well as sophisticated cross talks amongst these cardiogenic molecules. Having said that, it is not surprising to mention that cardiovascular malformations are the largest cause of human birth defects. The majority of these congenital heart diseases (CHD) are either due to incomplete septation of atria, ventricles, and the atrioventricular canal [24], or defective valve formation [30]. These anomalies range from mild subclinical defects like the patent foramen oval (PFO), to more severe life threatening malformations like tricuspid atresia. Mild defects, which usually do not initially affect heart function, can later lead to impaired exercise tolerance like atrial septal defects (ASDs) and pose as cardiovascular risk factors if left undiagnosed [31,32].

1.1.2.2. Causes and Embryology

1.1.2.2.1. Genetic Factors

Most CHDs diagnosed usually have some sort of familial history, although sporadic cases are not that uncommon. Recent advances in molecular screening and better understanding of the mechanisms governing heart development have helped immensely in deciphering the genetic causes behind CHDs. It is now well documented that heart defects can often be linked to several gene loci and that phenotypes usually vary within families having a CHD linked to a particular mutation. Of course, there are some exceptions especially when the mutation is a dominant autosomal trait, like Holt-Oram Syndrome

(HOS), where defects are caused by mutations in TBX5. Moreover, even within the same family, HOS heart symptoms severity varies from mild conduction system defects to more severe structural abnormalities. Therefore, understanding genotype-phenotype associations remain poorly understood and pose a major challenge. One possible explanation is the presence of modifier genes that could contribute to the severity of CHDs [30]. Mutations in a handful of genes encoding cardiogenic transcription factors, like NKX2-5, TBX5, and GATA4, can be linked to a large portion of human CHDs [24,30]. A better understanding of interacting partners, upstream regulators, and downstream effectors and targets will definitely help understand the different elements of diseases and unravel new perpetrator genes. One important observation is that one transcription factor can be linked to multiple defects and that each anomaly can be traced to several genes (**Table 1.1**).

1.1.2.2.2. Environmental Influence

As mentioned earlier, the symptoms linked to a particular CHD can vary within the same family, which clearly stresses the importance of environmental factors. Understanding these gene-environment interactions will further our current understanding of CHDs and enhance patient care and prevention [30]. For example, an embryo intrauterine environment is usually decided by maternal factors including lifestyle, health status, medication, exposure to environmental chemicals, and maternal genotype. These factors will definitely influence the tightly regulated heart development and can explain partly the heterogeneity of CHDs [33].

Table 1.1: Some Cardiac Transcription Factors and Their Associated CHDs.

Transcription Factor	Cardiac Expression	Role	CHD	References
NKX-2.5	Pancardiac	Cardiac specification and patterning Conduction system development	ASD, VSD, Conduction	24,30
GATA-4	Pancardiac	Cardiac differentiation Ventral morphogenesis	ASD, VSD	24,30,53
GATA-5	Pancardiac	Cardiac differentiation Ventral morphogenesis	ASD, VSD	24,28

ASD: Atrial Septal Defect; **VSD:** Ventricular Septal Defect.

1.1.3. Role of GATA Transcription Factors

Vertebrate heart formation is clearly an interplay of complex processes that involve an array of transcription regulators such as the GATA family. This evolutionary conserved family of transcription factors has emerged as a pivotal player in heart development [34,35]. Members of the GATA family specifically and efficiently bind to the DNA consensus (T/AGATAA/G), known as the GATA element [36]. In vertebrates, six GATA family members have been identified to date [34,35,37], of which, GATA-1, -2 and -3 are important transcription regulators of hematopoietic cells differentiation and development [36,38,39]. The other three, GATA-4, -5, and -6, are associated with cardiogenesis, as well as gastrointestinal and gut derived tissues formation [34,35,37,40]. In the following section, the regulation and role of the latter three factors in heart development will be summarized.

1.1.3.1. Protein Structure and Expression Patterns

All GATA proteins identified to date have a DNA binding domain comprised of two highly conserved zinc fingers (ZnFs) with a Cys-X_2-Cys-X_{17}-Cys-X_2-Cys consensus, X being any amino acid [41]. The C-terminal ZnF along with an adjacent basic domain are necessary for sequence recognition and binding to the DNA motif. On the other hand, the N-

terminal ZnF is only required for sequence specificity and stability of protein-DNA interaction and does not bind DNA directly [41,42]. Moreover, both the N-terminal and the C-terminal ZnFs can interact with other regulatory proteins, like KLF-13, NKX2.5 and MEF-2 [43-45]. GATA-4, which is the most extensively studied cardiac GATA factor, contains a nuclear localization domain (NLS) [42]. In addition, the N-terminal of GATA-4 contains two separate transcription activation domains (TAD1 and TAD2), which share a significant homology with the other cardiac GATA factors [42]. The three GATA proteins expressed in the heart share a great structural homology which may explain the observed functional redundancy and similarities in transcription activation mechanisms [42,46,47]. GATA genes genomic structures are highly conserved; for instance, all vertebrate GATA genes possess one or more non-coding exon(s) and the zinc fingers are encoded on two separate exons (**Figure 1.2**).

In addition to their expression in the developing heart, cardiac-expressed GATA factors are also expressed in many endodermal and mesodermal derivatives [35,37,42,46]. In the mouse embryos, cardiac GATA factors are all expressed in the precardiac mesoderm as early as embryonic day E7-7.5 [46-48]. The expression patterns of GATA-4 and GATA-6 are almost identical and their levels are highest in the posterior regions of the forming heart [46,48]. However, their expression patterns start diverging as cardiogenesis progresses, and by E11.5, GATA-4 transcripts are far more abundant throughout the developing atria and ventricles. Furthermore, between E12.5 and E13.5, GATA-6 but not GATA-4 transcripts are detectable in the vascular smooth muscles of the caval veins and dorsal aorta [49]. The cardiac expression of these two factors persists into the adult heart, with mainly GATA-4 expressed in the endocardium and myocardium [46]. Although the expression pattern of GATA-5 initially overlaps with that of the other two factors, by E12.5, its expression becomes largely

restricted to the atrial endocardium. And by E16.5, GATA-5 transcripts are undetectable within the mouse heart [47].

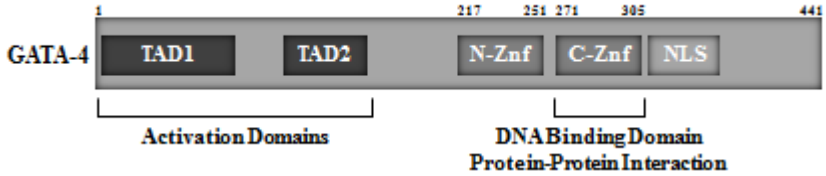


Figure 1.2. GATA-4 Schematic Structure. TAD, transactivation domain; **N-Znf**, N-terminal Zinc finger; **C-Znf**, C-terminal Zinc finger; **NLS**, nuclear localization domain (Morrisey 1997, Charron 1999, Molkentin 2000).

1.1.3.2. Function in Cardiac Specification, Differentiation and Morphogenesis

As mentioned above, GATA factors are co-expressed within the developing heart**,** and given their highly similar DNA-binding domains, it would not be surprising to see some functional redundancy in cells in which more than one family member is expressed [34,37]. Furthermore, GATA-4 and -6 essential roles in extracardiac tissues early in embryogenesis have complicated the study of GATA factors in mammalian heart development. For instance, GATA-4 null as well as GATA-6 null mice die prior to, or during early cardiogenesis. Surprisingly, these embryos are able to develop cardiomyocytes and hence the ablation of GATA-4 or GATA-6 is not sufficient to block heart formation initiation or precardiac cells differentiation [49,50]. To the contrary, GATA-5 knockout mice are viable, although recently we reported a significant incidence of bicuspid aortic valve (BAV) in these mice as well as in mice with the endocardial-specific inactivation of GATA-5, associated to a defective endocardial cell differentiation [51]. On the other hand, gain-of-function experiments have shown that ectopic expression of either GATA-4 or -5 is sufficient to induce contractile proteins that are cardiac specific [52].

The formation of the vertebrate multi-chambered heart requires a series of morphogenetic events orchestrated by various transcription factors like the GATA factors. In contrast to the inability of GATA-4 ablation to inhibit cardiac formation initiation, GATA-4 null rescued embryos display a variety of cardiac morphogenetic defects and usually die by E10 [53]. On the other hand, GATA-6 null rescued embryos, which also die by E10.5 and show impaired cardiovascular function, suffer a severe block to liver development [54]. In addition, data from conditional ablation studies in mice underline the important role played by GATA-4 in regulating cardiac myocyte proliferation and right ventricle and atrioventricular canal morphogenesis [55].

1.1.3.3. Interaction with Other Transcription factors

Complex interactions between various transcription factors orchestrate gene expression in a spatio-temporal manner to ensure proper heart formation. GATA factors are key players, and can interact with various transcription factors with either a net positive or a net negative effect on their transcriptional activation strength. This ensures specificity within cells in which the interacting factors are co-expressed.

A physical interaction exists between the C-terminal ZnFs of GATA-4 or -5 and NKX2-5, translated into a positive synergistic activation of a subset of cardiac promoters. On the other hand, GATA-4 or -6 but not -5 can interact with MEF-2 proteins via the C-terminal ZnF and cooperatively activate gene transcription [44,45]. Other positive C-terminal ZnF interactions include SRF [56], NFAT-3 [57], d-HAND [58], and many more. Interestingly, both ZnFs of GATA-4 are needed for its cooperative interaction with GATA-6 on the ANF and BNP promoters [34]. GATA-4 can also synergize with some transcription factors through domains that are distinct from the ZnFs [59]. Conversely, many factors like FOG-2 can interact

with GATA factors and negatively modulate their transactivation ability [60]. In essence, GATA factors serve as key nuclear effectors of various growth and differentiation processes and their differential interactions with other proteins confer spatio-temporal functional specificity.

1.1.3.4. Regulation of the Activity of GATA Factors

GATA factors activity can be either regulated at gene expression level, posttranslationally, or upon the binding of modulatory cofactors. Complex gene regulation mechanisms control the likewise intricate GATA factors expression patterns. Important aspects of these regulatory mechanisms are the presence of more than one transcription initiation site as well as the existence of enhancers that modulate and direct their spatial and stage dependent expression. For instance, mouse *Gata-6*gene is flanked by regions that contain few Sp1 potential binding sites, several GATA elements, two E-box elements and an NK element (NKE) [61]. Moreover, three enhancer regions has been associated with *GATA-6* gene flanking region enriched with GATA and MEF binding sites [62]. As for *GATA-4* gene regulation, studies in zebrafish identified a 14.8 kb genomic fragment, in which T-box enriched distal sequences direct expression to heart posterior regions and proximal ones are sufficient for its expression in anterior regions of the heart [63].

GATA-4 activity can be modulated by a number of posttranslational modifications, like the phosphorylation of Thr100 and Ser105 within the N-terminal transactivation domain by ERK and p38 MAPK [24]. Ser105 phosphorylation, which enhances both GATA-4 DNA binding and transactivational activity, plays an essential role in the cardiac myocytes hypertrophic growth response and survival [24,64]. Moreover, GATA-4 activity has been reported to be increased upon phosphorylation by PKC and PKA [65,66].

Conversely, phosphorylation of the N-terminal by GSK-3β has a negative impact on GATA-4 activity apparently by stimulating GATA-4 nuclear export [67]. Furthermore, GATA-4 acetylation by p300 has been shown to mediate embryonic stem cells differentiation into cardiac myocytes as well as to play a role in primary cardiomyocytes hypertrophy [67,68]. Surprisingly, there have been no reports on the posttranslational modification of either GATA-5 or GATA-6 [59].

1.2. Mammalian Postnatal Heart

1.2.1. Anatomy and Function

The mammalian adult heart is a four-chambered restless organ that pumps deoxygenated blood to the lungs and oxygenated blood to all body organs. In this section I will focus on the anatomy of mammalian heart, mainly mouse and human, and describe the functions of its different components (**Figure 1.3**).

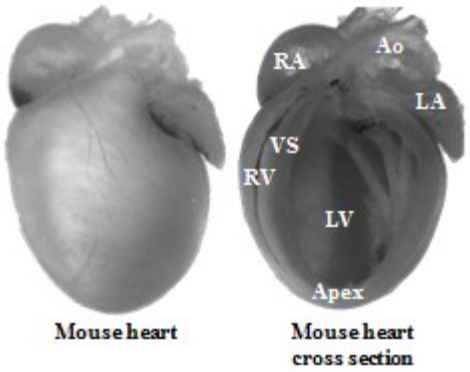


Figure 1.3. A Mouse Heart. The four chambered mouse heart is made of the right atrium (**RA**), right ventricle (**RV**), left atrium (**LA**), and left ventricle (**LV**). **Ao**, aorta; **VS**, ventricular septum.

1.2.1.1. Gross Anatomy

The human and mouse postnatal hearts are very similar anatomically. Both are four-chambered with the two atria separated by an interatrial septum (IAS) and the two ventricles

by the interventricular septum (IVS). A small atrioventricular septum (AVS) located between the IAS and IVS separates the subaortic left ventriclular outlet segment from the right ventricle. The human AVS is known as the membranous septum owing to its thin fibrous structure, while the mouse one is mostly thick and muscular [69,70]. Each atrium is connected to its respective ventricle via the atrioventricular junction (AVJ) where the atrioventricular valves are situated. The left atrium (LA) is separated from the left ventricle (LV) by the two-leaflets mitral valve (MV, bicuspid), while the right atrium (RA) is separated from the right ventricle (RV) by the three-leaflets tricuspid valve (TV). These valves are connected to papillary muscles via numerous chordae tendineae, which are more pronounced in the human heart than in the mouse heart. The ventriclular inner lining is characterized by myocardial protrusions, the trabeculae (trabeculae carneae). The thickness and shape of the IVS is slightly different between human and mouse; for instance, the human massive muscular IVS thickness can exceed that of the free wall of the left ventricle and morphologically has a very wide base at the AV valves attachments [70]. On the other hand, the mouse IVS is not as big and compact and usually its base narrows toward the AV septum [69].

The heart serves as the engine of the circulatory system and therefore, major blood vessels connect the heart to the rest of the body. These vessels are either arteries that take blood away from the heart ventricles or veins that empty into the heart atria. The aorta, an artery, carries oxygenated blood from the left ventricle to the entire body organs. On the other hand, the pulmonary artery transports deoxygenated blood from the right ventricle to the lungs. As for veins, both the superior and inferior caval veins transporting deoxygenated blood from body organs empty into the right atrium of the heart, while four pulmonary veins empty O_2 rich blood into the left atrium to complete the cycle. Anatomically, the venous

components of the human and the mouse cardiovascular systems are slightly different. Whereas the four pulmonary veins connect individually to the left atrium of the human heart, they join into a single pulmonary confluence just before emptying into the mouse left atrium [69,70].

Finally, the heart has its own drainage system known as the coronary circulation. Coronary arteries supply the heart with oxygenated blood while coronary veins collect blood from the heart. Two major arteries originate from the right and left aortic sinuses known as the right coronary artery (RCA) and the left coronary artery (LCA), respectively. The LCA bifurcates into the left anterior descending (LAD) and left circumflex (LCX) branches and all supply most of the left atrium, left ventricle, IVS, and AV bundles. On the other hand, the RCA, a single large artery, supplies the right atrium, right ventricle, IVS, and the sinoatrial (SA) and AV nodes. Furthermore, deoxygenated blood from the left side and most of the right side of the heart is drained by the great cardiac vein, and middle and small right cardiac veins, respectively. These veins drain into the coronary sinus, a short but wide venous channel that opens between the inferior vena cava (IVC) orifice and the right AV orifice. The right ventricle venous return drains directly into the right atrium [71].

1.2.1.2. Heart Chambers and Their Specialized Functions

As mentioned earlier the mammalian heart has four chambers, namely, RA, RV, LA, and LV. The RA internal wall is smooth posteriorly, and ridgelike and muscular anteriorly. It receives deoxygenated blood from the SVC, IVC, and coronary sinus. Moreover, the sinus node or the sinoatrial (SA) node, the heart pacemaker, is located within the RA wall near the SVC orifice, while the atrioventricular (AV) node is located on the central fibrous body right atrial side in the AV septum muscular portion. The RA drains

blood to the RV across the tricuspid valve, which is situated in the inflow tract (sinus) of the RV. Blood is then discharged into the pulmonary artery across the semilunar pulmonic valve located in the outflow tract (infundibulum). The sinus and the infundibulum of the RV are situated far from each other and both contain coarse trabeculations. On the left side of the heart, the LA has a narrow and long appendage that is usually its only trabeculated structure. The LA receives oxygenated blood from the four pulmonary veins and empties into the LV across the mitral valve contained within the large sinus portion. Then blood is ejected across the aortic valve into the aorta located in the small left ventricular infundibulum. The trabeculated sinus and the smooth infundibulum of the LV are closely juxtaposed. The thick muscular LV free wall generates the power needed to pump blood, against resistance in the arterial system, to the rest of the body [72].

1.2.1.3. Cellular Composition of the Heart

The heart is not a homogeneous tissue made of one type of cells; rather, it is a heterogeneous organ composed of a variety of specialized cells. In addition to the cardiac myocytes, which make up one third of the total cell number, the heart is composed of fibroblasts, endothelial cells, phagocytes, vascular smooth muscle cells, epithelial cells, nerve fibers and a surrounding extracellular matrix [73-75].

The developing heart initially consists of developing muscle cells, but later in embryonic life the myocardium assumes its heterogeneous structure. The majority of the mature myocardium is comprised of cardiac myocytes, the contractile cells of the heart. In addition, fibroblasts, which are also abundant, play an important role in myocardial repair and are hugely responsible for post-myocardial infarction (MI) scar tissue formation [73,76,77]. Furthermore, the heart is innervated and thus nerve fibers are also present. These nerve fibers

are usually in conjunction with the specialized conduction system and serve to regulate heart rate and myocardial contraction. Additionally, immune cells like phagocytes, which engulf debris, are often seen around blood vessels and between fibers. The heart is a highly vascularized organ and thus it is rich in vascular endothelial and smooth muscle cells. On the other hand, the epicardium is also composed of different cell types including epithelial squamous or low cuboidal cells, fibroblasts, mesenchymal cells, and nervous fibers. Finally, the endocardium which lines the interior cavities of the heart, is composed of a layer of connective tissue covered with a sheet of endothelial cells. These endothelial cells are continuous with and largely similar to the endothelial cells of the vascular system [73].

1.2.2. Cardiac Physiology

1.2.2.1. The Heart as a Muscle

The heart performance as a pump is largely dependent on the contractile activity of its function as a striated muscle. Furthermore, heart muscle contraction is a reflection of the summated and integrated function of its different contractile elements. In the following section I will be describing the myocardial ultrastructure before discussing the physiology of cardiac contraction.

1.2.2.1.1. Microanatomy of the Myocardium

The heart muscle is made of many interconnecting, branching cells (fibers) usually 5-10 μM in diameter. These fibers are connected end to end by modified cell membranes known as the intercalated disks. On the other hand, the cytoplasmic membrane along their longitudinal portions is called the sarcolemma. The electrical resistance across the intercalated disk is pretty low and thus the myocardium acts as a functional syncytium.

Irregular rodlike fibrils run the length of the fiber and are in turn made of regular repeating sarcomeres giving the fibrils their characteristic cross-banded appearance. The sarcomere, the basic unit of contraction in the myocardium, is made of a thicker set of contractile proteins (myofilaments) composed of myosin, and a thinner set of myofilaments composed primarily of actin. These myofilaments are fairly rigid, but a series of repetitive reactions between specialized sites along the two sets lead to force development and sarcomere shortening. Moreover, numerous mitochondria are located between the fibrils and serve as the energy generators for contraction. A fine network of microtubules, the sarcoplasmic reticula, are crucial to the mechanism of excitation contraction mechanism, which will be described in details later [78].

Two dark lines, termed the Z lines, delineate each sarcomere, which in turn is centrally occupied by a dark area, the A-band, flanked by two lighter zones termed the I-bands. The thicker myosin filaments run the length of the A band, and the thin filaments extend from the Z line, form the I-band, and partly overlap the myosin filaments in the lateral portions of the A-band. The length of these myofilaments is always constant and does not vary during contraction. Upon activation, specialized sites along the two sets of myofilaments repetitively interact causing the thin filaments to slide inward essentially shortening the sarcomere and, hence, the muscle [78] (**Figure 1.4**).

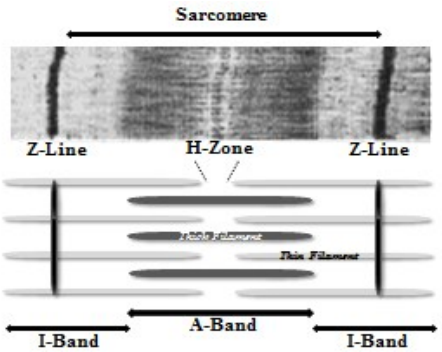


Figure 1.4. The Sarcomere.

1.2.2.1.2. Conduction System

The heart conduction system, made of dedicated cardiomyocytes, is responsible for generating and conducting the electrical impulse. It should always be distinguished from the working myocardium whose primary function is contraction. As mentioned briefly in the heart development section, the conduction system encompasses various components with different functions. The sinoatrial (SA) node generates the impulse and thus is considered the heart pacemaker. The impulse is then conducted via the atrial myocardium toward the atrio-ventricular (AV) node. After a very short delay, the AV node hastily transmits the impulse via the bundle branches and peripheral Purkinje network (PPN) ensuring a synchronized activation of the ventricular myocardium [12].

Nodal myocytes are unique and their phenotype is usually dubbed as embryonic because of their electrical and contractile characteristics. Despite that, these cells are physiologically highly specialized and therefore, should not be considered as remnants of the embryonic myocardium. The ventricular conduction system is formed of two component parts; the first of which is made of the atrioventricular bundle and its branches, and is positioned on top and on both sides of the ventricular crest, penetrating the compact myocardium. This part receives the depolarization impulse from the AV node and then transmits it to the ventricles. On the other hand, the second part of the ventricular conduction system surrounds the ventricular subaortic outlet as well as the right atrioventricular junction [12].

The SA node is primarily associated with the characteristic automatic contraction of the cardiac muscle. It generates the electrical signal that subsequently triggers the depolarization and eventually the contraction of the cardiac muscle by a process known as the excitation-contraction coupling. The intrinsic firing of the SA node is controlled by three

voltage-gated channels: a nonspecific cation funny channel (HCN), the L-Type Ca^{+2} channel (dihydropyridine receptor, DHPR), and a K^+ channel. The slow diastolic depolarization (DD) characteristic of the SA node is due to funny currents (I_f) through HCN channels, which are oddly activated on hyperpolarization with an activation threshold of -40/-50 mV. The inward depolarizing I_f induces the opening of the L-type Ca^{+2} channels and an influx of calcium ions further depolarizes the membrane. Finally, the depolarization-dependent K^+ channels open and an efflux of potassium ions (Ik) triggers repolarization [79].

1.2.2.1.3. Excitation Contraction Coupling

As mentioned earlier, the process by which the depolarization of the myocyte membrane triggers contraction is called the cardiac excitation–contraction coupling. Ca^{+2}, as a second messenger, plays a pivotal role in linking the electrical signal to the activation of myofilaments and hence contraction [80]. By a process termed calcium-induced calcium release (CICR) [81-83], Ca^{+2} entering the cell as an inward ICa triggers Ca^{+2} release from the sarcoplasmic reticulum (SR) via the ryanodine receptors (RyR). This causes a rise in free intracellular Ca^{+2} concentration ($[Ca^{+2}]i$), allowing it to bind to troponin C of the thin myofilaments. Ca^{+2} binding induces a conformational change in troponin C, freeing myosin binding sites on the actin filaments, previously hidden by tropomyosin. Then myosin binds to the newly freed binding sites on actin, releasing inorganic phosphorus and triggering a sliding motion characteristic of muscle contraction (**Figure 1.5**). Afterwards, ATP binds to myosin, which in turn releases actin and hydrolyzes ATP in order to return to its resting conformation. Finally, Ca^{+2} dissociates from troponin, due to a decline in intracellular calcium, reinstating the block by tropomyosin. The decrease of Ca^{+2} intracellularly involves

an SR Ca^{+2}-ATPase (SERCA), a sarcolemmal Na^{+}/Ca^{+2} exchanger (NCX), a sarcolemmal Ca^{+2}-ATPase, and a mitochondrial Ca^{+2} uniporter [80,82,83].

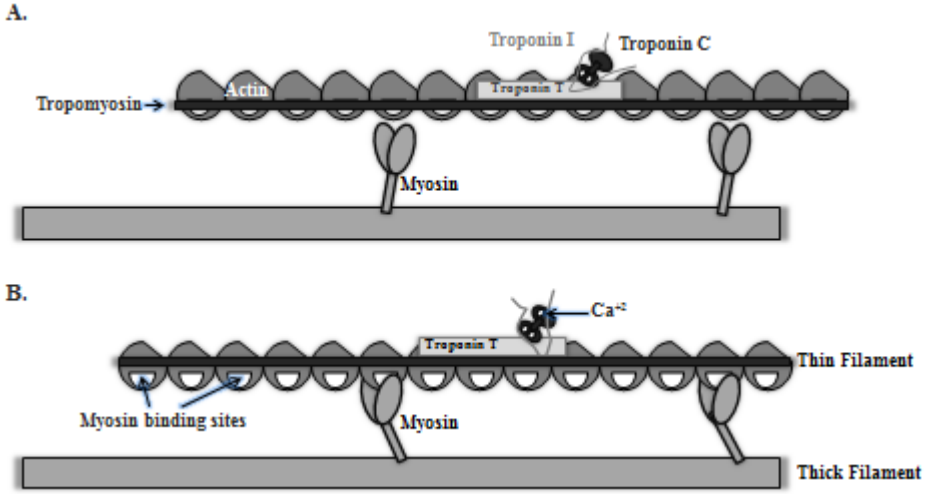


Figure 1.5. Cardiac Muscle Contraction Apparatus. A. Relaxed cardiac muscle in the absence of Ca^{+2}. **B.** Activated cardiac muscle in the presence of Ca^{+2}.

1.2.2.1.4. Cardiac Indices and Echocardiography

Cardiac structure and function are highly interdependent, and any change affecting either one of them can easily affect the other. Therefore and in order to assess the overall heart status, an array of both systole- and diastole- specific indices has been formulated. These indices can be assessed using a variety of techniques, like electrocardiograms (ECG) and echocardiography. Echocardiography is one of the routinely used techniques in human clinical studies, and has recently been miniaturized to assess physiologic changes in normal and genetically modified rodents. Nowadays, this powerful technique is currently the most commonly used tool to assess mouse cardiac structure [84]. For example, changes in LV mass can easily be monitored and assessed by M-mode echocardiography (**Figure 1.6**). LV mass can be calculated using the following formula:

$$LV\ mass\ (g) = 1.05\ [(3LVEDD+3IVSd+3LVPWd)-(3LVEDD)]$$

Where LVEDD is left ventricular end-diastolic dimension, IVSd is interventricular septum thickness at diastole, and LVPWd is left ventricular posterior wall thickness at diastole. This formula has been proven to correlate with gross morphometric heart weight. LV mass is usually corrected to animal body weight or to femur length. Moreover, the gravimetric analysis of mouse hearts like heart weight (HW) to body weight (BW) ratio is always an essential quality control measurement for echocardiography obtained LV mass [85,86].

Figure 1.6. M-mode Echocardiography. An M-mode image of a mouse heart obtained using the Vevo 2100. **IVSs**, interventricular septal thickness at systole; **IVSd**, interventricular septal thickness at diastole; **LVESD**, left ventricular end-systolic dimension; **LVEDD**, left ventricular end-diastolic dimension; **LVPWs**, left ventricular posterior wall thickness at systole; **LVPWd**, left ventricular posterior wall thickness at diastole.

Echocardiography is also used to assess the overall left ventricle (LV) function in both human and mouse. Many parameters can be essential in determining global heart function. For instance, fractional shortening (FS), defined as the fraction of diastolic dimension lost during systole [87], can be used to measure LV function in the absence of

segmental wall-motion abnormalities [84]. FS can be calculated from the maximal diastolic (LV end-diastolic dimension, LVEDD) and systolic dimensions (LV end-systolic dimension, LVESD) obtained using M-mode imaging. The formula for calculating FS is:

$$FS\,(\%) = [(LVEDD - LVESD)/LVEDD] \times 100$$

Ejection fraction (EF), another highly reproducible parameter usually representing the blood fraction ejected by the left ventricle during systole can also be calculated by the following formula:

$$EF\,(\%) = [(LVEDD^2 - LVESD^2)/LVEDD^2] \times 100$$

Another form of echocardiography that is usually used to determine stroke volume (SV) and cardiac output (CO) is Doppler echocardiography. To calculate the CO defined as the volume of blood pumped by the heart every single minute; heart rate (HR), aortic outflow velocity-time integral (Ao-VTI, cm), and aortic diameter (Ao-Root, mm) should be all determined.

$$SV = 1000 \times Ao\text{-}VTI \times \pi \times (Ao\text{-}Root/20)^2$$

$$CO = SV \times HR$$

Moreover, Doppler echocardiography is mostly used to assess ventricular diastolic function in both human beings and rodents. One of the most studied diastolic function parameters is the mitral valve flow analysis. Therefore, the degree of diastolic dysfunction can be evaluated by obtaining a variety of measurements which include, the peak E (early filling wave) and the peak A (atrial contraction wave) wave velocities, their E/A ratio, and the isovolumic relaxation time [84].

1.2.2.2. Heart Pathophysiology and Diseases

1.2.2.2.1. Definition and Introduction

Summing up, the heart is an extremely complex indispensable organ with specialized compartments formed of a variety of dedicated cell types, which work in harmony and under extreme coordination to ensure the proper distribution of blood and hence O_2 to the rest of the body. Moreover, the heart as a continuously beating muscle requires a huge amount of O_2 and nutrients, and thus, a constant supply of blood via the coronary system should be maintained to all heart regions at all times. Therefore, any factor or event disturbing the proper structure or function of one or more of its components or affecting heart blood supply will eventually lead to heart disease and in many circumstances sudden death. Nowadays, heart disease as a group of conditions and anomalies is the leading cause of death in the western world [88]. Apart from the congenital heart diseases described earlier, the human adult heart disease can be a direct cause of coronary artery disease, cardiac arrhythmias, cardiomyopathies, valve disorders, and many more. In the next section, I will try to define, indicate the causes of, and briefly describe treatments for some of these conditions.

1.2.2.2.2. Types, Causes, and Treatments

1.2.2.2.2.1. Coronary Artery Disease

Coronary artery disease (CAD), also known as coronary heart disease (CHD), is the most common form of heart disease and is the number one cause of death in the United States [89]. CAD could lead to angina (chest pain), myocardial ischemia, and myocardial infarction and thus sudden death [90]. Atherosclerosis of coronary arteries is at the basis of CAD, and the rupture of atherosclerotic plaques is believed to be the main culprit behind

myocardial infarction (MI). Thrombus formation following atherosclerotic plaques activation and rupture can block one of the major coronary arteries, preventing blood flow to a certain heart region and inducing cardiomyocyte loss and scar formation characteristic of MI [76,77]. Currently CAD patients undergo stenting or standard balloon angioplasty to increase the lumen of atherosclerotic arteries [91]. Recent knowledge on the role of inflammation in CAD offered new prospects for treatments using immunosuppressants, anti-inflammatory drugs, and even vaccines [77].

1.2.2.2.2.2. Cardiac Arrhythmias

Ion channels play a crucial role in heart physiology and therefore genetic or sporadic defects or ion channelopathies, affecting their functions lead to a group of pathophysiological conditions commonly known as cardiac arrhythmias [92]. Atrial fibrillation (AF), an erratic activation of the atria, is the most common form of arrhythmias with a prevalence close to 1% [92,93]. Usually, localized electrical discharges arising from areas other than the sinus node cause the ectopic impulses at the basis of AF. Therapies for AF focus on the management of symptoms and the prevention of complications that will possibly require treatment of any associated cardiac or endocrine disease [93]. On the other hand, arrhythmias can also be conduction disorders, like atrioventricular blocks, or ventricular arrhythmias, like the long QT syndrome [92].

1.2.2.2.2.3. Cardiomyopathies

Cardiomyopathies are a set of myocardial diseases associated with cardiac dysfunction. A cardiomyopathy can either be a dilated cardiomyopathy (DCM), a

hypertrophic cardiomyopathy (HCM), a restrictive cardiomyopathy, an arrhythmogenic right ventricular cardiomyopathy (ARVC), or just an unclassified cardiomyopathy [94-96].

Dilated cardiomyopathy (DCM) is a clinical condition of heart failure characterized by dilatation and impaired systolic function of the left ventricle or both ventricles. DCM can be familial or genetic, toxic or alcoholic, viral or immune, or just idiopathic in nature [94,95]. DCM patients usually have high levels of tumor necrosis factor-α (TNF-α), and therefore, are currently treated with the immunomodulating agent Pentoxifylline with an improvement in overall heart function [94]. On the other hand, restrictive cardiomyopathy is a condition characterized by reduced ventricular diastolic volumes and eventual diastolic dysfunction with a preserved systolic function [94,96]. For example, endomyocardial fibrosis (EMF), a form of restrictive cardiomyopathy, is characterized by dense fibrous tissue in the endocardium that distorts the papillary muscles and hampers ventricular diastole [95].

The third type of cardiomyopathy is hypertrophic cardiomyopathy (HCM), a fairly common disease that is clinically diagnosed by unexplained ventricular hypertrophy [94,95,97]. Pathologically, HCM is identified by disarrayed hypertrophic cardiac myocytes and increased interstitial fibrosis [97]. HCM is the major cause of sudden cardiac death in the young especially the athletes [96,97]. It is now well established that HCM is an autosomal dominant disorder caused by mutations in genes encoding for sarcomeric proteins [95,97]. HCM treatments focus on reducing heart failure symptoms and complications, and because many HCM patients have diastolic dysfunction requiring higher filling pressures, cautious usage of diuretics is recommended [96].

The last form of cardiomyopathy is arrhythmogenic right ventricular cardiomyopathy (ARVC), which is an autosomal, dominant inherited disorder characterized by a dilated right ventricle with a reduced systolic function [95,96]. Microscopically, it is characterized by the loss

of right ventricle myocytes and the partial or complete replacement with fatty and fibrous tissue [95]. ARVC commonly leads to syncope and ventricular arrhythmias, but rarely to heart failure and sudden death [96].

1.2.2.3. Cardiac Remodeling and Heart Failure

1.2.2.3.1. Definition and Introduction

Heart failure, an outcome of most of the cardiac illnesses mentioned earlier, is a devastating disease and currently the number one cause of death in the industrialized world [88,98]. In many of these conditions, hypertrophy, defined as an increase in heart mass, is the initial heart response designed to sustain normal function. However, if prolonged, hypertrophic growth due to a persistent stress would eventually lead to heart failure and even sudden death [88]. In this section I will try to define and briefly describe the heart hypertrophic response.

Cardiac hypertrophy is usually associated with almost all heart failure forms and conditions [88,98]. Conversely, the heart of an athlete is an exception to this relation, where the hypertrophic response is induced by chronic exercise training. This kind of hypertrophy, commonly known as physiologic hypertrophy, is associated with preserved or even enhanced heart function. On the other hand, heart failure-associated hypertrophy also known as pathological hypertrophy occurs in response to persistent stressors, including valve disease, hypertension, genetic mutations, and myocardial infarction [98]. The latter abnormalities are almost always associated with myocyte death, ventricular wall fibrosis, and myocardial remodeling [88]. Myocardial remodeling, which is at the basis of cardiac hypertrophy, usually accompanies disease conditions and is triggered by either mechanical stretch, ischemia, hormones, or drugs [74].

Pathological hypertrophy can be either concentric or eccentric in nature [98,99]. Concentric hypertrophy, characterized by thick walls and small cavities, is the result of increased systolic wall stress induced by pressure overload [98]. It is mediated by the generation of new sarcomeres in parallel resulting in an increased width to length ratio of the cardiomyocytes [74]. On the other hand, eccentric hypertrophy characterized by relatively thin walls and dilated cavities, is a consequence of increased diastolic wall stress triggered by volume overload [98]. Eccentric hypertrophy is associated with the lengthening of cardiac myocytes mediated by the addition of sarcomeres in series [74]. In both types of pathological hypertrophy the addition of new sarcomeres originally is compensatory and helps in preserving normal heart function. However, and if the stress persists the hypertrophied heart would eventually decompensate, leading to cardiac heart failure [98] (**Figure 1.7**).

1.2.2.3.2. Molecular Mechanisms of Cardiac Hypertrophy

At the cellular level, cardiac hypertrophy is accompanied by myocyte growth, increased protein synthesis, sarcomeric reorganization, and fetal cardiac genes reinduction [88]. These genes include atrial natriuretic peptide (ANP) [100], B-type natriuretic peptide (BNP) [101], and genes of contractile proteins fetal isoforms, such as skeletal α-actin [102] and β-myosin heavy chain (β-MHC) [103]. This is accompanied by the downregulation of some highly expressed adult cardiac genes, such as α-MHC and SERCA [98]. Cardiac hypertrophy can be promoted via numerous extracellular ligands, especially, those of G-protein coupled receptors (GPCRs), such as angiotensin, endothelin, and adrenergic agonists 88.

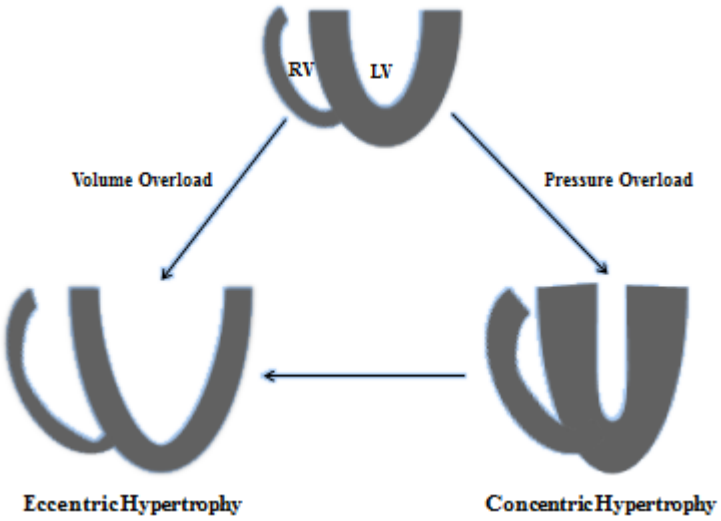


Figure 1.7. Pathologic Cardiac Hypertrophy. Pathologic hypertrophy can be concentric or eccentric. Concentric hypertrophy is caused by pressure overload, while eccentric hypertrophy is caused by volume overload. Modified from [98].

The upregulation of the natriuretic peptide genes (ANP and BNP) is one of the early hallmarks of cardiac stress, and their blood levels are clinically used as markers of cardiac stress and heart failure [100,101,104]. Another important marker of cardiac hypertrophy is the switch from the adult α-MHC isoform to the fetal β-MHC isoform. The latter isoform has less efficient ATPase activity and thus partly contributes to the decreased myofibrillar activity and compromised contractility [88]. Furthermore, the accompanying downregulation of SERCA and other proteins are directly associated to the abnormalities in calcium handling seen in stressed cardiac cells [105]. Finally, a number of transcription factors have been shown to play critical roles in the genetic reprogramming of stressed cardiomyocytes. For instance, GATA-4 posttranslational modification by several stress-related protein kinases has been shown to be crucial for the induction of the cardiac hypertrophic program [57,65,106,107]. Other transcription factors implicated in cardiac hypertrophy include the nuclear factor of activated T-cells (NFAT) [57], MEF2 [108], SRF [109], the zing finger transcription factor zfp260 [85], and the cAMP-responsive transcription factor CREB [110]. The activities of most of these transcription

factors are usually enhanced via posttranslational mechanisms that comprise several kinases and histone acetylases and deacetylases (HATs and HDACs). In essence, stress-induced cardiomyocyte hypertrophy is characterized by changes in gene expression patterns governed by a complex mesh of signaling networks and pathways [57,65,88,105-108].

1.3. Cardiomyocyte Death and Survival

1.3.1. Introduction

The majority of heart myocytes are terminally differentiated and incapable of proliferating. However, in response to growth and stress stimuli, the heart hypertrophies via the enlargement and remodeling of individual cardiac myocytes [111,112]. Whereas initially physiologic, the prolongation of this remodeling process leads to pathologic changes and ultimately heart failure [113]. Moreover, pathologic hypertrophy and cardiac remodeling are frequently triggered or accompanied by cardiomyocyte loss, a common observation in ischemic heart disease and drug-related cardiac injury [114]. Interestingly, loss of cardiac myocytes is sufficient to cause cardiac injury and heart failure, as demonstrated by several research groups [115,116]. On the other hand, it has been shown that enhancing cardiac myocyte survival is protective against heart failure and efficiently counterbalances chemotherapy-induced cardiotoxicity [117-119].

1.3.2. Cell Death Mechanisms in the Heart

Cardiomyocyte loss leading to heart failure could happen through one or a combination of three different death mechanisms, namely, necrosis, apoptosis, and autophagy. Therefore, it is extremely necessary to understand these death mechanisms in order to decipher survival pathways and to identify potential therapeutic targets [120,121].

1.3.2.1. Necrosis

Necrosis, or cell death, is characterized by the dilatation of the cytoplasmic organelles, the rupture of the plasma membrane, and inflammation [122]. Until recently, necrosis has been considered to be accidental and uncontrolled. Nowadays, it is clear that necrotic cell death is programmed and well controlled [120, 123]. Lately, it has been established that the programmed necrosis plays a key role in heart disease, with the accompanying inflammation contributing to remodeling and contractile failure progression [121].

Mitochondria play an important role and the opening of the mitochondrial permeability transition (MPT) pore is a defining molecular feature of programmed necrosis. The MPT pore, also termed mitochondrial depolarization, opens in response to increased mitochondrial Ca^{+2}, oxidative stress, inorganic phosphate, and alkaline PH. The opening of this channel induces a depolarization of the mitochondrial membrane and the shutdown of ATP production triggering necrosis. For instance, oxidative stress, which is implicated in many cardiac pathologies, induces necrosis via the MPT pore opening and ATP depletion [124]. Finally, the MPT pore consists of a voltage-dependent anion channel, an adenine nucleotide translocator (ANT), cyclophilin D (CypD) and other smaller molecules [125].

1.3.2.2. Apoptosis

1.3.2.2.1. Introduction

Apoptosis, or programmed cell death, is an energy-requiring process that culminates in cell shrinkage, intracellular proteins proteolysis, mitochondrial function compromise, and DNA fragmentation. Apoptosis follows well-defined time-dependent pathways, which eventually lead to the activation of caspases necessary to dismantle intracellular components [126].

There are two major apoptotic pathways, the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is triggered in response to ligands binding to membrane death receptors of the *tumor necrosis factor receptor family* (TNF-α), known as the Fas receptors [126,127]. Binding of the Fas-ligands to the Fas receptors initiates the formation of a protein complex called the multi-protein death-inducing signaling complex (DISC). Conformational changes in the DISC complex will then activate caspase 8, a central component of the extrinsic death pathway [127]. On the other hand, the intrinsic pathway involves the mitochondria and is activated in response to signals originating inside the cell. A key step in the intrinsic pathway is the disturbance of the mitochondrial membrane integrity, which is usually regulated by the Bcl-2 protein family. The latter family of proteins encompasses anti-apoptotic proteins, like BCL-2 and BCL-$X_{L,}$ and pro-apoptotic proteins, such as, BAX, BAD, and BIM. The activation of the pro-apoptotic proteins leads to the mitochondrial permeability transition pore (MPTP) formation and the subsequent release of cytochrome C (CYT-C). The latter along with APAF-1, dATP, and procaspase-9 form a macromolecular complex called the apoptosome, leading to the activation of procaspase-9. Caspase-9 (Casp-9) activation is supported by Smac/DIABLO, which is also released by the mitochondria to inhibit the antiapoptotic activity of the inhibitor of apoptosis protein (IAP). In addition, mitochondria release endonuclease G and apoptosis-inducing factor (AIF), which promote the characteristic chromatin condensation and large-scale DNA fragmentation [128]. Moreover, Casp-9 activation will eventually activate caspase-3 (Casp-3), which is technically the ultimate apoptosis 'enforcer' being activated by both the extrinsic and intrinsic pathways. Finally, key caspase 3-dependent substrates get activated leading to the distinctive features of apoptotic cell death [126,127] (**Figure 1.8**).

In the normal myocardium apoptosis is extremely rare with rates of 1 in 10,000-100,000 [129]. However, it is significantly increased in many cardiac conditions, such as cardiomyopathies and decompensated hypertrophy [120]. Nowadays, multiple cardiomyocyte cell death inducing stimuli have been identified, including reactive oxygen species (ROS), proinflammatory cytokines, and cytoskeletal disorders. Apoptotic stress signals can trigger the activation of some G-protein coupled receptors, or induce an increased production of ROS [121]. The subsequent stimulation of these G-protein coupled receptors activates kinases like apoptosis signal regulating kinase 1 (ASK-1) and c-Jun N-terminal kinase (JNK) [120,121]. Conversely, apoptotic cardiac myocyte death is counterbalanced by prosurvival signaling pathways converging on kinases like the protein kinase B PKB/AKT and the proto-oncogene serine-threonine protein kinase (PIM-1) [121,130,131].

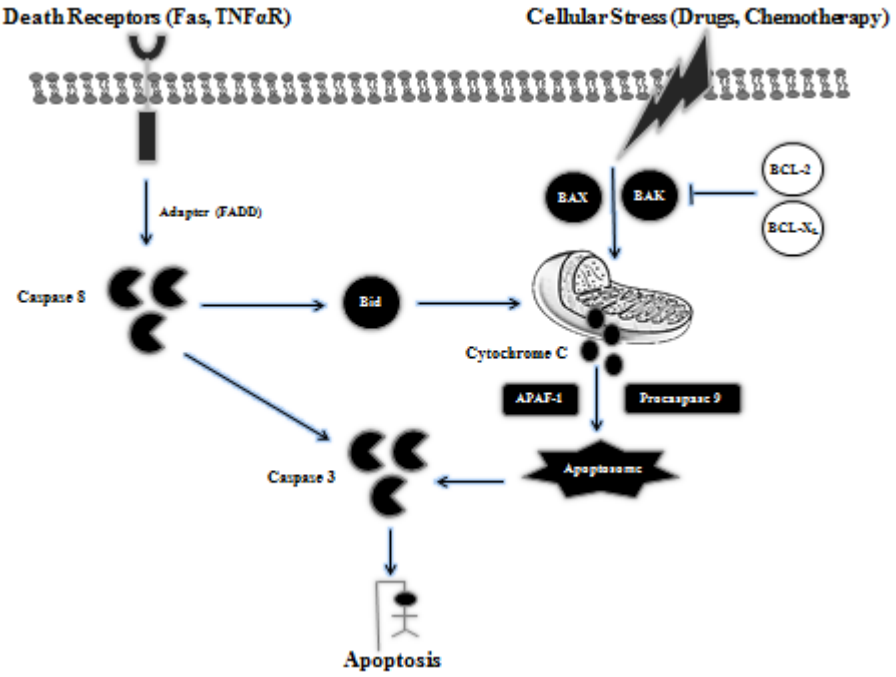


Figure 1.8. Apoptosis. Apoptotic cell death can be triggered via the extrinsic (Death receptors) pathway or the intrinsic (Cellular stress) pathway.

1.3.2.2.2. Bcl-2 Protein Family

As mentioned above, the Bcl-2 protein family control over the intrinsic apoptotic pathway is dictated by the balance between its proapoptotic and antiapoptotic members. Bcl-2 family members share at least one Bcl-2 homology domain (BH) necessary for protein-protein interactions. Proapoptotic members, which include BCL-2, BCL-X_L, BCL-W, MCL-1, A-1, and BOO/DIVA, have as many as four BH domains (BH1-BH4). On the other hand, proapoptotic members have less BH regions; for instance, BAX and BAK, critical for mitochondrial pore formation and subsequent CYT-C release, have only three BH domains. Other proapoptotic proteins including: BIM, PUMA, and BID are known to bind and inhibit the prosurvival family members via their lone BH domain, the BH3 [127]. Apoptosis inducing stresses are sensed by Bcl-2 family proteins, which then translocate to the outer mitochondrial membrane and interact with other family members to regulate the release of proapoptotic molecules. Interestingly, Bcl-2 family proteins functional activities depend on their intracellular localization, with some targeting both the ER and the nuclear membranes to exert additional or alternate functions [128].

The antiapoptotic Bcl-2 members potently inhibit apoptosis through the hetero-dimerization with and hence the sequestration of the proapoptotic family members [132]. Whereas, BCL-2 is always bound to intracellular membranes, BCL-X_L can be either membrane-bound, or free in the cytosol [133,134]. Both BCL-2 and BCL-X_L stabilize the MPT pore and potently block the release of mitochondrial apoptogenic factors [134,135]. Furthermore, endoplasmic reticulum bound BCL-2 and BCL-X_L increase its permeability to calcium, counterbalancing Ca^{+2} role in apoptosis [136]. In the heart, antiapoptotic Bcl-2 proteins are cardioprotective and both BCL-2 and BCL-X_L have been shown to play prosurvival roles in cardiomyocytes [117,118,134]. On the other hand, the proapoptotic BAX and BID are mostly

cytosolic and usually translocate to the mitochondria in stress conditions [133]. Upon its activation, BAX undergoes a conformational change, inserts via its C-terminal into the outer mitochondrial membrane, and then oligomerizes [137]. Furthermore, the BH3-only BID is part of the extrinsic receptor pathway, and once activated by caspase 8, translocates to the mitochondria bridging the intrinsic and extrinsic pathways. Moreover, BID could either directly contribute to the outer mitochondrial membrane permeabilization [138], or it could augment BAX signalling either by its direct interaction with BAX/BAK [139], or through its scavenging of BCL-2 and BCL-X_L [140]. In the heart, both BID and BAX have been shown to play crucial roles in many cardiac conditions [141,142].

1.3.2.3. Autophagy

Autophagy or self-digestion, a mechanism utilized by the cell for bulk degradation of proteins and organelles during starvation, is a third process by which myocyte survival could be affected. Autophagy is usually activated in many heart conditions such as hemodynamic overload and myocardial ischemia [120,121,143]. This process is tightly controlled by autophagy-related genes, which are mostly involved in autophagosome formation. Autophagy utilizes a non-selective degradation system, in clear contrast to the ubiquitin-proteasome degradation system. Development, adaptation to nutrient deprivation, clearance of proteins and organelles, elimination of micro-organisms, prevention of aging, induction of cell death, suppression of tumors, and presentation of antigens are some of the recently described roles of autophagy. Although autophagy appears to regulate both cell survival and death, it is not yet clear whether it is a sign of impaired cardiac myocyte repair or it is a death pathway for compromised myocytes [121]. However, increasing data indicate that constitutive autophagy is a homeostatic mechanism essential for maintaining cell size as well as normal cardiac

structure and function, and that an upregulated autophagy in failing hearts is a protective adaptive response against hemodynamic stress [143]. On the other hand, excessive autophagic activity can promote cell death, apoptosis, and even necrosis.

Finally, many new studies reported the presence of a crosstalk between apoptosis, necrosis, and autophagy. For instance, signaling pathways induced by common stressors like ROS were found to regulate both apoptosis and autophagy [121]. Furthermore, members of the beclin and the Bcl-2 families seem to play a critical role in the crosstalk between the apoptotic and autophagic pathways. Beclin-1 is a mammalian autophagy gene that directly interacts with many anti-apoptotic Bcl-2 family proteins such as BCL-2 and BCL-X_L [144].

1.3.3. Cell Survival Pathways in the Heart

1.3.3.1. Introduction

In the heart, the death mechanisms described earlier are modulated by several survival signaling pathways including the MAPK/ERK, PI3K/AKT, and JAK/STAT pathways. These pathways, which are activated by both extracellular and intracellular signals, such as hormones and drugs, exert their prosurvival effects by antagonizing those of pro-death factors mentioned in the previous sections.

1.3.3.2. Survival Signalling Pathways

1.3.3.2.1. MAPKs Pathway

Mitogen activated protein kinases (MAPKs) are a group of kinases acting in succession to regulate cell growth, differentiation, and survival. Three major branches make up the MAPKs pathway and are named after their terminal effector kinases: the c-Jun NH_2 terminal kinases (JNKs), the extracellular signal-regulated kinases (ERKs), and p38. These

terminal kinases are usually activated by dual phosphorylation on a threonine and a tyrosine residue (T-X-Y). Upon their activation, they phosphorylate a wide array of cytoplasmic, nuclear, and mitochondrial targets to regulate a variety of cellular processes including cell death [145] (**Figure 1.9**).

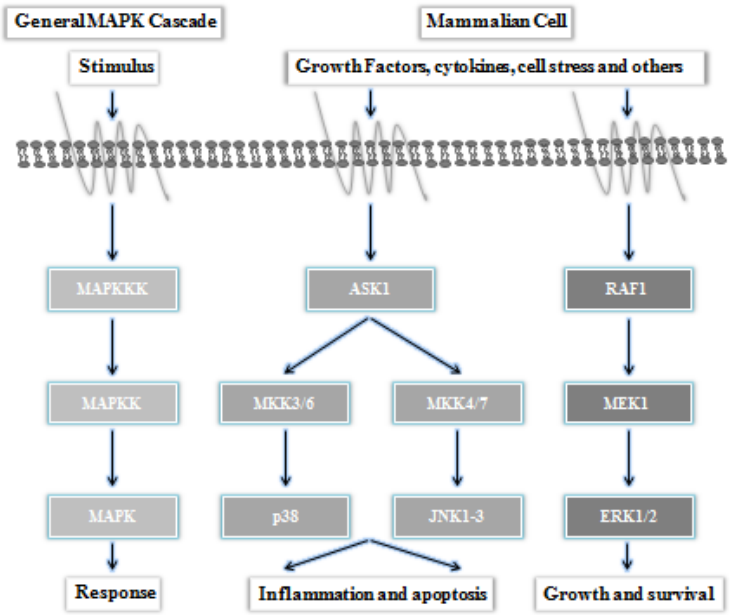


Figure 1.9. Mammalian MAPK Pathways. Growth factors, cytokines and various signals acting via seven transmembrane receptors induce the different MAPK signaling pathways.

JNK signaling starts with the activation of low molecular weight GTP-binding proteins (Ras/Rac/Cdc42), which later induce MAPK kinase kinases (MEKKs) activation. Later, MEKKs promote the activation of the MAPK kinases (MKKs) that in turn phosphorylate and activate JNKs. Signals promoting JNKs activation range from stress to mitogenic in nature and usually result in the regulation of the activities of many transcription factors like c-Jun, activating transcription factor-2 (ATF-2), NFAT, and p53. In cardiac cells, JNKs are activated in response to angiotensin II infusion, mechanical stretch and $G\alpha_q$-coupled receptor agonists like phenylephrine (PE). Recently, JNKs were found to play critical roles in the regulation of cell death especially the apoptosis mitochondrial pathway.

These findings demonstrate that JNKs have both pro- and anti-apoptotic effects, although experimental models that prove they induce pro-death signals are far more numerous [145,146].

On the other hand, the most common ERK signaling pathway commences with the activation of small G-proteins (Ras, Rac, Rho, etc..) leading to the activation of Raf kinases, then MEKs, culminating in ERK1/2 activation. In the heart, ERKs have been shown to be part of prosurvival signals in response to a variety of stressors like oxidative stress, hypoxia, and anthracycline exposure. For instance, ERK-1/2 activation was found to augment cellular viability by phosphorylating and activating either p90 ribosomal S6 kinases (RSKs) or GATA-4. RSKs are known to promote survival through their direct phosphorylation and inactivation of the proapoptotic BAD protein, while GATA-4 promotes survival through its positive effects on BCL-2 and BCL-X_L expression [117,146]. Finally, p38 MAPK is thought to play a role in cardiac myocyte apoptosis though findings are still contradictory. The majority of studies so far demonstrate a proapoptotic role of p38 MAPK in cardiomyocytes, but one cannot neglect some convincing findings regarding its prosurvival effects. Therefore, p38 MAPK might be either proapoptotic or antiapoptotic depending on the cell type and condition [145].

1.3.3.2.2. PKB/AKT Pathway

Protein kinase B (PKB), commonly known as AKT, is a serine/threonine kinase implicated in a number of cellular functions including energy metabolism and cellular survival. AKT role in cell survival is believed to be mediated by the preservation of mitochondrial integrity where not only it counteracts the function of proapoptotic Bcl-2 family proteins but also inhibits the MPT pore formation [131]. In the heart, the activation of phosphoinositol 3-kinase (PI3K) in response to stimulated receptor tyrosine kinases [131,147],

G-protein coupled receptors (GPCRs), and glycoprotein 130 [148,149], triggers the translocation of AKT to the plasma membrane where it becomes activated upon its phosphorylation at T308 by phosphoinositide-dependent kinase-1 (PDK-1) [131,150,151]. The activated AKT can then phosphorylate an array of targets including mTOR, FOXO, and GSK-3β in the cytosol [131,152], or translocate to other subcellular compartments like the nucleus and mitochondria where it plays a crucial role in maintaining mitochondrial integrity (**Figure 1.10**) [153].

Glycogen synthase kinase-3β (GSK-3β), important for glycogen synthesis, is now recognized as a key player in many other cellular processes. In the heart, GSK-3β, which is a basally active kinase, is both a suppressor of the hypertrophic response as well as a proapoptotic signal. However, phosphorylation by AKT at Ser9 inhibits its kinase activity and therefore its proapoptotic effects at the mitochondrial level. Moreover, GSK-3β has been found to regulate through phosphorylation some Bcl-2 family members like MCL-1 and BAX. The phosphorylation of MCL-1 targets it to degradation, while the phosphorylation of BAX induces an activating conformational change [131]. Finally, GSK-3β phosphorylates many transcription factors modulating their actions on gene expression [154]. For instance, the phosphorylation of GATA-4 by GSK-3β was found to promote the former's export from the nucleus, possibly inhibiting its prosurvival effects [67].

AKT can promote survival through its inhibition of proapoptotic Bcl-2 family members like BAX and BAD. AKT direct phosphorylation of BAD at Ser136 induces its dissociation from BCL-X_L, freeing the latter [155]. On the other hand, the phosphorylation of BAX at Ser184 prevents the conformational change necessary for its translocation to the mitochondria [156]. To the contrary, AKT can induce the upregulation of many antiapoptotic Bcl-2 family members [131,157]. For instance, AKT was found to play a role in the prevention of both BCL-2 and BCL-X_L downregulation in a variety of cardiac conditions. Moreover, AKT

downstream targets like mTOR complex-1 (mTORC-1) can activate the expression of antiapoptotic Bcl-2 family members, like MCL-1, promoting cell survival. Therefore, AKT is cardioprotective and exerts its prosurvival effects through the phosphorylation of a wide array of targets implicated in cellular stress and death pathways [131].

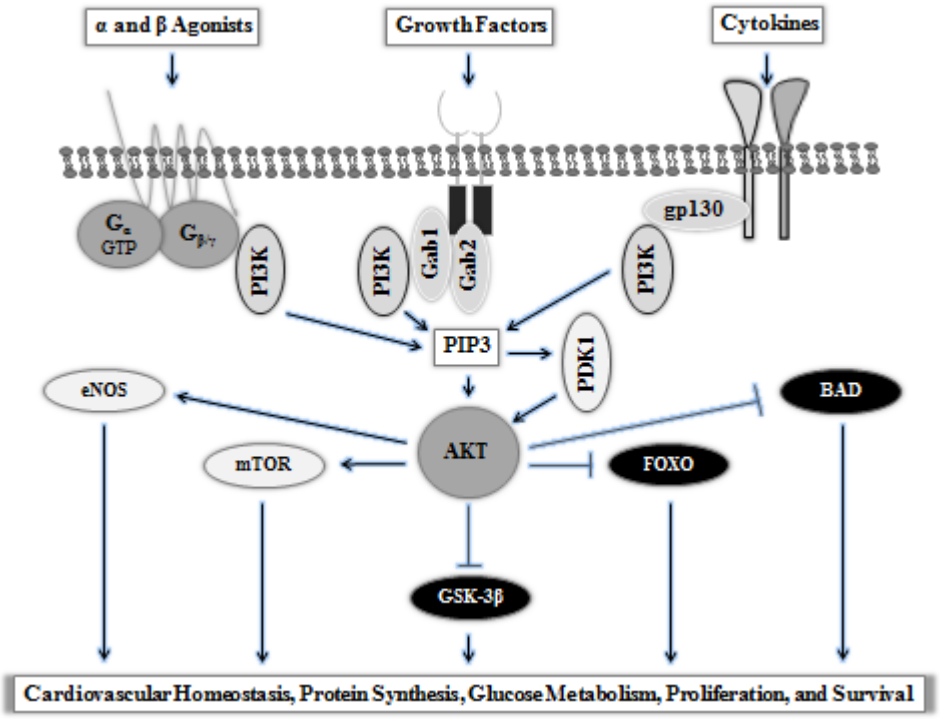


Figure 1.10. PKB/AKT Pathway. Growth factors, adrenergic agonists, and cytokines lead to the activation of the AKT pathway in cardiac cells.

1.3.3.2.3. JAK/STAT Pathway

In the heart, Janus kinases (JAKs), which are tyrosine kinases, are activated by a variety of signals including GPCRs and IL-6 cytokine family. Activated JAKs directly phosphorylate and activate the signal transducers and activators of transcription (STATs) family of transcription factors. Many studies examining myocardial cell death have demonstrated a prosurvival role of JAK/STAT signaling. The JAK/STAT prosurvival effects are still obscure but are most likely due to their transcriptional regulation of apoptotic

proteins like BCL-X_L and BAX [145]. For instance, JAK activation was found to be associated with an increase in BCL-X_L and a decrease in BAX levels [158].

1.3.4. ER Stress Response in the Heart

The existence of a functional endoplasmic reticulum (ER) system critical in cardiac physiology and pathology has only recently received attention in the cardiovascular community. The ER membrane performs numerous essential functions, including protein folding and maturation, regulation of calcium homeostasis, and lipid and sterol synthesis. Any perturbation affecting one of its major functions induces the ER stress response, also known as the unfolded protein response (UPR) [159,160]. The UPR is originally an adaptive response triggered to restore homeostasis by suppressing protein synthesis and upregulating protein chaperones designed to handle the accumulation of misfolded proteins. Although the early UPR events are prosurvival, the persistence of stress eventually activates death pathways culminating in apoptosis [160].

The activation of the ER stress response involves three different pathways controlled by three different ER integral membrane proteins: dsRNA-activated protein kinase-like ER kinase (PERK), inositol-requiring kinase-1 (IRE-1), and activating transcription factor-6 (ATF-6). The combination of these proteins in association with BiP/glucose regulated protein-78 (BiP/GRP-78), a molecular chaperone central to many of the ER functions, results in inhibition of protein synthesis, increased expression of ER chaperones, and upregulation of proteins involved in the ER-associated degradation (ERAD). Stress and the accumulation of unfolded proteins trigger the dissociation of the stress sensing BiP/GRP-78 from one or all three proteins inducing their activation. Then activated PERK phosphorylates the translation initiator factor eIF-2α resulting in the inhibition of protein

synthesis. On the other hand, an activated ATF-6 translocates to the Golgi and undergoes cleavage to yield the N-ATF-6, a soluble transcription factor. The latter would eventually induce the expression of more than 30 genes including BiP/GRP-78, the transcription factor X-box binding protein-1 (XBP-1), and ERAD-associated proteins. Finally the activation of IRE-1 induces its homodimerization, autophosphorylation, and activation of its endoribonuclease activity, which cleaves 28S rRNA to inhibit translation, as well as splices XBP-1 mRNA into its active form. XBP1s protein binds to several promoters upregulating genes involved in ERAD. In addition, IRE-1 can recruit tumour necrosis factor receptor-associated factor-2 (TRAF-2) possibly linking the ER stress response to apoptosis [160].

In summary, the ER stress response occurs in two phases; the first is physiologic and involves the two main UPR transcription factors, N-ATF-6 and XBP-1. On the other hand, the second phase is pathologic and involves the PERK pathway, which triggers proapoptotic signals responsible for cell loss in disease conditions. Finally, in the heart, ER stress appears to play a major role in cardiovascular homeostasis, as well as in pathologic conditions such as hypoxia, hypertrophy, and drug-induced toxicity. Therefore, this pathway could serve as a potential therapeutic target for the treatment of cardiac insults and diseases [160].

1.4. Anti-Neoplastic Drug-Induced Cardiotoxicity

1.4.1. Cancer

Cancer is a malignant growth or tumor caused by the uncontrolled proliferation of abnormal cells. These cancerous cells have their genomes altered at multiple sites producing mutations that activate oncogenes and repress tumor suppressor genes. In general, cancer cells have defective proliferation and homeostasis regulating circuits. Nowadays,

more than a hundred different types of tumors targeting almost all body organs have been described [161]. The increased cancer incidence and the associated huge mortality rate amongst patients coupled to the complexity of the disease provoked intense research into deciphering its mechanisms, which essentially paved the way for the current multi-billion anticancer drugs industry.

Physiologically, cancer cells acquire novel capabilities that enable them to evade the body's built-in anticancer defense mechanisms. These alterations, which dictate the degree of malignant growth, include: growth signals self-sufficiency, antigrowth signals insensitivity, apoptosis evasion, indefinite proliferation potential, continuous angiogenesis, and tissue invasion and metastasis [161]. Tumor cells and in clear contrast to normal cells, show a reduced dependence on exogenous mitogenic growth signals. They have developed a growth stimulation autonomy that is typically achieved by altering either extracellular growth signals, their transcellular transducers, or the intracellular pathways responsible for relaying and translating the growth signal into action. Furthermore, cancer cells evade antigrowth signals that are known to block proliferation by either forcing cells into the quiescent (G_0) state (e.g. retinoblastoma protein pathway) or inducing their entry into postmitotic differentiated states (e.g. Mad-Max pathway) [162-164]. A third characteristic of cancer cells is their ability to resist apoptosis and hence cell death. These cells can evade apoptosis by either the loss of a proapoptotic regulator like the *p53* tumor suppressor gene [165], or the upregulation of factors central to prosurvival pathways, such as the PI3K/AKT pathway [166,167].

Mammalian cells and in addition to their dependence on environmental signals carry an intrinsic autonomous antiproliferation program. The disruption of this program is key for their ability to expand to macroscopic life threatening growths. Immortalized tumor

cells acquire an ability to multiply without limit, breaching the mortality barrier of telomere shortening [161]. This successive erosion of telomeres with each multiplication round eventually leads to karyotypic disarray and cell death. Cancer cells avoid this by actively maintaining their telomeres mostly through the upregulation of the telomerase enzyme, which preserves the telomeres length above a certain threshold permitting the unlimited multiplication of cancer cells [168]. Indefinite proliferation leading to a large number of cells means a greater demand for oxygen and nutrients, and hence a successful tumor formation requires the sustained growth of blood vessels, a process known as angiogenesis. Tumors activate the angiogenic switch by inducing the angiogenesis initiation signals like the vascular endothelial growth factor (VEGF) and inhibiting antiangiogenic factors such as thrombospondin-1 [169,170]. This ability to induce and sustain angiogenesis is believed to be acquired in mid-stage lesions, *a priori* to full blown tumor appearance [161]. The last characteristic of tumor cells is their ability to escape the primary cancer mass and invade adjacent or distant tissues where nutrients and space are not limiting. This metastasis process, which is the cause of most of human cancer fatalities, is exceedingly complex and remains incompletely understood [171]. Invasion and metastasis involve changes in the physical interactions of cells to their microenvironment as well as the activation of proteases. Proteins responsible for cell to cell interactions (CAMs), and cell to matrix associations (integrins) are hugely altered in cells with invasive and metastatic potentials. For instance, E-cadherin, an adhesion molecule ubiquitously expressed on epithelial cells, is one of the most commonly altered proteins in invasive types of cancers [172,173]. Finally, these six capabilities acquired by cancer cells along with their associated cellular pathways are currently the targets of antineoplastic therapies [161], which I will discuss in the next section.

1.4.2. Anticancer Treatments

As mentioned above, cancer is not a single disease with a lone cause, rather it is a huge number of variable diseases with complex origins. Therefore, anticancer treatments are numerous and their individual success rates depend on the type and degree of the malignancy in question. Cancer therapies can be broadly divided into surgery, chemotherapy, radiation therapy, and biological therapy. Cancer surgery, which is defined as an operation to remove a tumor, is the basis of cancer therapy and is recommended in many cases in which the abnormal growth is localized and has not metastasized yet. It might be the only cancer treatment or it may be supplemented with other treatments. Chemotherapy, on the other hand, involves a group of cytotoxic drugs, like anthracyclines, that kill rapidly dividing cells by interfering with cell division and DNA synthesis. Unfortunately and in addition to destroying cancer cells, chemotherapy harms healthy dividing cells especially those lining the gut and forming the hair follicle. Chemotherapy, which is performed to either cure or control cancers, can be used alone or along with either radiation therapy, surgery, or biological therapy depending on the type and degree of the malignant tumor. On the other hand, radiotherapy, which usually accompanies surgery and/or chemotherapy, is defined as the use of high energy radiation to kill tumors by interfering with the DNA of cancer cells. Again radiation therapy can kill normal cells adjacent to the targeted tumor leading to side effects and thus careful planning is crucial prior to treatment [174].

Given the difficulties, limited success rate, and side effects associated with the afore-mentioned anticancer therapies, a fourth treatment modality, namely biological therapy, has been gaining ground in the past 20 years. Biological therapy, which is designed to boost anticancer immune responses, is based on the observation that the incidence of tumors is higher in immunosuppressed patients and the identification of cancer-specific antigens and

lymphocytes. These days, bone marrow transplantation and monoclonal antibodies targeting tumor cells are examples of widely and efficiently used immunotherapies. Although progress in this field has been slow and still faces major hurdles like the development of valid assays that monitor immune responses, recent evidence on the clinical potential and efficacy of several anticancer immunotherapeutic drugs has promoted the development of immunotherapy. Moreover, new promising research data are increasingly showing a beneficial effect of the combination of other treatment modalities with immunotherapy [175].

The last major and most promising field of cancer treatment is the so called targeted therapy, where a group of antibodies and small-molecule kinase inhibitors are designed to more or less specifically target key proteins involved in growth and proliferation pathways. Currently, 11 FDA-approved agents exist and many more are waiting for approval, offering new hope to many patients with cancers that are unresponsive to other treatment modalities. Interestingly, the high mutation rate affecting protein kinases in cancers and the subsequent cancer-driving roles played by these altered kinases triggered the search for molecules that can specifically inhibit these pro-growth kinases. Today, targeted therapeutics, like Imatinib mesylate (Gleevec), have drastically transformed the treatment of solid tumors and some blood malignancies. The PI3K pathway plays a crucial role in cancers where major factors in this pathway are usually mutated or amplified, thus making it a perfect target in cancer therapy. Therefore these days, the pharmaceutical industry is actively pursuing agents that can inhibit either receptor tyrosine kinases upstream of, or pro-growth kinases associated to the PI3K pathway, with several agents already in clinical trials. Another aspect of cancer is the dysregulation of cell cycle regulators, and hence targeting these factors is currently a major focus in cancer research. In fact, numerous cyclin-D kinases

(CDKs) inhibitors, which target these proliferation essential kinases, are in development with many in clinical trials [176].

1.4.3. Cardiotoxicity of Cancer Therapy

Despite the recent successes, cancer therapy progress continues to be hindered by the cardiotoxicity associated with many anticancer treatments. Antineoplastic therapy-induced cardiotoxicity is currently an alarming clinical problem faced by cardiologists, hence the urgent need to deal with such a critical issue. Heart cells, particularly cardiomyocytes, are prone to temporary or permanent injury upon exposure to toxic agents, like recreational drugs, alcohol, and therapeutic agents. Moreover, the fact that both cancer incidence and heart disease prevalence increase in the elderly population further compounds the issue at hand. The realization that cancer therapy might aggravate an underlying cardiac problem or create a new one did not become a concern for oncologists and cardiologists until the early 1970s. The cardiotoxicity of anthracyclines, bacterial antibiotics used in chemotherapy, was the first to be described raising this issue to the forefront of discussion. Although much of the literature focuses on anthracycline-related cardiomyopathy, other forms of cardiotoxicity that impair cardiac function are also common. For instance, drugs negatively affecting the vasculature and the pericardium can result in ischemia and changes in blood pressure, as well as an imbalance in liquid equilibrium and pericardial thickening, respectively. Finally, anticancer treatment can exacerbate or induce cardiac arrhythmias and other cardiac conditions in many patients [176-179].

In anthracycline-induced cardiomyopathy, the prototypical example of anticancer-related cardiotoxicity, LV systolic dysfunction was found to be dose dependent and damage was permanent and irreversible eventually leading to heart failure and cardiac

death. Anthracyclines and other drugs that commonly cause irreversible cell destruction are known as type I agents [117, 177]. On the other hand, many novel drugs that also cause cardiomyopathy, like the monoclonal antibody trastuzumab, were found to induce a reversible less severe damage and are thus called type II agents. This agent-induced toxicity is not dose dependent and in most cases reversible upon drug withdrawal [177]. The cardiotoxicity of doxorubicin, an anthracycline, and imatinib, a tyrosine kinase inhibitor, being the focus of this thesis, will be discussed in the coming sections.

1.4.4. Doxorubicin

1.4.4.1. History and Clinical Use

Doxorubicin (DOX) is one of several anthracycline antibiotics that have been effectively used as anticancer agents in the past 50 years. Anthracyclines are commonly used in the treatment of breast cancer, leukemia, lymphoma, and sarcomas. They induce cell death through mechanisms that involve DNA intercalation, topoisomerase II inhibition, as well as replication and transcription inhibition. Nowadays, the use of anthracyclines is limited due to their well-established negative effects on the heart, especially cardiomyocytes often resulting in cardiomyopathy [177].

Doxorubicin (Adriamycin, Bedford Laboratories, Bedford, OH) has been used as an anticancer agent since the late 1960s. Tumors treated with this anthracycline include esophageal and breast carcinomas, osteosarcoma, soft tissue sarcoma, Kaposi's sarcoma, and Hodgkin's and non-Hodgkin's lymphomas [179]. Moreover, in many breast cancer patients doxorubicin is used in conjunction with trastuzumab, a monoclonal antibody against the oncogenic human epidermal growth factor receptor 2 (HER-2, ErbB-2) [180]. Unfortunately,

the use of DOX has been subdued by reports of fatal cardiotoxic events in cancer patients on DOX chemotherapy [117,118,181].

1.4.4.2. Doxorubicin-Induced Cardiotoxicity

Today DOX-induced cardiotoxicity has been well established and as mentioned above has limited the use of this drug in cancer therapy. Mechanistically, DOX is known to form a complex with iron, increasing free radical production and inducing oxidative damage characterized by membrane disruption and large scale cellular dysfunction. Cardiomyocytes in particular are highly susceptible to oxidative damage, first because of their limited regenerative capacity, and second due to their lack of essential oxidative stress enzymes. Other mechanisms that could aid in cardiotoxicity induction include DNA damage, mitochondrial DNA mutations accumulation, calcium handling alterations, and dysregulation of important cardiac transcription factors. This cardiotoxicity is dose dependent and partially irreversible damage occurs with each drug administration leading to characteristic structural changes that can be assessed using electron microscopy. These structural irregularities can range from vacuolation and contractile elements disarray to cell death [177,182,183].

Generally, doxorubicin-induced cardiomyopathy occurs within 1 month to 1 year, and risk factors include older age, hypertension, diabetes, previous cardiac disease, and simultaneous treatment with other anticancer drugs [179]. On the other hand, many strategies have been developed to reduce this risk, like the use of DOX analogues, liposomal delivery systems, and the co-administration of cardioprotective chemicals. These approaches have been more or less successful with the highest success rate associated with dosage limitation and alternative drug delivery methods [177]. Treatment of this drug-induced cardiomyopathy include angiotensin-converting enzyme (ACE) inhibitors, diuretics, beta-blockers, and

digoxin. Surprisingly and despite the dose-dependent negative effects on the heart, DOX continues to be used in cancer therapy due to its efficacy in the treatment of many tumors [177,179,182].

1.4.4.3. Mechanisms of Cardiotoxicity and the Role of GATA-4

Over the past couple of decades, research on DOX-induced cardiotoxicity failed to completely elucidate the precise mechanisms underlying this toxicity. However, most studies agree on the pivotal role played by DOX-induced oxidative stress, given that DOX chemical structure possesses an inherent tendency to generate free radicals during its metabolism. For instance, DOX conversion to an unstable semiquinone that favors ROS generation was recently described. Moreover, mitochondrial DNA damage caused by DOX directly or by ROS was found to cause respiratory chain failure and increased ROS production [182]. On the other hand, DOX cardiotoxicity could be aided by a variety of contributors including, calcium handling dysregulation, adrenergic dysfunction, and inhibition of cardiomyocyte-specific gene(s) expression, like GATA-4. In fact, GATA-4, a known key regulator of heart development and an essential postnatal myocytes survival factor, is depleted rapidly in response to DOX treatment [117]. Interestingly, the inhibition of *GATA-4* gene transcription by Daunorubicin, another anthracycline, was recently found to involve a p53-dependent mechanism [119]. Furthermore, GATA-4 downregulation was associated with concomitant decrease in both proapoptotic *BCL-X_L* and *BCL-2* gene expression, leading to increased apoptotic cell death. Conversely, enhancing GATA-4 activity by the α1 adrenergic agonist phenylephrine or the adenovirus-mediated overexpression of GATA-4, prevented DOX-induced apoptosis in cardiomyocytes mainly via the transcriptional activation of both *BCL-X_L* and *BCL-2* [117,118].

Most of the cellular events mentioned above lead to cardiomyocyte death mainly through apoptosis and necrosis, but other cell death forms, like autophagy, might also be plausible. DOX-induced oxidative stress and the subsequent effect on cytosolic calcium homeostasis lead to mitochondrial calcium overload, which in turn triggers mitochondrial permeability transition (MPT). MPT results in loss of membrane potential, swelling, and outer membrane rupture, subsequently, releasing cytochrome c and initiating the intrinsic apoptosis pathway [182]. On the other hand, several studies indicated that cardiomyocyte death in DOX-induced cardiac dysfunction can be partly through a Fas-mediated extrinsic apoptosis pathway [184]. Interestingly, necrosis or the increasingly popular programmed necrosis could play a major role in DOX-induced cardiotoxicity. Mechanistically, ATP depletion caused by mitochondrial uncoupling could favor necrotic rather than apoptotic cell death [185]. Finally, DOX was also found to dramatically increase autophagic fluxes in cardiomyocytes that essentially contribute to its cardiotoxicity. Once again, increased GATA-4 expression inhibited DOX-induced autophagy and reduced cell death, reinstating the central role of GATA-4 as a cardioprotective prosurvival signal in adult cardiomyocytes [118].

1.4.5. Kinase Inhibitors and Cardiotoxicity

1.4.5.1. Introduction

Kinase inhibitors (KIs) are small molecule inhibitors that usually compete with ATP for binding to the kinase ATP pocket. KIs binding blocks the phospho-transferase activity of an oncogenic kinase preventing the phosphorylation of downstream substrates. These kinase inhibitors must bind the pocket with a very high affinity at low cellular concentrations (nM to μM), given the abundance of ATP in a cell (mM). It is relatively easy

to make these KIs due to the fact that the ATP pocket is highly conserved across the more than 500 different human kinases. Not surprisingly, type I inhibitors, which target the ATP pocket only, are less selective and thus lead to off-target effects and potential toxicity. This poor selectivity is addressed in type II inhibitors like Imatinib, which usually recognize other regions of the kinase in addition to the ATP pocket. Type II inhibitors are thus more selective and typically more potent because they can bind and inhibit the kinase in both its active and inactive conformations. Finally, type III inhibitors like the MEK inhibitors, target kinase-specific non-conserved regions different than the ATP pocket, and therefore, are excellent selective KIs. Unfortunately, type III inhibitors design-associated difficulties and less effectiveness make them a small minority of therapeutic agents in development. Furthermore, the superior effectiveness of more selective inhibitors over non-selective ones has been challenged in a variety of oncological and inflammatory diseases, because non-selective KIs can target other kinases that might be essential for disease progression and would typically lead to a better anticancer efficacy. Moreover, non-selective KIs can be used in more cancers and thus are more lucrative from a pharmaceutical firm point of view [186].

1.4.5.2. Imatinib Mesylate and Tyrosine Kinase Inhibitors

The human kenome is composed of 518 kinases, out of which around 90 are tyrosine kinases (TKs). Tyrosine kinases can be receptor tyrosine kinases (RTKs) or non-receptor TKs. TKs are usually heavily mutated or amplified in cancers and therefore the development of therapeutic TK inhibitors (TKIs) has exploded recently. Imatinib mesylate (Gleevec, Novartis), was the first TKI to reach market following FDA approval in 2001. Imatinib approval as an anticancer drug paved the way for several tyrosine-kinase-targeted therapies [187]. Imatinib targets the tyrosine kinase activity of the BCR-ABL fusion protein

resulting from a chromosomal translocation, the Philadelphia chromosome [188]. This fusion protein contains a part of the BCR non-kinase domain and the non-receptor c-ABL tyrosine kinase domain. The BCR-ABL fusion protein dimerization cross phosphorylates ABL kinase domain leading to its constitutive activation. This constitutively active kinase activates antiapoptotic pathways including, the RAS-RAF-ERK pathway, the PI3K-AKT pathway, and the JAK-STAT pathway, ultimately enhancing cell division and inhibiting DNA repair leading to chronic myelogenous leukemia (CML) [189]. CML is characterized by the uncontrolled and increased production of abnormal bone marrow myeloid cells, which usually accumulate in blood [190]. During the pre-Imatinib era, CML was treated by chemotherapy, interferon, and bone marrow transplantation but with variable successes (reviewed in [191]). The discovery of Imatinib has revolutionized the treatment of CML patients, with an outstanding 70% cytogenic remission and initial minimal side effects, namely peripheral edema and dyspnea [192]. In addition to its inhibition of BCR-ABL, Imatinib was found to inhibit the receptor for stem cell factor (c-KIT), usually overexpressed in gastrointestinal stromal tumors (GISTs), and platelet derived growth factor receptors (PDGFRs), which play roles in other cancers including GIST and glioblastoma [186]. Recently, Imatinib use has been extended to the treatment of more than 10 solid cancers including gastrointestinal stromal tumors and prostate cancer [193-195].

1.4.5.3. Imatinib-Induced Cardiotoxicity

More recently, numerous reports on the unexpected effect of Imatinib therapy on the development of congestive heart failure (CHF) have emerged [187,188,196-198]. These data are highly controversial given the results of IRIS (International Randomized Study of Interferon versus STI571, Imatinib), where the overall incidence of CHF was about 1% in both the

imatinib and the interferon arms [199-201]. This discrepancy could be attributed to the lack of accurate and extensive cardiac function monitoring in most cancer clinical trials, and/or to variables that can affect cardiovascular parameters like age, sex, or obesity. When tested on cultured cardiomyocytes and in mice, Imatinib was found to induce cell death associated with early apoptotic features, and to lead to compromised cardiac function, proving that it has the potential to cause CHF. In the same study, imatinib-induced cardiomyocyte death was found to be triggered or at least associated with a dysregulated mitochondrial function [188], an anomaly aggravated in aging hearts [202].

Mechanistically, Imatinib-induced cardiotoxicity was reported to be partly due to its direct inhibition of c-ABL in cardiomyocytes, where the use of an Imatinib-resistant mutant, ABL T3151, partially rescued these cells. Furthermore, treated cardiac myocytes had increased phosphorylation of the eukaryotic translation initiation factor 2α (eIF-2α), a substrate of PRK-like ER kinase (PERK), an important player in the endoplasmic reticulum stress response [188]. A prolonged ER stress recruits the proapoptotic Jun-N-terminal kinases (JNKs) eventually leading to cell death [203]. Moreover, Imatinib was found to induce an upregulation of the proapoptotic protein kinase Cδ (PKC-δ), which could possibly have a role in its cardiotoxicity [188,204]. Unfortunately, the pathways implicated in Imatinib-induced cardiac toxicity are still not fully understood. For example, the pathway linking ABL inhibition to the ER stress response is not clear yet.

1.5. Hypothesis and Objectives

Cancer drugs induced cardiotoxicity leading to cardiomyocyte death and heart complications is an important health related issue but the underlying mechanisms remain poorly understood. Unveiling these mechanisms is crucial for overcoming this toxicity. Postnatal cardiac myocytes are terminally differentiated and the heart has limited regenerative capacity; as such loss of myocytes is sufficient to cause heart failure. As mentioned earlier, previous work from the lab has shown that interfering with myocyte loss protects against heart failure. The objective of my PhD is to elucidate the factors and pathways that promote myocyte survival and to determine the molecular basis of drug induced cardiotoxicity. Specifically, my project addresses the cardiotoxicity of the antineoplastic drugs Imatinib mesylate and doxorubicin (DOX). It will focus on the role of GATA-4 in cell survival and the pathways involved.

My hypothesis is that GATA-4 is a central regulator of cardiomyocyte survival and that the negative effects induced by both Imatinib and doxorubicin converge on pathways involving GATA-4 upstream regulators, partners, and downstream targets.

Our Specific aims were:

1. Understanding the Mechanism of Imatinib Cardiotoxicity
2. Identifying GATA-4 Upstream Regulators in Cardiomyocyte Survival
3. Identifying GATA-4 prosurvival Partners

The proposed work would help establish a plausible mechanism for Imatinib cardiotoxicity and should provide answers on the pathways involved. Moreover, It will further our understanding of the role of GATA-4, its upstream regulators, partners, and downstream targets in cardiomyocyte survival.

Chapter One
Age dependent Imatinib mesylate induced cardiac toxicity

Wael Maharsy, Anne Aries, Omar Mansour, Hiba Komati, and Mona Nemer

Contributions:

In this Chapter, I performed all the *in vivo* and *ex vivo* work done with Imatinib and wrote the chapter. Genetically modified mice were generated by previous members in the laboratory.

2. Chapter 1

2.1. Abstract

Postnatal cardiomyocytes have limited proliferative capacities and their loss leads ultimately to heart failure. This effect on myocyte cell survival underlies, in part, the cardiotoxicity of some antineoplastic drugs. Cardiotoxicity of Imatinib mesylate, the first of a new generation of highly selective anticancer drugs, has been reported in patients, some progressing to congestive heart failure. This controversial finding represents a major clinical challenge that could limit effective drug use. Understanding the mechanisms of drug induced cardiotoxicity and risk factors is crucial for prevention of cardiovascular complications in cancer patients. We used genetically engineered mice (C57BL/6) and cultured primary rat ventricular cardiac myocytes to analyze the mechanisms of Imatinib cardiotoxicity. We found that Imatinib (200 mg/Kg/day for 5 weeks) exerts its negative effects on murine cardiac function and structure through mitochondrial-dependent myocyte death. We also found that Imatinib reduces levels of transcription factor GATA4 and its prosurvival target genes Bcl-2 and Bcl-XL. GATA4 haploinsufficient mice were more susceptible to drug induced cardiotoxicity. Enhancing mitochondrial integrity by myocyte-specific upregulation of Bcl-2 protected against Imatinib cardiotoxicity. Importantly, Imatinib cardiotoxicity was more severe in older mice, in part due to an age-dependent increase in oxidative stress as confirmed in vitro in H2O2-stressed primary rat ventricular cardiomyocytes. The results indicate that Imatinib action on the heart involves mitochondrial impairment and mitochondrial-dependent cell death that can be further aggravated by oxidative stress. This in turn offers a possible explanation for the current conflicting data regarding Imatinib-induced cardiotoxicity in cancer patients and suggests that cardiac monitoring of older patients receiving Imatinib therapy may be especially warranted.

2.2. Introduction

Postnatal myocytes have limited regenerative capacity and their loss leads to cardiac remodeling that involves fibrosis and enlargement of the remaining myocytes[205]. This process, termed pathologic hypertrophy, which ultimately leads to heart failure [113,205], is often triggered by or accompanies ischemic heart disease and is also observed in response to some antitumor therapies. For example, the anthracycline group of antineoplastic drugs, have been shown to induce cardiotoxicity in cancer patients (reviewed in [177]). This latent anthracycline cardiotoxicity is thought to be due to dysregulated mitochondrial biogenesis and increased myocyte apoptosis. Doxorubicin causes decreased expression of the anti-apoptotic genes *BCL-2* and *BCL-X* [117]. In fact, loss of myocytes is sufficient to cause heart failure as shown in several experimental models [115,116]. Conversely, previous work from our lab has shown that interfering with myocyte loss protects against heart failure and prevents chemotherapy-induced cardiotoxicity [117].

Imatinib mesylate (Imatinib) was the first of a new generation of highly selective anticancer drugs that paved the way for several novel tyrosine-kinase-targeted therapies [187,206]. Imatinib targets the tyrosine kinase activity of the BCR-Abl fusion protein resulting from a chromosomal translocation, the Philadelphia chromosome [207]. This constitutively active gene results in dysregulated cell cycle, enhanced cell division, and impaired DNA repair leading to chronic myelogenous leukemia (CML) [208]. The introduction of Imatinib has revolutionized the treatment of CML patients, with an outstanding 70% cytogenic remission and initial minimal side effects, namely peripheral edema and dyspnea [209]. More recently, Imatinib use has been extended to the treatment of other solid cancers including gastrointestinal stromal tumors and prostate cancer [193-195].

Recently, several reports on the effect of Imatinib therapy on the development of congestive heart failure (CHF) have emerged [187,188,196-198]. The finding that Imatinib may be cardiotoxic was unexpected given the results of IRIS (International Randomized Study of Interferon versus STI571), where the overall incidence of CHF was about 1% in both the imatinib and the interferon arms [199-201]. This discrepancy could be attributed to the lack of accurate and extensive cardiac function monitoring in most cancer clinical trials, and/or to variables that can affect cardiovascular parameters like age, sex, or obesity. It is also important to point out that cardiac toxicity could be latent as seen with Doxorubicin where cardiac dysfunction can occur years after the end of drug usage [177]. Mechanistically, the latent effects could be the result of subclinical alterations of cardiac gene expression that ultimately impair the adaptive stress response of the heart [117]. In the case of Imatinib, an earlier study suggested that Imatinib-induced cardiac dysfunction may be mitochondrial-dependent [188]. At present, the effects of and the mechanisms underlying Imatinib action on the heart remain incompletely understood. In the present work we analyzed the mechanism of Imatinib action on the heart and investigated the impact of aging on Imatinib-induced cardiotoxicity. We found that Imatinib induces cardiomyocyte loss via the activation of the mitochondrial apoptotic pathway and that prevention of mitochondrial dysfunction through upregulation of Bcl-2 protects against Imatinib cardiotoxicity. Remarkably, clinical evidence of cardiotoxicity was found to be age-dependent and more overt in aging mice. These results provide new insights into the mechanism of Imatinib action on the heart and offer a possible explanation for the current controversy regarding Imatinib-induced cardiotoxicity in cancer patients.

2.3. Materials and Methods

2.3.1. Cell Cultures: Procedures with neonatal cardiomyocytes were as previously described [34]. Imatinib mesylate (Gleevec®, Novartis) 100 mg tablets were dissolved in water and repeatedly centrifuged at 2,500g to produce highly purified material. Primary cardiomyocytes were treated with vehicle or Imatinib at varying concentrations (0.5 μM, 1 μM, 2.5 μM, and 5 μM). Hydrogen peroxide (H_2O_2) was added to culture plates at a final concentration of 25 μM.

2.3.2. *In Vivo* Experiments: Mice were handled in accordance with institutional guidelines for animal care. Experiments were approved by the institutional Animal Care Committee of the University of Ottawa. *Gata4+/-* mice were previously described [210]. *Bcl-2* overexpressing mice were generated using an SV40 expression vector containing the human *Bcl-2* cDNA under the control of the alpha MHC promoter [210] to specifically direct expression to cardiomyocytes. hBcl-2 expression in transgenic mice was verified using northern and western blots. Mice were treated with Imatinib mesylate (200 mg/kg/d) or vehicle for 5 weeks as previously described [188]. At the end of the experimental protocol, mice were anesthetized with 12-15 μl/g i.p Avertin (2.5% solution) and sacrificed. For M-mode echocardiography, mice were anesthetized (2.0% isoflurane, 80 ml/min 100% O_2), their anterior chests were shaved, and two-dimensional guided M-mode echocardiography was performed using a Visual-Sonics VEVO 770 and a 30-MHz linear array transducer as described by Aries et al. [117].

2.3.3. Immunohistochemistry and Terminal Deoxynucleotidyltransferase Mediated dUTP End labeling (TUNEL): Immunohistochemical studies were performed as described [28,211]. The ANF antibody [(T4014, RGG-9103) (dilution 1:1000)] was purchased from Peninsula Laboratories (San Carlos, California) . A Zeiss AxioImager.A2 light microscope

(Objectives: Zeiss EC Plan-Neufluar; Camera: Zeiss AxioCam MRC)was used for image acquisition. The TUNEL assay was utilized to detect apoptotic nuclei using an Apoptag kit (Intergen, Purchase, NY) and counterstained with methyl green. Analysis was done as described [117].

2.3.4. Blots Analysis: Northern on RNA samples and Western on cytoplasmic extracts from homogenized mice tissues were performed as reported previously [34]. The Bcl-2 antibody [(Ab-2; PC-68) (dilution 1:1000)] was purchased from Oncogene Research Products (San Diego, California).

2.3.5. Real-Time PCR: Total RNA was isolated from mice tissues with Trizol (Invitrogen). Transcript levels for the various cardiac markers were determined by real-time PCR carried out as described by Debrus et al. [211].

2.3.6. Statistical Analysis: Data are reported as means ± the standard error of the mean (SEM). A student unpaired t test was used to compare any two groups, while the one-way analysis of variance (ANOVA) test was used to compare multiple groups. In all cases, a $P < 0.05$ was considered as an index of statistical significance.

2.4. Results

2.4.1. Imatinib Negatively Affects Murine Cardiac Function and Structure

To analyze the effect of Imatinib on the heart, wild type mice (150d, N= 6 per group) were treated for 5 weeks with Imatinib (200mg/Kg/day) or vehicle alone and cardiac function was assessed using echocardiography. No significant difference in the body weights of the 2 groups was noted. Mice treated with Imatinib had a reduced cardiac output, as well as a mildly reduced left ventricular wall thickness (LVmass/Body Weight) (**Figure 2.1A and 2.1B**). In addition, Imatinib induced a reduction in the LV mean gradient probably due to impaired cardiac relaxation, characteristic of diastolic dysfunction (**Figure 2.1C**). Ejection fraction and fractional shortening were not significantly changed upon Imatinib treatment (not shown). The decrease in LV wall thickness was confirmed by histologic examination of Mason Trichrome stained tissue sections (**Figure 2.1D**).

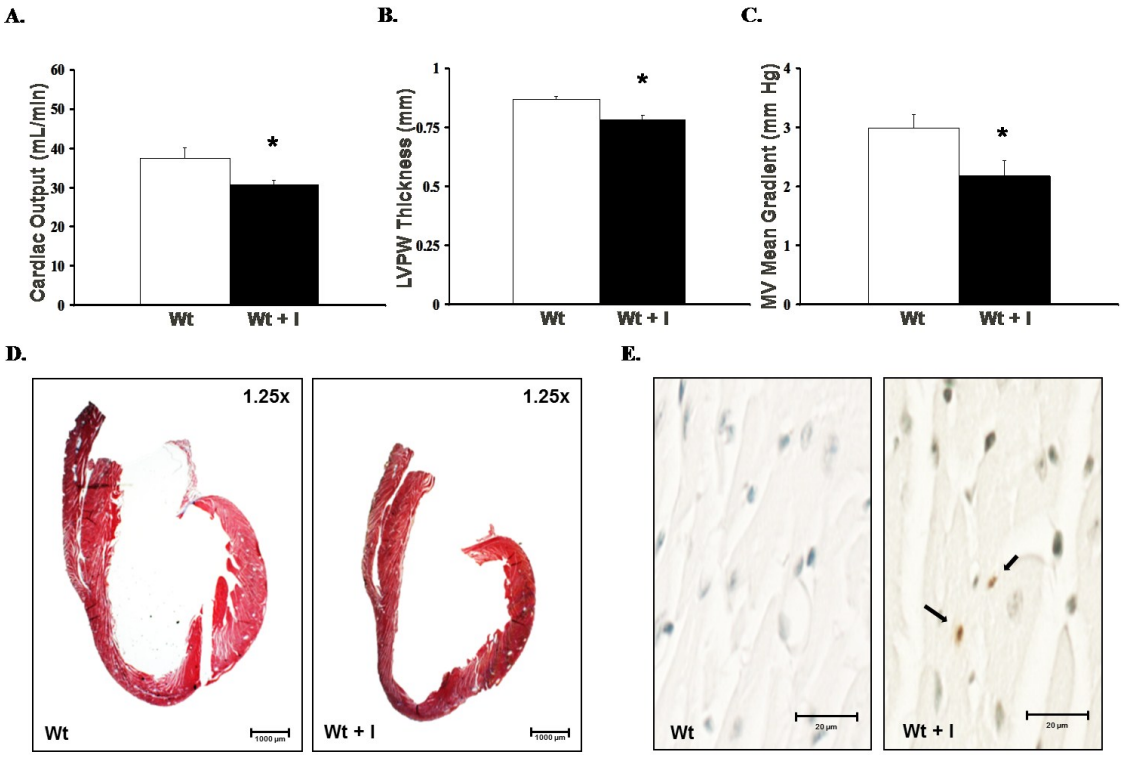


Figure 2.1. Imatinib induces cardiotoxicity in mice A. Graph representing cardiac output (CO) in wild type mice (Wt) and wild type mice treated with Imatinib (Wt + I) as obtained by echocardiography. **B.** Graph representing LV posterior wall thickness in Wt and Wt + I mice. **C.** Graph representing mitral valve mean gradient in Wt and Wt + I mice. The data shown are the mean of measurements obtained from four different mice (150d) per group <0.05. **D.** Representative Trichrome-stained heart sections from Wt and Wt + I mice. **E.** Representative TUNEL images with arrows indicating brown stained positive nuclei. N= 6.

As reduction in LV mass may be indicative of myocyte loss, TUNEL assays were performed on heart sections to assess cell death. As shown in **Figure 2.1D**, Imatinib treatment induced an increase in TUNEL positive nuclei in treated mice as compared to the vehicle treated ones (1.049% +/- 0.078 vs. 0.300% +/- 0.011, $p < 0.02$).

2.4.2. Imatinib-Induced Cardiotoxicity is Age Dependent

Given the above and the fact that mitochondrial integrity and biogenesis are affected in aging hearts [202], we investigated the impact of aging on the severity of Imatinib-induced cardiotoxicity. Older mice (450d) were treated with vehicle or Imatinib and their cardiac parameters compared to those of younger adult mice (150d). After 5 weeks of Imatinib (Gleevec) administration, echocardiography showed that both young and old mice treated with Imatinib had an increased left ventricular volume at diastole (**Figure 2.2A**).

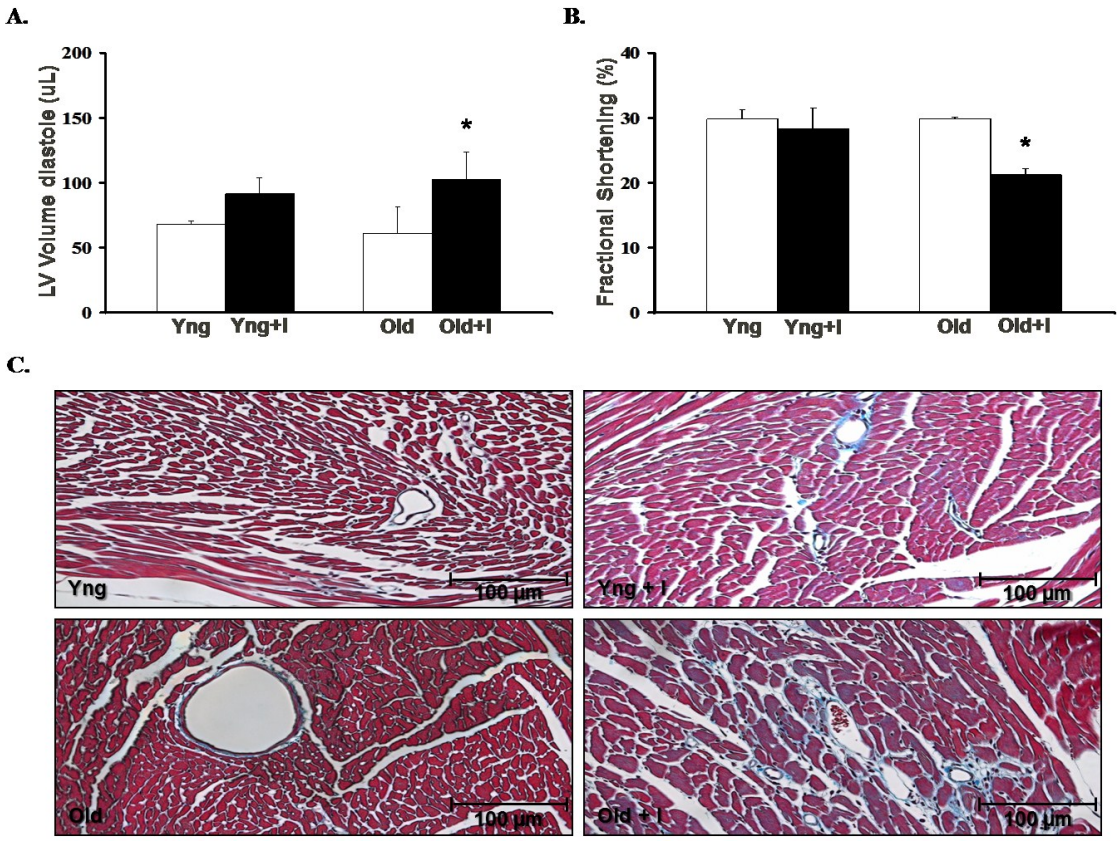
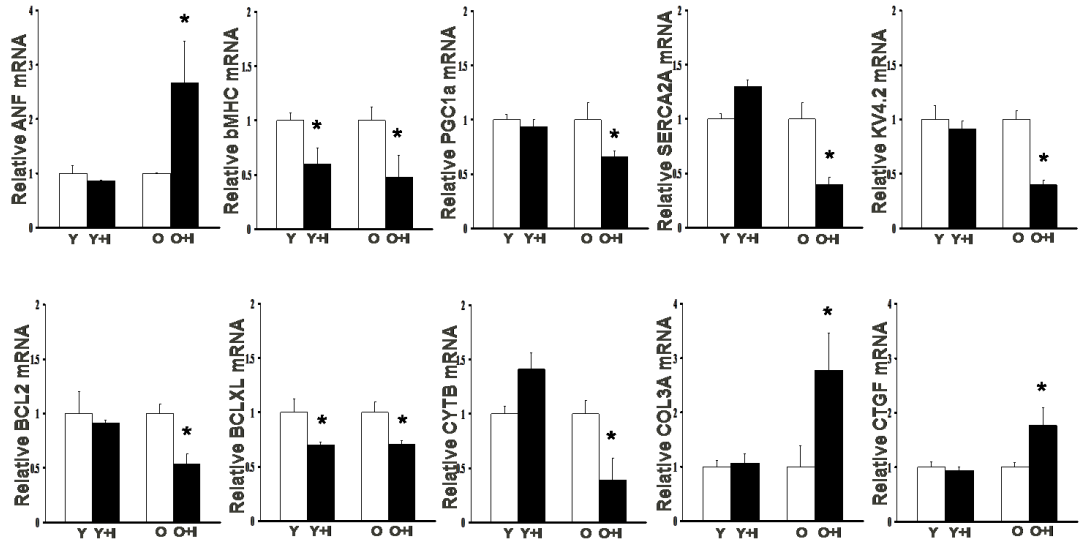


Figure 2.2. Imatinib-Induced Cardiotoxicity is Age Dependent. A. Echocardiography results showing changes in left ventricular chamber diastolic volume in young (Yng, 150d) and old (Old, 450d) mice treated with vehicle or Imatinib (200 mg/Kg/d). **B.** Echocardiography results showing changes in fractional shortening (FS) in the source groups. **C.** Trichrome stained left ventricle sections from young and old mice treated with vehicle or Imatinib (200 mg/Kg/d) note excessive remodeling in old treated hearts. N= 6.

Fractional shortening, reflective of contractility, was only significantly reduced in the Imatinib treated older mice group (**Figure 2.2B**). Trichrome staining revealed increased myocyte loss and fibrosis in the left ventricles of treated old mice; younger mice heart sections had little if any increase in fibrosis (**Figure 2.2C**). Consistent with the differential effects of Imatinib on the 2 age groups, differential changes in gene expression were observed in old vs. young Imatinib treated mice (**Figure 2.3A**). Real-Time PCR (QPCR) analysis performed on cDNA samples obtained from mice ventricles showed similar downregulation of certain genes such as bMHC and Bcl-X_L in both treated groups. Other genes were differentially regulated in older mice. For example, important ion channels like Kv4.2 and SERCA2A were differentially downregulated in the treated older mice. Interestingly, mitochondrial integrity markers, like PGC-1 and CYTB mRNA levels, were reduced only in the ventricles of older treated mice. Moreover, the cardiac stress marker ANF and some profibrotic genes, like COL3A and CTGF, were only upregulated in old mice treated with Imatinib. These changes in gene expression are consistent with an exaggerated stress response and cardiac remodeling of older Imatinib-treated mice. This could be due to impaired homeostatic mechanisms predisposing aging cardiomyocytes to drug-induced cardiac injury. A closer look at gene expression differences between young and old mice (**Figure 2.3B**) showed an upregulation of stress response genes like bMHC, SERCA2A, and CYTB in the older population. Moreover, pro-death genes like Beclin1, an autophagy marker, and BAX, were also differentially upregulated in older mice. Finally, the hypoxia responsive gene HIF1α level increased in older mice confirming previous reports describing the injury-susceptible hypoxic state of aging cardiomyocytes.

A.


Relative ANF mRNA
Relative bMHC mRNA
Relative PGC1a mRNA
Relative SERCA2A mRNA
Relative KV4.2 mRNA
Relative BCL2 mRNA
Relative BCLXL mRNA
Relative CYTB mRNA
Relative COL3A mRNA
Relative CTGF mRNA
Y Y+I O O+I

B.

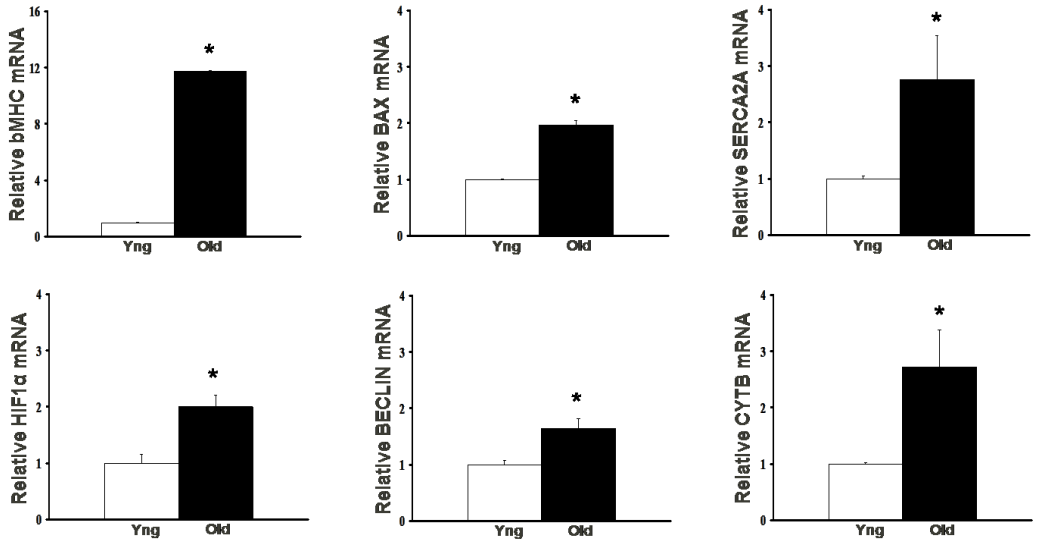

Relative bMHC mRNA
Relative BAX mRNA
Relative SERCA2A mRNA
Relative HIF1α mRNA
Relative BECLIN mRNA
Relative CYTB mRNA
Yng Old

Figure 2.3. Impaired regulation of stress response genes in older mice. A. Graphs showing QPCR results for ANF, bMHC, PGC1a, SERCA2A, KV4.2, Bcl-2, Bcl-X_L, CYTB, COL3A1, and CTGF mRNA levels in mice ventricles from all four subgroups. **B.** Graphs showing QPCR results for bMHC, BAX, SERCA2A, HIF1α , Beclin, and CYTB mRNA levels in mice ventricles from young and old mice. The data expressed are corrected to S16 mRNA levels (internal control) and values obtained from the non-treated ventricular samples were assigned an arbitrary value of 1. ‘’*’’ indicates statistical significance where $p < 0.05$. N=6.

2.4.3. GATA4 Downregulation Is Associated With Imatinib-Induced Cardiotoxicity

Previously, we showed that GATA4, an important regulator of cardiomyocyte differentiation and heart development promotes cardiomyocyte survival and prevents anthracycline-induced cardiotoxicity [117]. We checked whether Imatinib treatment alters GATA4 levels which would in turn affect myocyte survival. Staining of histological sections with a specific GATA4 antibody revealed that GATA4 protein levels (brown nuclei) were downregulated in Imatinib treated mice versus their control littermates (**Figure 2.4A**). This effect was also observed in cultured primary cardiomyocytes (**Figure 2.4B**) where GATA4 downregulation was observed 18hrs post treatment. As control, GATA6 protein levels were monitored and found to remain unchanged following Imatinib treatment (Figure 4B). Given these findings, we tested whether GATA4 haploinsufficient mice are more sensitive to Imatinib-induced cardiac toxicity. *GATA4*$^{+/-}$ mice (N= 7) were treated for 5 weeks with Imatinib and cardiac function was assessed using echocardiography. No significant difference was noted in the body weights of the treated and non-treated mice. Whereas only two wild type mice treated with Imatinib had ejection fractions below 45% (suggestive of heart failure), five *GATA4*$^{+/-}$ mice showed signs of heart failure with ejection fractions less than 45% (**Figure 2.4C**). These *GATA4*$^{+/-}$ mice showed a more significant increase in left ventricular diameter compared to the wild type mice, which is typical of dilated cardiomyopathy (**Figure 2.4D and 2.4E**). These results are suggestive of an important role for GATA4 in Imatinib-induced cardiac toxicity.

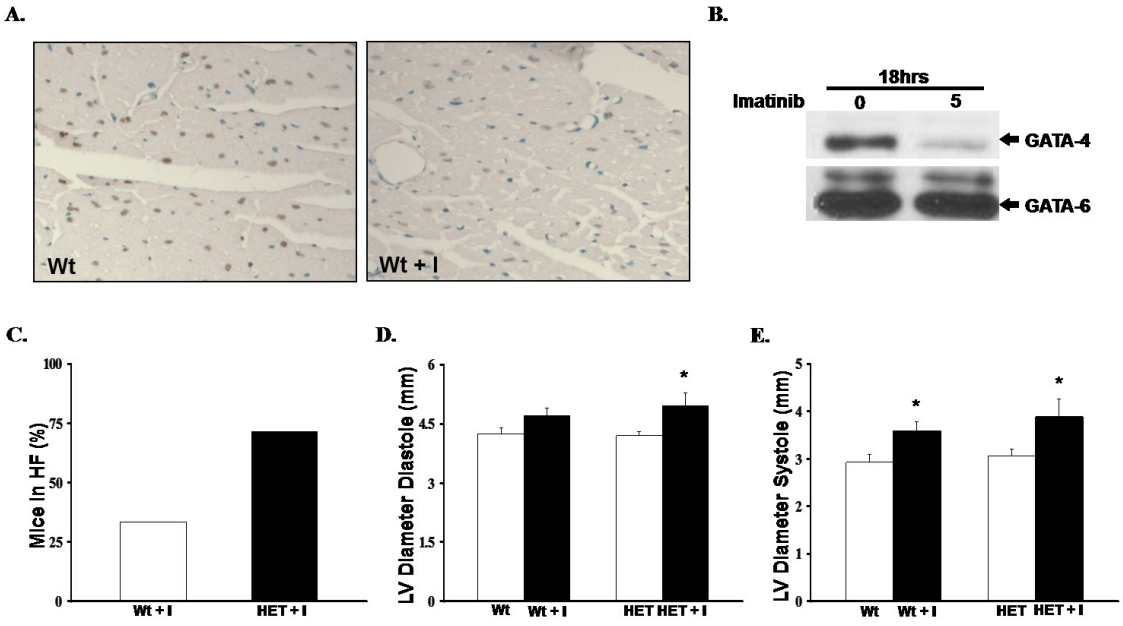

A.
Wt
Wt + I
B.
18hrs
Imatinib
0
5
GATA-4
GATA-6
C.
Mice In HF (%)
100
75
50
25
0
Wt + I
HET + I
D.
LV Diameter Diastole (mm)
6
4.5
3
1.5
0
*
Wt
Wt + I
HET
HET + I
E.
LV Diameter Systole (mm)
5
4
3
2
1
0
*
*
Wt
Wt + I
HET
HET + I

Figure 4. GATA4 is implicated in Imatinib induced cardiotoxicity. A. Histological sections stained with a GATA4 antibody and counterstained with methyl green. Note the nuclear distribution of GATA4 (brown nuclei). **B.** Western blot analysis of GATA4 protein levels in primary cardiac myocytes treated with 5 μM Imatinib for 18 hrs. **C.** % of mice in heart failure (HF) post Imatinib treatment (Ejection Fraction < 45%). **D. & E.** Graphs representing LV Diameter in both diastole and systole in Wt, Wt + I, GATA4 haploinsufficient (HET), and HET + I mice. The data shown are the mean of measurements obtained from seven different mice per group. ‘’***’’** indicates statistical significance where $p < 0.05$. N= 7.

2.4.4. Bcl-2 Protein Overexpression in Mice Hearts is Protective

We and others have previously shown that GATA4 is an upstream regulator of Bcl-2 and Bcl-XL, two antiapoptotic proteins. Bcl-2 and Bcl-X inhibit apoptosis by counterbalancing the effects of the proapoptotic BAX and BAD proteins, preventing mitochondrial uncoupling and cytochrome C release [212,213]. Thus, the ratio of pro/anti-apoptotic proteins is critical to mitochondrial integrity and function. Since Bcl-2 expression was significantly down regulated by Imatinib in older mice, we tested whether myocyte specific Bcl-2 overexpression can rescue Imatinib cardiotoxicity. Transgenic mice overexpressing human Bcl-2 under the control of the α-myosin heavy chain promoter were analyzed. These mice overexpress Bcl-2 specifically in the heart as confirmed by Northern and Western blots (**Figure 2.5A**). Imatinib-induced reductions in both the LV mass to body weight ratio and the MV mean gradient were attenuated in the Bcl-2 transgenic mice (**Figure 2.5B and 2.5D**). Consistent with the above data, Atrial Natriuretic factor (ANF) an important cardiac stress marker [214] was elevated in heart tissues from the Imatinib-treated wild type mice but not in those from Bcl-2 transgenics (**Figure 2.5C**). TUNEL assays performed on mice heart sections as expected showed an increased percentage of positive nuclei in wild type mice treated with either Imatinib or Doxorubicin, an anticancer drug known to be cardiotoxic. On the other hand, mice overexpressing Bcl-2 had no significant increase in the number of positive nuclei following Imatinib or Doxorubicin treatments (**Figure 2.5E**).

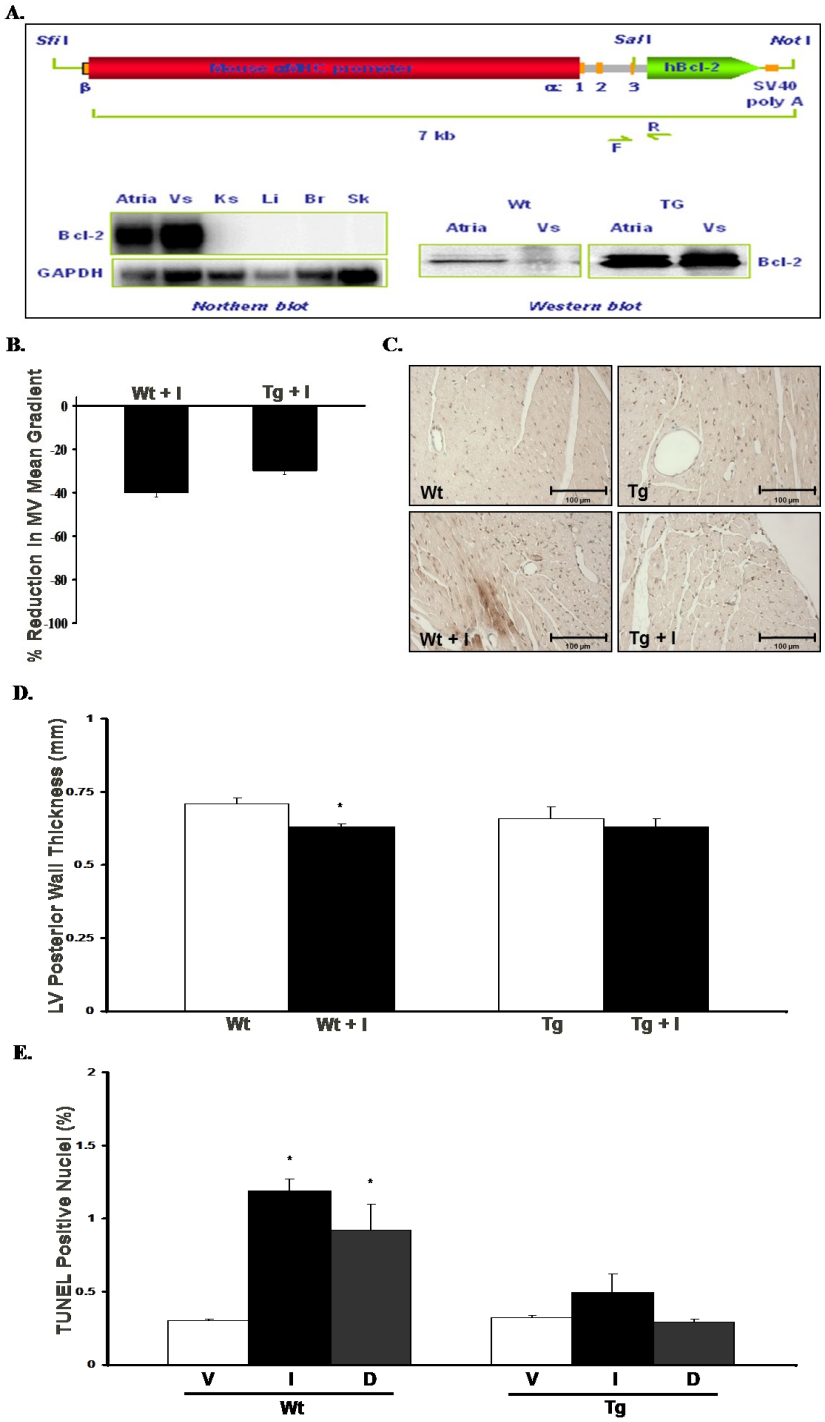

A.
Sfi I
Sal I
Not I
β
α: 1 2 3
hBcl-2
SV40 poly A
7 kb
R
F
Atria Vs Ks Li Br Sk
Bcl-2
GAPDH
Northern blot
Wt
TG
Atria
Vs
Atria
Vs
Bcl-2
Western blot
B.
% Reduction In MV Mean Gradient
Wt + I
Tg + I
0
-20
-40
-60
-80
-100
C.
Wt
Tg
Wt + I
Tg + I
D.
LV Posterior Wall Thickness (mm)
1
0.75
0.5
0.25
0
Wt
Wt + I
Tg
Tg + I
E.
TUNEL Positive Nuclei (%)
2
1.5
1
0.5
0
V
I
D
Wt
V
I
D
Tg

Figure 2.5. Bcl-2 Overexpression is Cardioprotective. **A.** Human Bcl-2 cDNA construct used to generate the cardiomyocyte specific Bcl-2 overexpressing mice (upper panel). The specific expression of Bcl-2 was confirmed using northern (bottom right panel, cropped) and western blots (bottom left panel, cropped). Vs.=Ventricles; Ks=Kidneys; Li=Liver; Br=Brain; SK=Skeletal Muscle. **B.** Graph representing percentage decrease in mitral valve mean gradient compared to the non-treated controls as obtained by echocardiography in wild type (WT) and Bcl-2 transgenic (Tg). **C.** Histological sections from mice from the four different subgroups stained with ANF antibody and counterstained with methyl green. Note the cytoplasmic presence of the ANF stress marker (brown) only in wt treated with Imatinib. **D.** Graph representing left ventricular posterior wall thickness (LVPW) obtained by echocardiographic analysis. ‘’*’’ indicates statistical significance where $p < 0.05$. **E.** Percentage of TUNEL positive nuclei in Wt and Bcl-2 (Tg) mice treated with vehicle (V), Imatinib (I), or doxorubicin (D). The data shown are the mean of experiments done on histological sections from four different mice (150d) with ten fields counted per experiment.

2.4.5. Imatinib-Induced Cell Death is Exacerbated in H_2O_2 Treated Cardiomyocytes

To assess whether myocyte cell death was a direct effect of the drug, we used primary cardiomyocytes cultures to carry out a time course and dose response analysis of Imatinib-induced myocyte cell death. TUNEL assays performed on ventricular myocytes showed that Imatinib concentrations as low as 0.5uM were able to induce cell death 18 hours post treatment (**Figure 2.6A**). Furthermore, the time course study revealed that Imatinib effects on cell survival can be observed as early as 3 hours post treatment with a 2 fold increase in TUNEL positive cells (**Figure 2.6B**). Increased oxidative stress is a hallmark of aging hearts [202]. We tested whether increased oxidative stress reproduces the effects of aging on Imatinib-induced cardiotoxicity by cotreating primary cardiac myocytes with H_2O_2 and Imatinib (0.5μM and 2.5μM). TUNEL assays revealed a higher percentage of cell death in cardiomyocytes cotreated with H2O2 (25μM) and Imatinib than in cardiomyocytes treated with either H2O2 or Imatinib alone (**Figure 2.6D**). Together the data indicate that Imatinib treatment has profound effects on cardiomyocyte gene expression which in turn can alter cardiomyocyte survival, contractility and stress response.

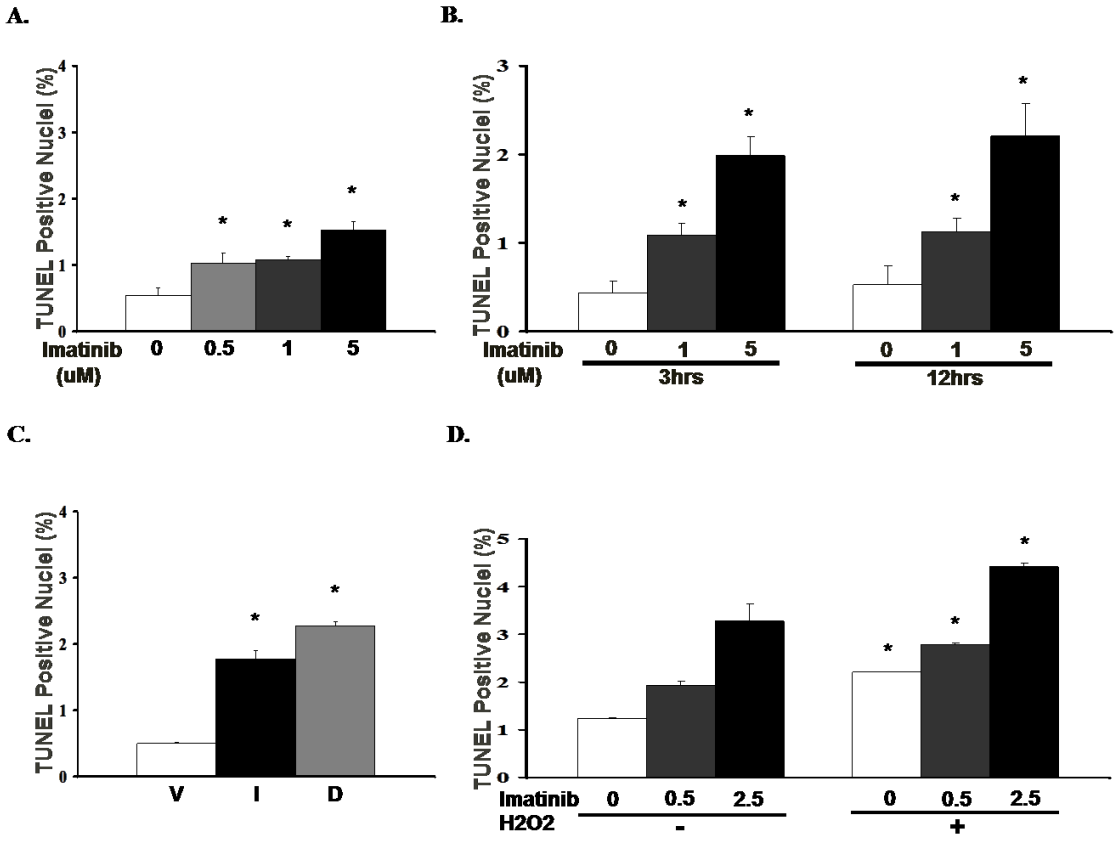

A.
TUNEL Positive Nuclei (%)
Imatinib (uM)
0
0.5
1
5
B.
TUNEL Positive Nuclei (%)
Imatinib (uM)
0
1
5
3hrs
0
1
5
12hrs
C.
TUNEL Positive Nuclei (%)
V
I
D
D.
TUNEL Positive Nuclei (%)
Imatinib
H2O2
0
0.5
2.5
-
0
0.5
2.5
+

Figure 2.6. H_2O_2 Treated Cardiomyocytes Are More Sensitive to Imatinib Toxicity. **A.** Dose Response: TUNEL results obtained after rat primary cardiomyocytes (CMCs) were treated with 0 μM, 0.5 μM, 1 μM, and 5 μM Imatinib for 18hrs. **B.** Time Course: TUNEL results obtained after CMCs were treated with 1 μM or 5 μM Imatinib for 3hrs or 12hrs. **C.** TUNEL results comparing percentage of cell death in vehicle, Imatinib, and doxorubicin treated CMCs. **D.** Quantification showing TUNEL results obtained after CMCs were treated with either vehicle or H2O2 (25 μM) for 24hrs and then treated with different doses of Imatinib (0 μM, 0.5 μM, and 2. 5 μM) for 18hrs. The data shown are the mean of 3 different experiments done, with ten fields counted per experiment. ‘’*’’ indicates statistical significance where $p < 0.05$ vs. the respective control. The results indicate that Imatinib directly affects cardiomyocyte survival.

2.5. Discussion

Chemotherapy-induced cardiotoxicity is a major impediment to the effective use of several anticancer drugs and has been most extensively studied in the case of anthracyclines [215]. Drug-induced cardiotoxicity ranges from acute effects on ion channels - resulting in mild to life threatening arrhythmias - to latent irreversible cardiomyopathies and heart failure. Because of the latter, undesirable cardiac effects of drugs often emerge after several years in clinical use. Recently, unexpected cardiotoxicity of the new generation of antineoplastic "designer drugs" has emerged [187,188,196-198,216]. These drugs usually target a defective tyrosine kinase receptor inhibiting its oncogenic effects [217]. Imatinib mesylate was the first of such highly specific anticancer drugs to be approved by the FDA [187], after results from the IRIS clinical study confirmed its potency in the treatment of CML patients with minimal side effects and an estimated 1% CHF incidence [199-201]. Current research is focusing on the potential use of Imatinib in prostate cancer, typically a disease of older males [194,194]. Imatinib-induced cardiotoxicity is presently controversial, possibly due to the modest cardiac monitoring and the relatively short-term nature of such anticancer clinical studies. Moreover, few studies have directly analyzed its effects on cardiac cells in well-defined experimental settings.

In the present work, we investigated the mechanism of Imatinib action on the heart and the impact of age on the cardiac effects induced. Our results both *in vivo* and *in vitro* in cultured cardiomyocytes confirm and extend a previous study that reported Imatinib-induced cardiotoxicity in mice [188] and provide mechanistic insight into Imatinib action in the heart. The altered cardiac physiology in mice receiving Imatinib suggests that diastolic dysfunction may be an early manifestation of cardiotoxicity that could ultimately lead to heart failure. Moreover, reduced left ventricular mass and increased cell death suggest that Imatinib affects

myocyte cell survival, an effect that could be irreversible and cause delayed cardiac manifestations as in the case of anthracycline cardiotoxicity.

Our results also indicate that Imatinib interferes with mitochondrial function and that aging exacerbates Imatinib induced cardiac dysfunction. Heart function and structure severely deteriorated in older mice compared to their younger counterparts and was accompanied by differential changes in gene expression including upregulation of the cardiac stress marker ANF and the profibrotic genes COL3A and CTGF. The downregulation of the PGC1a and CYTB in the ventricles of older treated mice further underscored the impaired mitochondrial function hypothesis as the underlying cause of Imatinib dependent cardiac dysfunction. The upregulated expression of genes implicated in various stress pathways in older mice may explain the susceptibility of aging cardiomyocytes to Imatinib-induced injury.

GATA4, which has a well-established role in cardiomyocyte differentiation and heart development, has emerged as a critical regulator of cardiomyocyte survival and adaptive stress response of the adult heart [117]. GATA4 prevents anthracycline induced cardiotoxicity, in part through the upregulation of Bcl-2 and Bcl-X [117,212]. Our data uncover a role for transcription factor GATA4 in Imatinib cardiotoxicity. Imatinib treatment significantly reduced GATA4 levels both in mice and in cultured cardiomyocytes consolidating its role as a survival factor. Not surprisingly, GATA4 haploinsufficient mice were clearly more susceptible to Imatinib cardiotoxicity and almost 75% of these mice showed signs of heart failure and dilated cardiomyopathy. It is therefore noteworthy that two chemically distinct anticancer drugs cause cardiotoxicity in part by targeting GATA4 in cardiomyocytes. This raises the intriguing possibility that cardiotoxic drugs may converge on the same nuclear pathway and point to GATA4 as a critical component therein. Bcl-2, a downstream target of

GATA4, is a potent antiapoptotic protein that counterbalances the effects of proapoptotic proteins like BAX and BAD, preventing mitochondrial uncoupling and the subsequent cytochrome c release [212,213]. Consistent with an effect of Imatinib on the mitochondrial intrinsic apoptotic pathway, overexpression of Bcl-2 in myocytes attenuated Imatinib cardiotoxicity. Finally, our *in vitro* results in cultured cardiomyocytes confirmed our *in vivo* work and pointed toward oxidative stress, a hallmark of aging hearts [202], as the principle perpetrator behind the hypersensitivity of older hearts to Imatinib.

Altogether these results show that Imatinib-induced cardiotoxicity involves mitochondrial impairment and increased cell death that can be further worsened by increased oxidative stress as occurs in aging myocytes. This offers a plausible explanation for the controversial findings regarding Imatinib action on the hearts of cancer patients and thus advocates for cardiac follow-up of cancer survivors who received Imatinib treatment. Finally, the fact that Imatinib use has been extended to the treatment of prostate cancer patients [194-195], a disease of older male subjects, suggests a closer cardiac monitoring of those patients during and after Imatinib therapy.

Chapter Two
Imatinib Cardiotoxicity, Role of a Novel AKT/GATA-4 Survival Pathway.

Hiba Komati, Wael Maharsy, Janie Beauregard, Mona Nemer.

Contributions:

In this Chapter, I performed the western blots and TUNEL assays on Imatinib treated cardiomyocytes including the infections and treatments. I also did all the *in vivo* work and wrote the chapter. Transgenic mice lines were previously developed in our laboratory.

3. Chapter 2:

3.1. Abstract

The emerging cardiotoxicity of several tyrosine kinase inhibitors may limit clinical use or efficacy of otherwise powerful anticancer agents. Understanding the molecular basis of cardiotoxicity is essential for developing preventive/protective approaches. Imatinib mesylate – the first small tyrosine kinase inhibitor to be FDA approved – induces cardiomyocyte mitochondrial impairment and cardiomyocyte death. We now report that the mechanism underlying this cardiotoxicity involves interference with an AKT-GATA-4 survival pathway. Treatment of cultured primary cardiac myocytes with Imatinib induces cell death as early as 1hr and cytoplasmic redistribution of transcription factor GATA-4. This is preceded by decreased phospho-AKT levels. Upregulation of activated AKT prevents both Imatinib-induced cell death and GATA-4 cytoplasmic accumulation. Mechanistically, we found that AKT binds to and phosphorylates GATA-4 enhancing its nuclear retention and transactivating properties. Consistent with an essential role for the AKT-GATA-4 pathway, insulin growth factor 1 (IGF-1), a known prosurvival growth factor and upstream regulator of AKT, was found to require GATA-4 for its cardioprotective action. Finally, adenoviral transfer experiments revealed that the severity of Imatinib cardiotoxicity is GATA-4 dose dependent and overexpression of GATA-4 prevents drug toxicity. In conclusion, Imatinib-induced cardiac toxicity involves interference with growth factors- AKT- GATA-4 survival signals. The work identifies new pharmacologic targets for cardioprotection in oncology settings.

3.2. Introduction

Despite recent breakthroughs, the progress of cancer therapy continues to be hindered by the cardiotoxicity associated with many anticancer treatments. Antineoplastic therapy-induced cardiotoxicity is currently an alarming clinical problem faced by both oncologists and cardiologists, and hence the urgent need to deal with such a critical issue [176-179]. Postnatal cardiac myocytes are prone to temporary or permanent injury upon exposure to therapeutic agents [177] and their loss is sufficient to cause heart failure, as shown in several experimental models [115,116]. Imatinib mesylate (Gleevec) is a highly selective anticancer drug targeting the tyrosine kinase activity of the BCR-ABL fusion protein in chronic myelogenous leukemia (CML) patients [188]. This constitutively active BCR-ABL kinase activates antiapoptotic pathways including, the RAS-RAF-ERK pathway, the PI3K-AKT pathway, and the JAK-STAT pathway, ultimately enhancing cell division and inhibiting DNA repair leading to chronic myelogenous leukemia (CML) [208,218]. Imatinib, which has revolutionized the treatment of CML patients [209] also inhibits the receptor for stem cell factor (c-KIT), usually overexpressed in gastrointestinal stromal tumors (GISTs), and platelet derived growth factor receptors (PDGFRs), which play roles in other cancers including GIST and glioblastoma [186]. Unfortunately, an increasing number of highly controversial reports on the unexpected Imatinib-induced cardiotoxicity started to surface [187,188,196-198].

Protein kinase B (PKB) or AKT is a serine/threonine kinase implicated in a number of cellular functions including cellular survival. AKT role in cell survival is mediated by the preservation of mitochondrial integrity where it counteracts the function of proapoptotic Bcl-2 family proteins and inhibits mitochondrial uncoupling [131]. In the heart, the activation of phosphoinositol 3-kinase (PI3K) in response to stimulated receptor tyrosine kinases [131,147] or G-protein coupled receptors (GPCRs) [219], triggers plasma membrane translocation and

subsequent phosphorylation of AKT at T308 by phosphoinositide-dependent kinase-1 (PDK-1) [150,151,219]. Activated AKT can then phosphorylate an array of targets [131,152], or translocate to other subcellular compartments like the nucleus and mitochondria [153].

We earlier confirmed Imatinib-induced apoptotic cell death in both primary cardiac myocytes and mice hearts [188]. We also found that this anomaly was aggravated by oxidative stress and was thus more pronounced in older mice. In this study we closely investigated the effects of Imatinib on two potential targets, AKT and GATA-4. The latter is a known key regulator of heart development and an essential postnatal myocyte survival factor that is usually depleted in cardiac cells treated with antineoplastic cardiotoxic agents [117,119]. We found that Imatinib-induced cell death is rapid and probably multifaceted. The expected rapid inhibition of c-ABL was accompanied by the decreased phosphorylation of AKT. AKT upregulation counterbalanced Imatinib-induced cell death in a GATA-4 dependent manner. Our results suggest that Imatinib targets a novel AKT/GATA-4 prosurvival pathway leading to its sustained cardiotoxic effects. In essence, in this study we report the direct interaction between two key players in cardiomyocyte survival, and offer a better explanation for some of the mechanistic aspects of Imatinib-induced cardiotoxicity.

3.3. Materials and Methods

3.3.1. Cell Cultures: Procedures with neonatal cardiomyocytes including protein extractions were as previously described [34]. Imatinib mesylate (Gleevec®, Novartis) 100 mg tablets were dissolved in water and repeatedly centrifuged at 2,500g to produce highly purified material [188]. Primary cardiomyocytes were treated with vehicle or Imatinib at varying concentrations (1 μM and 5 μM) [188]. The cloning and production of adeno-LacZ, adeno-GATA-4 (G4), adeno-MyrAKT (MYR-AKT), and adeno-antisense GATA-4 (ASG4) were previously reported. Transfections and luciferase assays using ANF reporter plasmids were carried out as previously described [34,211]. Insulin-like Growth Factor (IGF-1) was purchased from Peprotech Inc., New Jersey, USA. AKT inhibitor (5μM) [220] and GSK inhibitor (5μM) [221] were added 30 minutes prior to Imatinib administration.

3.3.2. *In Vivo* Experiments: Mice were handled in accordance with institutional guidelines for animal care. Experiments were approved by the institutional Animal Care Committee of the University of Ottawa. *Gata-4+/-* mice were previously described in reference [49]. Mice were treated with Imatinib mesylate (200 mg/kg/d) or vehicle for 5 weeks as previously described [198]. At the end of the experimental protocol, mice were anesthetized with 12-15 μl/g i.p Avertin (2.5% solution) and sacrificed. For M-mode echocardiography, mice were anesthetized (2.0% isoflurane, 80 ml/min 100% O_2), their anterior chests were shaved, and two-dimensional guided M-mode echocardiography was performed using a Visual-Sonics VEVO 770 and a 30-MHz linear array transducer as described by Aries et al. [117].

3.3.3. Terminal Deoxynucleotidyltransferase Mediated dUTP End labeling (TUNEL): The TUNEL assay was utilized to detect apoptotic nuclei using an Apoptag kit (Intergen, Purchase, NY) and counterstained with methyl green. A Zeiss AxioImager.A2 light

microscope (Objectives: Zeiss EC Plan-Neufluar; Camera: Zeiss AxioCam MRC)was used for image acquisition.

3.3.4. Cytological Studies: Immunofluorescence was performed on cellular preparations as previously described [85], using a rabbit polyclonal rat GATA-4 antibody (dilution 1/1000), and sarcomeric alpha-Actinin antibody (1/500).

3.3.5. Western Blots Analysis: Nuclear and cytoplasmic extracts from cultured cells were prepared as reported previously [211]. Western blots were performed on nuclear extracts from infected cardiac myocytes as previously described [211]. All antibodies were used at a dilution of 1/1000 (Phospho-Ser262-AKT, phospho-c-ABL, phospho-BAD, GATA-4, AKT, BCL-X, and GAPDH). Visualization was done using anti-rabbit/mouse/goat horseradish peroxidase-conjugated antibodies (Sigma).

3.3.6. Kinase Assays: The recombinant proteins glutathione S-transferase (GST) and GST-GATA-4 were produced as described previously [24]. 5 μg of bacterially expressed protein were incubated with 20 ng of AKT kinase (Cell Signaling) in the reaction buffer (25 mM Tris [pH 7.5], 10 mM 6-Glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 5 μci ATP) at 30ºC for 30 min. The proteins were resolved by SDS-PAGE (10% w/v gels) and the blots exposed to X-ray films.

3.3.7. Pull-down Assays: The recombinant GST-GATA4 protein constructs were produced as previously described [24]. Pull down assays were carried out as previously described [24,85]. AKT was *in vitro* translated using the Promega TNT T7 Quick Coupled Transcription/Translation Systems kit (Cat. No: L1170) according to the manufacturers' protocol. All experiments were repeated at least twice with different protein preps.

4.3.7. Real-Time PCR: Total RNA was isolated from cells or mice tissues with Trizol (Invitrogen). The Q-PCR reaction was carried out as described by [211], using the appropriate

primers on a Rotor-GeneTM 6000 (Corbett, Australia). Analysis was done using the delta-delta CT quantitation method, with the ribosomal S-16 serving as the normalizer gene.

3.3.8. Statistical Analysis: Data are reported as means ± the standard error of the mean (SEM). A student unpaired t test was used to compare any two groups, while the one-way analysis of variance (ANOVA) test was used to compare multiple groups. In all cases, a $P < 0.05$ was considered as an index of statistical significance.

3.4. Results

3.4.1. Imatinib Affects GATA-4 Cellular Localization

The newly reported cardiotoxicity induced by Imatinib is currently a controversial issue that should be further investigated. After confirming Imatinib-induced cardiotoxicity in mice in the previous chapter, we tried to examine the impact of this antineoplastic drug on primary cardiomyocytes. Neonatal rat ventricular cardiomyocytes (CMCs) were treated with 5μM Imatinib for 1, 3, 6, and 18 hours. TUNEL assays performed on these treated myocytes showed that Imatinib induces cell death as early as 1 hour post treatment (**Figure 3.1A**). Previously, we showed that GATA4, an important regulator of cardiomyocyte differentiation and heart development, promotes cardiomyocyte survival and prevents anthracycline induced cardiotoxicity [115]. Therefore, it was interesting to check if a second class of antineoplastic drugs targets and depletes GATA-4 levels in cardiomyocytes. QPCR analysis did not show any effect of Imatinib on GATA-4 mRNA levels (**Figure 3.1B**). Western blot analysis showed that GATA4 nuclear protein levels were decreased at both 6 and 18 hours but not at 3 hours post treatment (**Figure 3.1C**). Finally, Imatinib-induced changes in nuclear GATA-4 protein levels would affect GATA-dependent transcription, and therefore, transgenic mice harboring a 3x-GATA-Luc reporter construct were treated with Imatinib (200mg/kg/day) for 5 weeks. Luciferase assays carried out on extracts from heart ventricles showed that the reporter activity was significantly reduced in the Imatinib treated mice as compared to the control non-treated ones (**Figure 3.1D**).

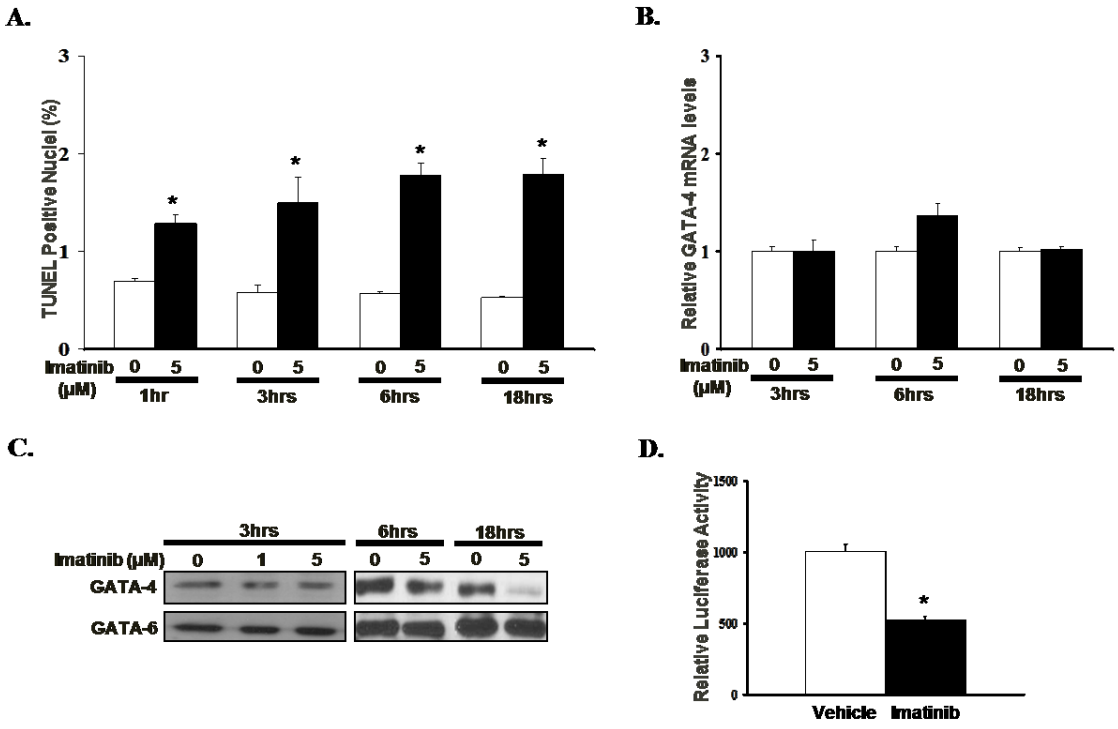

A.
TUNEL Positive Nuclei (%)
3
2
1
0
*
*
*
*
Imatinib (μM)
0 5
0 5
0 5
0 5
1hr
3hrs
6hrs
18hrs
B.
Relative GATA-4 mRNA levels
3
2
1
0
Imatinib (μM)
0 5
0 5
0 5
3hrs
6hrs
18hrs
C.
3hrs
6hrs
18hrs
Imatinib (μM)
0 1 5
0 5
0 5
GATA-4
GATA-6
D.
Relative Luciferase Activity
1500
1000
500
0
*
Vehicle
Imatinib

Figure 3.1. GATA-4 Is Implicated in Imatinib-induced Cardiotoxicity. A. TUNEL results obtained after CMCs were treated with 5 μM Imatinib for 1, 3, 6, or 18hrs. The data shown are the mean of 3 different experiments done with ten fields counted per experiment. **B.** Graph showing QPCR results for GATA-4 mRNA in treated and untreated cardiomyocytes for 3, 6, or 18 hrs. The data are expressed as the GATA-4 transcripts over S-16. The value obtained from untreated cardiomyocytes was assigned an arbitrary value of 1. **C.** GATA-4 protein levels in CMCs treated with 1 or 5 μM Imatinib for 3hrs or with 5 μM Imatinib for 6hrs and18hrs. **D.** Transgenic mice harboring a GATA-3x luciferase reporter (GATA-Luc) were treated with Imatinib (200mg/kg/day) for 5 weeks; luciferase activity was measured in heart ventricles. The graph shows the luciferase activity corrected to protein levels from ventricles of vehicle and Imatinib treated mice (N=3). ‘’*’’ indicates significance in comparison to the respective non-treated control, where $p < 0.05$.

The depletion of GATA-4 nuclear levels could be due to a decreased shuttling to the nucleus and therefore, immunofluorescence was used to assess the extent of GATA-4 nuclear localization in treated and non-treated cardiomyocytes. Time course analysis showed that Imatinib induces GATA-4 cytoplasmic accumulation starting 1 hour post treatment. At 6 hours post treatment, GATA-4 cytoplasmic levels exceed its nuclear levels, and at 18 hours, GATA-4 levels are greatly depleted from both compartments (**Figure 3.2**). Doxorubicin, a cardiotoxic anticancer drug, depleted GATA-4 nuclear levels with no increase in its cytoplasmic levels (**Figure 3.2**).

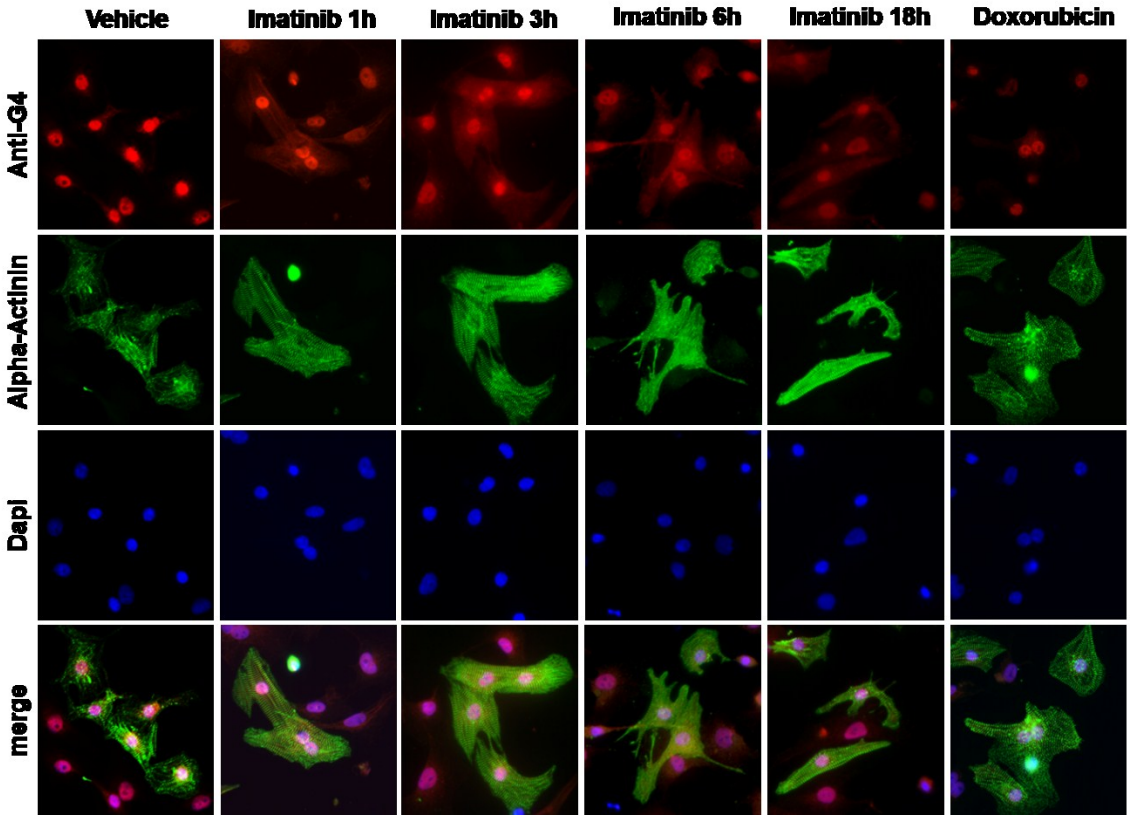


Figure 3.2. Imatinib Induces GATA-4 Cytoplasmic Accumulation. A. Immunofluorescence representative fields of CMCs treated with either vehicle, 1 μM Imatinib for 1, 3, 6, or 18hrs, or Doxorubicin. An anti-GATA-4 antibody was used to localize GATA-4 in cardiomyocytes specifically labeled via an alpha-Actinin antibody.

3.4.2. AKT-dependent GATA-4 Nuclear Translocation Counterbalances Imatinib Toxicity

The fact that cell death precedes the decrease in GATA-4 nuclear protein levels, lead us to test upstream prosurvival targets that might either affect GATA-4 activity and cellular localization, or just initiate cell death in a GATA-4 independent manner. Imatinib is known to inhibit the tyrosine kinase activity of c-ABL, and therefore, we tested if Imatinib cardiotoxicity is directly mediated by c-ABL and its downstream targets [188,208,218]. Western blot analysis, in agreement with previous reports [188], revealed a significant and rapid downregulation of the active phosphorylated form of c-ABL (**Figure 3.3A**). We then tried to investigate the role of AKT, a known c-ABL downstream target [188,208,218]. Western blot results clearly show a decrease in phospho-AKT to AKT protein ratio in cardiomyocytes treated with Imatinib, as early as 15 minutes (**Figure 3.3A**). The latter result confirms recent reports on the Imatinib-induced decreased AKT activity in non-cardiac cell types [222,223]. Finally, BCL-X levels, an important player in the mitochondrial apoptotic pathway, were not affected (**Figure 3.3A**). This is consistent with BCL-X characteristically slower transcriptional regulation in response to cellular stress [117,119].

Our results confirm previous findings on the decreased phosphorylation and activity of AKT upon Imatinib treatment [222,223]. Therefore, it was interesting to check if AKT upregulation would counterbalance the effects of Imatinib and protect myocytes. Primary ventricular cardiomyocytes were infected with an adenovirus expressing a constitutively active myristoylated form of AKT (Adeno-Myr-AKT). Western blot assays performed on cytoplasmic extracts from Adeno-Myr-AKT infected cells confirmed the overexpression of AKT as compared to the Adeno-LacZ infected ones (**Figure 3.3B, Upper Panel**). Interestingly, the upregulation of AKT was able to completely abolish Imatinib induced

cardiac myocyte death (**Figure 3.3B, Lower Panel**). These results prove that AKT is a key target in the mechanism by which Imatinib activates proapoptotic events in a cardiac cell. Based on the above results, AKT and GATA-4 seem to be implicated in Imatinib induced cardiotoxicity and therefore, it was interesting to investigate if these two survival factors are part of the same cardioprotective pathway. To check if AKT protective effects are GATA-4 dependent, cardiomyocytes were infected with either adeno-LacZ, adeno-antisense GATA-4 (ASG4), or both adeno-MYRAKT and adeno-ASG4 viruses. 48hrs post infection, cells were incubated with 5uM Imatinib for 18hrs. Interestingly, TUNEL assays performed on these cells revealed that the downregulation of GATA-4 (ASG4) severely impaired the prosurvival effects of AKT (MyrAKT) in both vehicle and Imatinib treated cardiomyocytes (**Figure 3.3C**). This strongly ascertains our hypothesis that AKT and GATA-4 are part of the same survival pathway. Interestingly, GATA4 seems to be a downstream effector of the protective AKT message in cardiac cells.

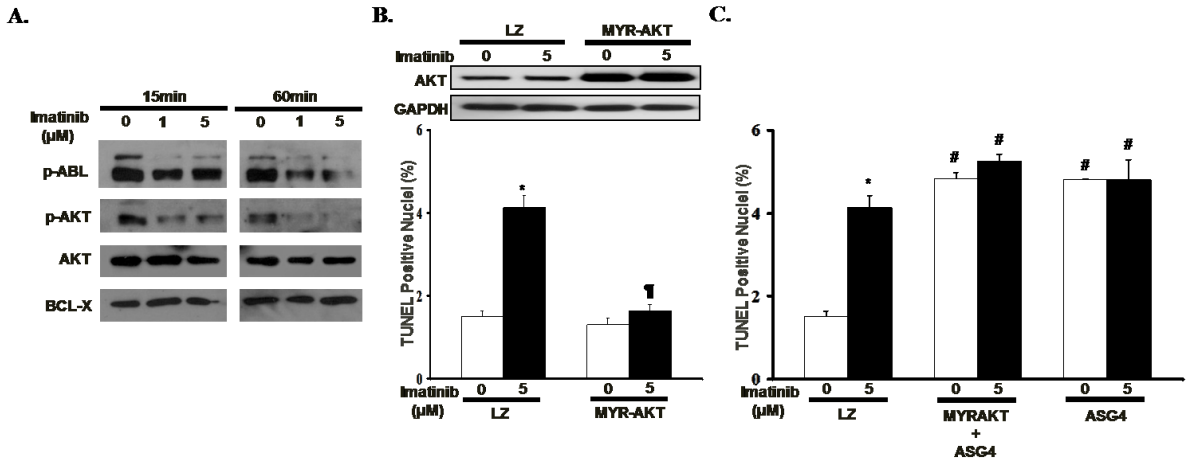


Figure 3.3. AKT Prevents Imatinib-induced Cell Death. A. Western blot analysis of phospho-cABL (p-ABL), phospho-AKT (AKT), total AKT (AKT), and BCL-X protein levels in CMCs treated with 1 and 5 μM Imatinib for 15 and 60 minutes. **B. Upper Panel:** AKT protein levels in CMCs infected with adeno-LacZ (LZ) or adeno-MyrAKT (MYR-AKT) and treated with vehicle or 5 μM Imatinib for 18hrs. **Lower Panel:** Quantification showing TUNEL results obtained after CMCs were infected either with adeno-LacZ (LZ), or adeno-MyrAKT (MYR-AKT) viruses, and treated with 5 μM Imatinib for 18hrs **C.** Quantification of TUNEL results obtained after CMCs were infected with either LacZ, adeno-antisense GATA-4 (ASG4), or both ASG4 and MYR-AKT. Cells were then treated with vehicle (-) or Imatinib (+) for 18hrs. The data shown are the mean of 3 different experiments done with ten fields counted per experiment. ‘’*’’ indicates significance in comparison to the respective non-treated control, ‘’#’’ indicates significance with respect to non-treated LZ control, and ‘’¶’’ indicates statistical significance with respect to the Imatinib-treated LZ control, where $p < 0.05$.

We then checked if the Imatinib-induced decrease in GATA-4 shuttling into the nucleus is AKT dependent. Cardiomyocytes were either infected with adeno-LacZ or adeno-MyrAKT, or treated with an AKT inhibitor (5μM) [220] or a GSK-3β inhibitor (5μM) [221], given the previously described GSK-3β effect on GATA-4 cellular localization [67]. Cells were then incubated with vehicle or 1 μM Imatinib for 6hrs. Immunofluorescence assays done on these cardiomyocytes clearly showed that AKT upregulation inhibits Imatinib-induced GATA-4 cytoplasmic accumulation (**Figure 4A and 4B**). Surprisingly, GSK-3β inhibition failed to prevent GATA-4 nuclear depletion seen in Imatinib treated cardiomyocytes (**Figure 4A and 4B**).

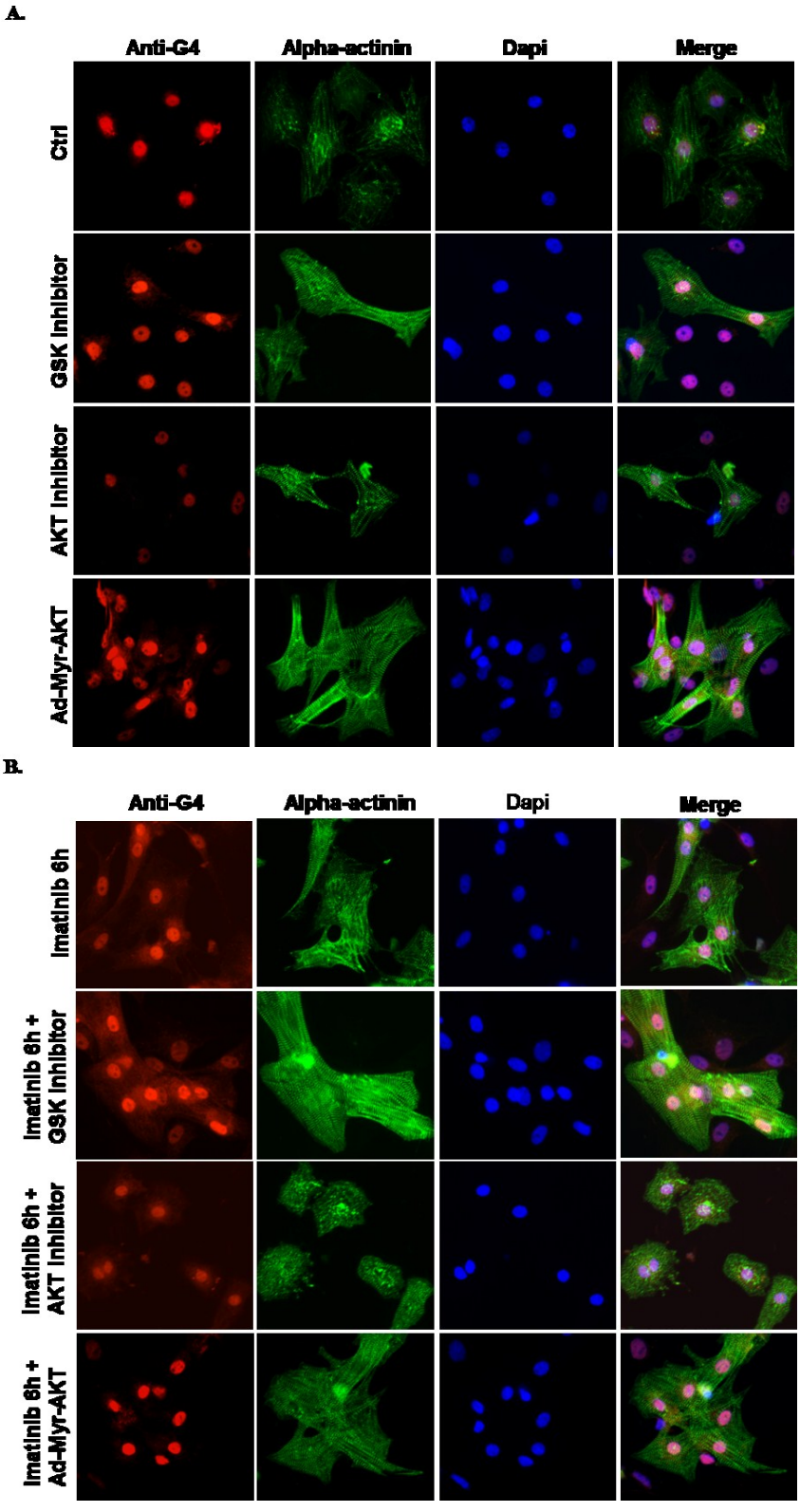

A.
Anti-G4
Alpha-actinin
Dapi
Merge
Ctrl
GSK Inhibitor
AKT Inhibitor
Ad-Myr-AKT
B.
Anti-G4
Alpha-actinin
Dapi
Merge
Imatinib 6h
Imatinib 6h + GSK Inhibitor
Imatinib 6h + AKT Inhibitor
Imatinib 6h + Ad-Myr-AKT

Figure 3.4. AKT Inhibits Imatinib-induced GATA-4 Cytoplasmic Accumulation. A. Immuno-fluorescent representative fields of CMCs treated with vehicle and either GSK-3β inhibitor or AKT inhibitor, or infected with adeno-MyrAKT virus. **B.** Same as A. but cells were additionally treated with 1 μM Imatinib for 6hrs. An anti-GATA-4 antibody was used to localize GATA-4 in cardiomyocytes specifically labeled via an alpha-Actinin antibody.

3.4.3. GATA-4 Is a Direct Phosphorylation Target of AKT

The above results indicate that GATA-4 is downstream of AKT, and therefore, we first investigated if the latter can affect the transcriptional activity of GATA-4. Luciferase assays performed on a minimal BNP promoter harboring 3 GATA sites showed that both wild type (WT) and constitutively active AKT (MYR) potentiate GATA-4 activity in a dose dependent manner (**Figure 3.5A**). Bioinformatical analysis of the GATA-4 protein sequence uncovered an AKT phosphorylation site on S109. We then investigated the effect of mutating this site on the AKT-dependent potentiation of GATA-4 activity. As expected, AKT was able to potentiate the activity of full length GATA-4 and a mutant S105A-GATA-4, S105 being a known MAPK phosphorylation site. Interestingly, AKT could not potentiate the activities of the C-terminal 201-440 GATA-4 and more importantly, the mutant S109A-GATA-4 (**Figure 3.5B**). **Figure 3.5C** is a dose response resulting from luciferase assays done on cells transfected with the different GATA-4 constructs. S109E-GATA-4 was the strongest activator, while both S109A-GATA-4 and 201-440-GATA-4 were weaker activators than full length GATA-4. These results confirmed that S109 is an AKT responsive site and we therefore checked whether AKT binds and phosphorylates GATA-4 on S109. *In vitro* kinase assay, where active AKT kinase was incubated with 1-207- , S105A-1-207, S109A-1-207, and 329-440-terminal GST GATA-4 fusion proteins and P^{32}, revealed that AKT can phosphorylate N-terminal GST-GATA-4 and mutant S105A-1-207-GST-GATA-4 (**Figure 3.5C**). On the other hand, AKT was not able to phosphorylate S109A-1-207-GST-GATA-4 and 329-440-GST GATA-4, confirming S109 as an AKT phosphorylation site (**Figure 3.5C**).

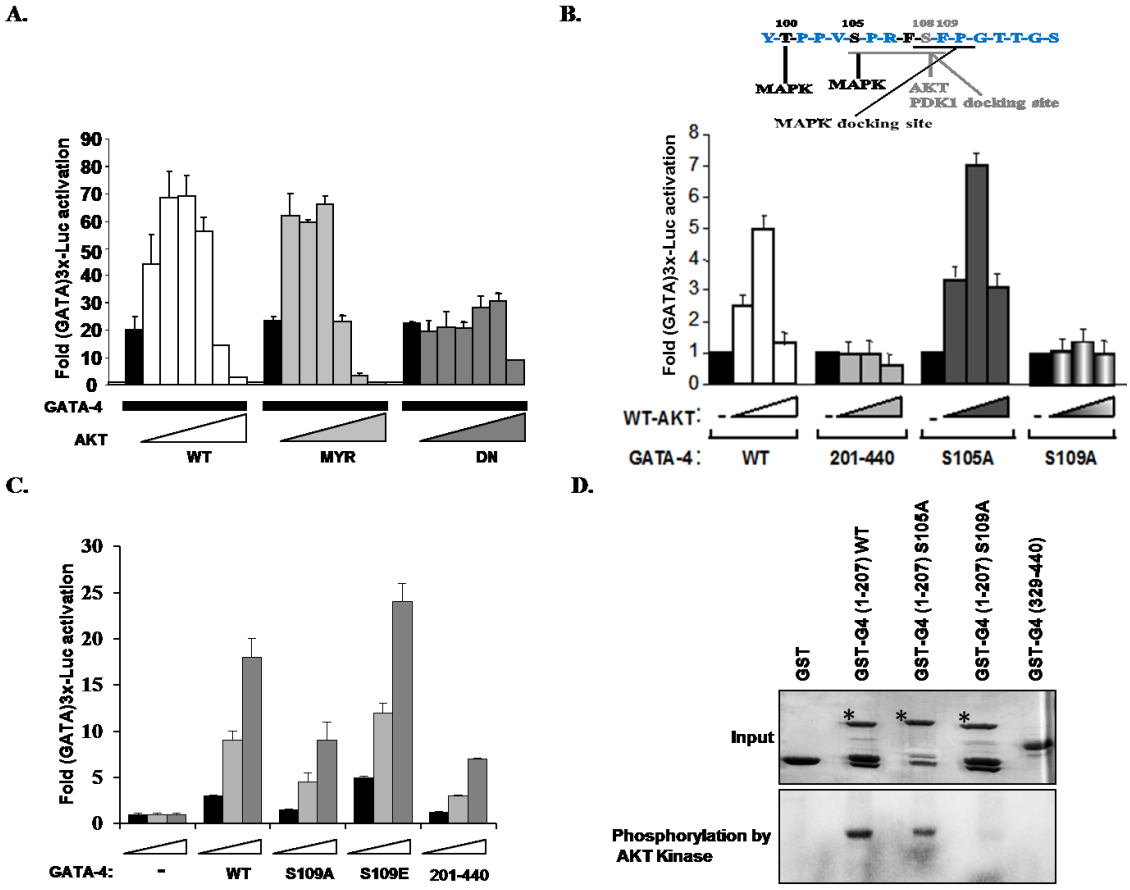

A.
Fold (GATA)3x-Luc activation
GATA-4
AKT
WT
MYR
DN
B.
100 105 108 109
Y-T-P-P-V-S-P-R-F-S-R-P-G-T-T-G-S
MAPK
MAPK
AKT
PDK1 docking site
MAPK docking site
Fold (GATA)3x-Luc activation
WT-AKT:
GATA-4:
WT
201-440
S105A
S109A
C.
Fold (GATA)3x-Luc activation
GATA-4:
-
WT
S109A
S109E
201-440
D.
GST
GST-G4 (1-207) WT
GST-G4 (1-207) S105A
GST-G4 (1-207) S109A
GST-G4 (329-440)
Input
Phosphorylation by AKT Kinase

Figure 3.5. AKT Directly Phosphorylates GATA-4. A. CMCs were transfected with a three GATA binding motifs promoter luciferase reporter [(GATA)3x-Luc] construct and a wild type GATA-4 expression vector. These cells were then infected with increasing concentrations of wild type adeno-AKT (WT), adeno-MyrAKT (MYR), or adeno-dominant negative-AKT (DN) viruses. **B.** CMCs were transfected with a three GATA binding motifs promoter luciferase reporter construct and either a wild type GATA-4 (WT), C-terminal GATA-4 (201-440), N-terminal **S105A**-GATA-4 (S105A), or N-terminal **S109A**-GATA-4 (S109A) expression vectors. These cells were then infected with increasing concentrations of wild type adeno-AKT (WT) virus. **C.** CMCs were transfected with a (GATA)3x-Luc reporter construct and increasing concentrations of either a wild type GATA-4 (WT), N-terminal **S109A**-GATA-4 (S109A), N-terminal **S109E**-GATA-4 (S109E), or C-terminal GATA-4 (201-440) expression vectors. **D.** *In vitro* AKT phosphorylation of GST-GATA-4 fusion proteins. Active AKT kinase was able to phosphorylate 1-207-GST-GATA-4 and **S105A**-1-207-GST-GATA-4 fusion proteins but not GST- alone, **S109A**-1-207-GST-GATA-4, or 329-440-GST-GATA-4.

3.4.4. IGF protective effects are mediated by GATA-4

IGF-1 is a growth factor known to promote cardiomyocyte growth and survival mainly via the PI3K/AKT pathway [99,224-226]. Therefore it was interesting to check if IGF-1 can rescue Imatinib-induced myocyte death. Cardiomyocytes were infected with adeno-LacZ and then treated with either Imatinib (1μM and 5μM), IGF-1 (100μM), or both for 18hrs. As seen in Figure 3.6A, treatment of primary cardiomyocytes with IGF-1 greatly attenuated Imatinib-induced cell death. Next, we checked if the IGF-1 prosurvival effects seen are GATA-4 dependent. Cells were infected with ASG4 adenovirus and cotreated with IGF-1 and Imatinib. The down regulation of GATA-4 in ASG4 infected cardiomyocytes completely abolished the cardioprotective effects of IGF-1 (**Figure 3.6A**). Western blot analysis revealed that IGF-1 can upregulate nuclear GATA-4 levels in non-treated cardiomyocytes, and attenuate its Imatinib-induced depletion (**Figure 3.6B**). GATA-6 levels remained unchanged under all experimental conditions (**Figure 3.6B**). Finally, immunofluorescence assays showed that IGF-1 completely rescues Imatinib-induced GATA-4 cytoplasmic accumulation (**Figure 3.6C**). Finally, we tested whether upregulating GATA-4 levels in cardiomyocytes would affect Imatinib cardiotoxicity. Neonate rat primary cardiomyocytes were infected with adeno-LacZ (LZ) or adeno-GATA-4 (G4) viruses. Cells were then treated with 5 μM Gleevec for 18hrs. As expected, Imatinib induced an increase in the percentage of positive nuclei in LZ-infected cardiac cells. On the other hand, GATA-4 upregulation efficiently attenuated Imatinib-induced cell death (**Figure 3.6D**).

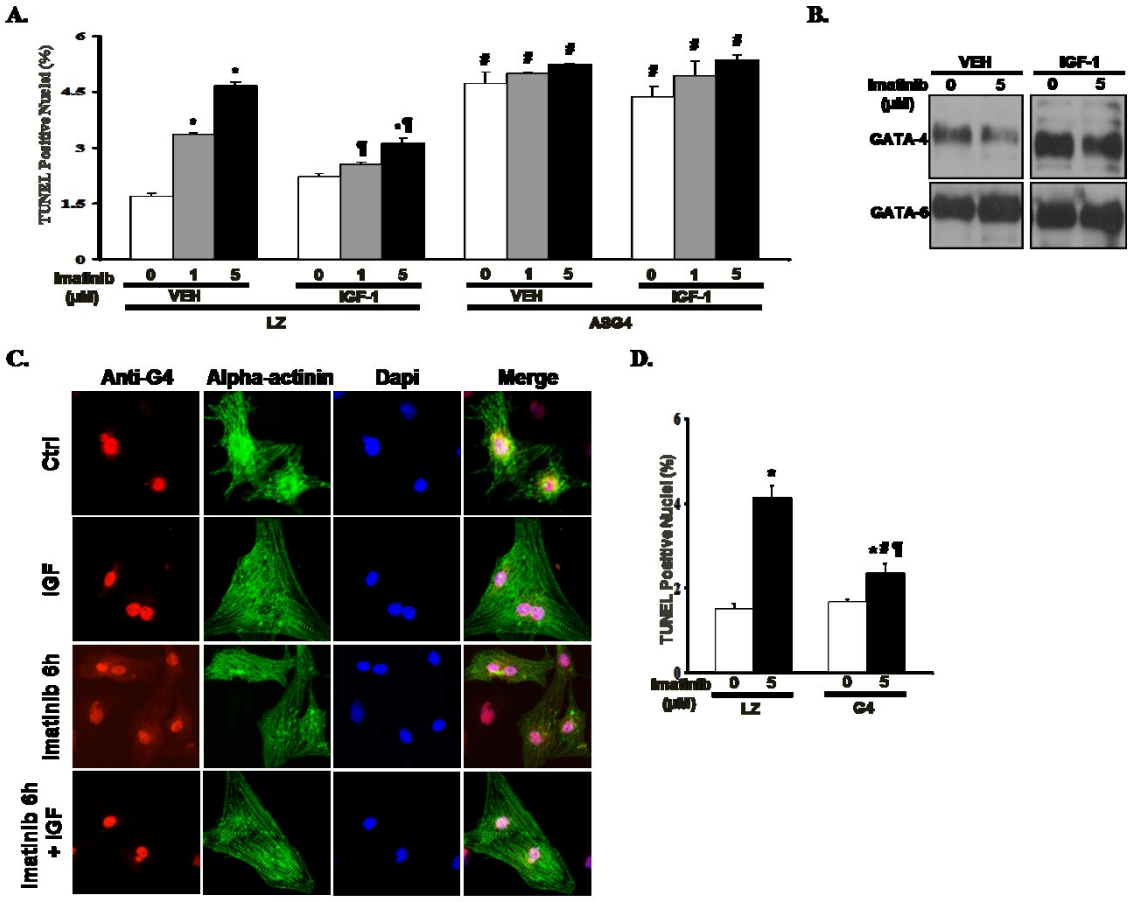

A.
TUNEL Positive Nuclei (%)
Imatinib (μM)
VEH
IGF-1
LZ
ASG4
B.
VEH
IGF-1
Imatinib (μM)
GATA-4
GATA-6
C.
Anti-G4
Alpha-actinin
Dapi
Merge
Ctrl
IGF
Imatinib 6h
Imatinib 6h + IGF
D.
TUNEL Positive Nuclei (%)
Imatinib (μM)
LZ
C4

Figure 3.6. Protective Effects of IGF-1 Are GATA-4 Dependent. A. TUNEL results showing the percentage of apoptotic nuclei after CMCs were infected either with adeno-LacZ (LZ) or adeno-antisense GATA-4 (ASG4) viruses, and then treated with 1 or 5 μM Imatinib +/- 100 μM IGF-1 for 18hrs. **B.** Western blot using GATA-4 and GATA-6 antibodies (1/2000) done on nuclear extracts from rat primary cardiomyocytes treated with vehicle, or IGF-I (100 μM) +/- Imatinib (5 μM). **C.** Immunofluorescent representative fields of CMCs treated with vehicle, IGF-1, Imatinib, or both for 6hrs. **D.** Quantification showing TUNEL results obtained after rat primary cardiomyocytes (CMCs) were infected either with Adeno-LacZ (LZ) or Adeno-GATA-4 (G4) viruses, and treated with 5 μM Imatinib for 18hrs. The data shown are the mean of 3 different experiments done with ten fields counted per experiment. . ‘’*’’ indicates significance in comparison to the respective non-treated control, ‘’#’’ indicates significance with respect to non-treated LZ control, and ‘’¶’’ indicates statistical significance with respect to the Imatinib-treated LZ control, where $p < 0.05$.

3.5. Discussion

Currently, anticancer therapy-induced cardiotoxicity is an alarming clinical problem facing both oncologists and cardiologists [176-179]. Imatinib mesylate (Gleevec) is a highly selective antineoplastic drug targeting the tyrosine kinase activity of the fusion protein, BCR-ABL, the causal agent in chronic myelogenous leukemia (CML) [188]. Unfortunately, an increasing number of highly controversial reports on the unexpected Imatinib-induced cardiotoxicity has started to surface [187,188,196-198]. Our results confirm the previously reported Imatinib-induced cell death [188]. This death is rapid and cardiomyocytes start dying as early as 1 hour post treatment. In this study we investigated the mechanism of Imatinib-induced cell death, specifically its effects on GATA-4, a prosurvival factor in the heart [117,119]. Imatinib clearly interferes with GATA-4 cellular localization as revealed by our Western Blots and immunostaining assays. Depleted GATA-4 nuclear levels along with its increased cytoplasmic levels are early events triggered by Imatinib and therefore might play a major role in inducing and sustaining the death process. This was further confirmed in mice hearts where GATA-4 activity was compromised. The fact that GATA-4 mRNA levels were not affected was suggestive that the observed Imatinib-induced dysregulation is at a posttranslational level. GATA-4 cellular localization and transcriptional activity are known to be heavily affected by posttranslational modifications imposed by various upstream factors [24,64-68]. Moreover, Imatinib is known to inhibit a variety of prosurvival kinases and therefore, it was interesting to check if one of those targets is an upstream regulator of GATA-4. Results show that Imatinib induces a decreased phosphorylation of both its direct target, c-ABL [187,192], and AKT, an important prosurvival kinase [131,152,155-157]. The latter phosphorylates and inhibits GSK-3β, which is known to bind GATA-4 and prevent its nuclear translocation [67]. Not surprisingly, AKT upregulation completely abolished the

negative effects exerted by Imatinib on cardiomyocyte survival, reinforcing its role as a key survival factor. This rescue was GATA-4 dependent and surprisingly, only AKT upregulation and not GSK-3β inhibition reversed Imatinib negative effects on GATA-4 cellular localization. Importantly, our results undoubtedly show that AKT directly phosphorylates GATA-4 on S109, potentiating its transcriptional activity. This novel AKT/GATA-4 interaction and the observation that Imatinib targets this prosurvival pathway has immense potentials in the basic science and clinical fields. For instance and since AKT is downstream many growth factor receptors, like the IGF-1R, it was expected to see that treating with IGF-1 rescues Imatinib-induced cardiomyocyte death in a GATA-4 dependent manner. In conclusion, our data reveal that Imatinib cardiotoxicity involves interference with the activation of AKT and GATA-4 mediated survival pathway(s). This will surely lead to the discovery of pharmaceutical inducers of this pathway that can limit or even reverse the cardiotoxicity of Imatinib or maybe other potentially cardiotoxic tyrosine kinases.

Chapter Three
Preventing DOX-induced Cardiotoxicity by PEX-1, a GATA-4 Partner

Hiba Komati, Wael Maharsy, Janie Beauregard, Marc Dagher, Jessica Rabski, Mona Nemer.

Contributions:

In this Chapter, I performed the Echocardiographic analysis, supervised Marc Dagher and Jessica Rabski, and wrote the chapter. Transgenic mice lines were developed by other members in the laboratory.

4. Chapter 3

4.1. Abstract

Doxorubicin is a potent chemotherapeutic agents used for the treatment of a wide variety of tumors; however, its usefulness is limited due to its associated cardiotoxicity. Doxorubicin induced-cardiotoxicity is a serious clinical problem as it leads to irreversible cardiomyopathy and myocyte loss. Identification of transcriptional mechanisms underlying doxorubicin action in the heart may provide new insights into the regulatory pathways of myocyte survival. We previously showed that GATA-4 is an essential survival factor of postnatal cardiomyocytes and required for prevention of doxorubicin-induced toxicity. More recently, we identified a novel transcription factor, phenylephrine-induced complex-1 (PEX-1), also known as ZFP260, as a GATA-4 partner in the adaptive hypertrophic response of the heart. This work reveals that overexpression of PEX-1 significantly attenuates doxorubicin-induced myocyte death as evidenced by the absence of TUNEL-positive nuclei. In contrast, downregulation of PEX-1 expression in myocytes showed decreased survival in the absence of treatment and an exaggerated response to DOX-induced cell death. We found that the mechanisms underlying the cardioprotective effects of GATA-4 and PEX-1 involve upregulation of the anti-apoptotic genes *BCL-2* and *BCL-X_L*. Importantly, this protective effect was further confirmed *in vivo*. Transgenic mice lines with inducible and cardiac-specific overexpression of GATA-4 and PEX-1 were treated with a single *i.p.* injection of DOX at a dosage of 15 mg/kg body weight. After one week, 2-D guided M-mode echocardiography (Vevo® 2100) was performed on these mice under conscious sedation. Importantly, transgenic mice showed significantly improved cardiac function and reduced apoptotic nuclei compared to control mice. This work suggests that targeting tissue specific transcription factors may be an effective approach to alleviate DOX toxicity.

4.2. Introduction

Heart failure is currently the number one death-causing illness in the industrialized world [88,89]. In many cases, the once physiologic hypertrophic response designed to sustain normal heart function in response to growth stimuli, gradually progresses to a pathologic hypertrophy that would ultimately lead to heart failure and sudden death [88,113,212]. The later pathologic hypertrophy is almost always triggered or accompanied by the loss of the mostly terminally differentiated cardiomyocytes [114]. Myocyte loss, which can also be triggered by some antineoplastic drugs, is sufficient to cause cardiac injury [115,116]. Enhancing the poorly understood cardiomyocyte survival program has been shown to be protective against drug induced cardiotoxicity [117-119]. This cardiotoxicity is currently a worrying clinical problem faced by cardiologists and oncologists, especially after a class of bacterial antibiotics used in chemotherapy, anthracyclines, was found to be cardiotoxic [176-179]. Doxorubicin (DOX), the most studied anthracycline, has been shown to promote oxidative stress-induced cardiac injury, which is usually aided by many contributors including the depletion of GATA-4 transcription factor. GATA-4 is a known key transcriptional regulator of heart development and growth, and has been lately described as a survival factor in postnatal cardiomyocytes [117]. Enhancing GATA-4 activity by phenylephrine, an α1-adrenergic agonist, or its adenoviral mediated overexpression, prevented DOX-induced apoptosis in cardiomyocytes mainly via the transcriptional activation of the antiapoptotic proteins, BCL-X_L and BCL-2 [117,118].

Recently, we identified ZFP260, or phenylephrine-induced complex-1 (PEX-1), as a novel collaborator of GATA-4. ZFP260 was found to play a pivotal role in both α1-adrenergic and endothelin-1 signaling in the myocardium [85,211]. Tamoxifen-induced overexpression of PEX-1 in adult mice hearts lead to an adaptive form of hypertrophy [85].

Interestingly, PEX-1 was found to physically and functionally interact with GATA-4 on promoters of several genes involved in cardiac hypertrophy [211]. In this paper we tried to investigate the role of PEX-1 in cardiac survival and DOX-induced cardiotoxicity. In mice, PEX-1 overexpression prevented the negative effects of DOX on cardiac structure and function. On the other hand, adenoviral PEX-1 upregulation in primary rat cardiomyocytes protected against cell death, inhibiting proapoptotic genes while inducing prosurvival ones like BCL-2, BCL-X_L. Finally, we found that PEX-1 and GATA-4 synergistically activate the expression of BCL-2 and BCL-X_L. Collectively, these results hint to a potential collaboration between PEX-1 and GATA-4 in prosurvival pathways that yet to be understood.

4.3. Materials and Methods

4.3.1. Animal Model: Mice were handled in accordance with institutional guidelines for research animal care. Experiments were approved by the institutional Animal Ethics Committee. The generation of CAT PEX-1 and alphaMHC/merCremer mice was described in [85]. 150 days old CAT-PEX1 and double-transgenic mice were treated with Tamoxifen as previously described [227,228]. One week post treatment, mice were anesthetized (2.0% isofluorane, 80 ml/min 100% O2), their anterior chests were shaved, and two-dimensional guided M-mode echocardiography was performed using a Visual-Sonics Vevo 2100 and a 30-MHz linear array transducer as described by [117]. The next day, mice were treated with a single i.p. injection of DOX (15mg/kg). A week after, mice hearts were imaged and the day after all mice were anesthetized with 2.2 µl/g i.p KXA cocktail (Ketamine 42.86 mg/ml, Xylazine 8.57 mg/ml, and Acepromazine 1.43 mg/ml) and either sacrificed for tissue collection, or heart-perfused for histological studies [117]. Genotyping was carried out using PCR and QPCR utilizing transgenes-specific oligonucleotides.

4.3.2. Cell Cultures: Procedures with neonatal cardiomyocytes were as previously described [34]. The cloning and production of adeno-LacZ, adeno-PEX-1, adeno-GATA-4, and adeno-antisense PEX-1 (ASPEX1) were previously reported [211]. NIH 3T3 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Transfections were performed using calcium-phosphate method as described in previous publications [34,117,211]. Cells were lysed 48 hours after transfection using the following lysis buffer: 100mMTris-HCl PH 7.9, 0.5% Nonidet P-40 And 1mM dithiothreitol (DTT). Luciferase activity was assayed using GlomaxTM 96 Microplate Luminometer (Promega). Fold activation was calculated according to the respective basal levels of promoter activity.

4.3.3. Histological and Cytological Studies: Mouse hearts were perfused with PBS-KCl, fixed with paraformaldehyde, and then paraffin-embedded. Sections were Trichrome-stained and were visualized at 1.25X and 63X magnifications.

4.3.4. Terminal Deoxynucleotidyltransferase Mediated dUTP Nick End labeling (TUNEL): The TUNEL assay was utilized to detect apoptotic nuclei using an Apoptag kit (Intergen, Purchase, NY) and counterstained with methyl green.

4.3.5. Real-Time PCR: Total RNA was isolated from cells or mice tissues with Trizol (Invitrogen). Transcript levels for the various genes were determined by real-time polymerase chain reaction (Q-PCR). The Q-PCR reaction was carried out as described by [211], using the appropriate primers on a Rotor-GeneTM 6000 (Corbett, Australia). Analysis was done using the delta-delta CT quantitation method, with the ribosomal S-16 serving as the normalizer gene.

4.3.6. Statistical Analysis: Data are reported as means ± the standard error of the mean (SEM). A Student unpaired t test was used to compare any two groups, while the one-way analysis of variance (ANOVA) test was used to compare multiple groups. In all cases, a $P < 0.05$ was considered as an index of statistical significance.

4.4. Results

4.4.1. PEX-1 is Protective Against DOX-Induced Cardiotoxicity in Mice.

In previous publications, PEX-1 was shown to be a GATA-4 collaborator in the heart hypertrophic response [85,211]. We recently reported that the conditional overexpression of PEX-1 in mice results in bigger hearts with preserved cardiac function, characteristic of adaptive hypertrophy [85]. On the other hand, GATA-4 is a survival factor that was found to prevent Doxorubicin (DOX) cardiotoxicity. The latter has been shown to induce dilation of the left ventricle and thinning of the ventricular walls. Therefore, it was interesting to see if PEX-1, like GATA-4, is a regulator of cardiomyocyte survival. α-MHC-merCremer mice were crossed with CAT-PEX-1 mice to obtain a double transgenic mouse line that overexpresses PEX-1 in cardiomyocytes upon Tamoxifen induction. 150 days old alphaMHC/merCremer or alphaMHC/merCremer-CAT-PEX-1 mice were treated with Tamoxifen (0.5mg/kg/day, ip) for five consecutive days. One week later, mice hearts were imaged and then treated with a single *i.p.* injection of vehicle or DOX (15mg/Kg). Finally a week after, mice were subjected to echocardiography and then sacrificed to harvest hearts for either immunohistochemical or QPCR analysis.

Echocardiographic analysis confirmed the negative effects of DOX on control mouse cardiac function. For instance, hearts were less efficient and greatly dilated as evidenced by the reduced ejection fraction (EF) (**Figure 4.1A**) and increased left ventricular internal dimension (**Figure 4.1B**). Interestingly, PEX-1 overexpressing mice hearts treated with DOX had a preserved cardiac function, and both EF and left ventricular intra-chamber diameter were not changed (**Figures 4.1A and 4.1B**). Furthermore, left ventricular mass (LV mass) corrected to body weight was as expected significantly decreased in control mice treated with DOX (**Figure 4.1C**). On the other hand, this index was expectedly higher in PEX-1

conditional transgenic mice [85], and remained so upon DOX administration (**Figure 4.1C**) confirming our hypothesis of PEX-1 being an integral part of survival pathways in the heart. Finally, Trichrome staining of mice heart sections clearly show that treated PEX-1 overexpressing hearts retained a healthy cardiomyocyte structure with minimal fibrosis, in severe contrast to the vacuolated and heavily fibrotic hearts of DOX-treated controls (**Figure 4.1D**).

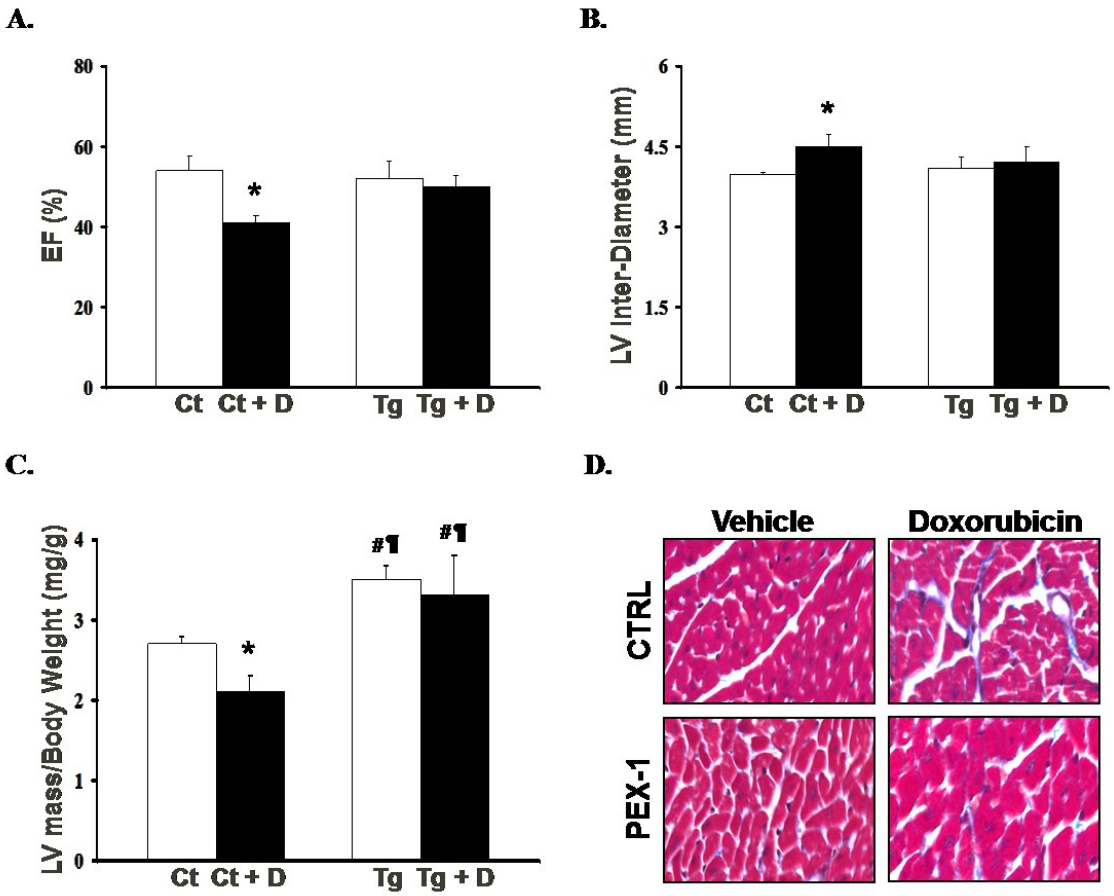


Figure 4.1. PEX-1 Overexpression Counterbalances DOX Cardiotoxicity in Mice. A. Graph representing ejection fraction (EF) in control merCremer mice (Ct) and double transgenic mice (Tg) treated with vehicle or with a single *i.p.* injection of DOX, as obtained by echocardiography. **B.** Graph representing LV inter-diameter in Ct, Ct + D, Tg, Tg + D mice. The data shown are the mean of measurements obtained from six different mice (150d) per group <0.05. **C.** Graph showing LV mass to body weight ratios in the different mice groups. **D.** 63X image of Trichrome stained hearts showing a population of ventricular cardiomyocytes. DOX treatment induced a decrease in the number of cardiomyocytes (in red) in control mice hearts and an increase in collagen fibers (in blue), indicative of fibrosis. PEX-1 overexpression protected the heart against the cardiomyocyte death as well as fibrosis.

4.4.2. PEX-1 Prevents Cardiomyocyte Death Induced by DOX.

As previously mentioned, GATA-4 efficiently protects against DOX-induced cardiotoxicity by preventing cardiomyocyte apoptosis [117]. Given the protective effects of PEX-1 overexpression *in vivo*, we checked if it can counterbalance DOX effects by inhibiting cell death. Cardiomyocytes were infected with either adeno-LacZ, adeno-GATA-4 (GATA-4), or adeno-PEX-1 viruses. 48hrs post infection, cells were incubated with DOX for 18hrs. TUNEL assays performed on these cells show that the upregulation of either PEX-1 or GATA-4 completely prevented cardiomyocyte death, which as anticipated increased in DOX-treated LacZ controls (**Figure 4.2**). This confirms that PEX-1and GATA-4 are both prosurvival factors with antiapoptotic properties.

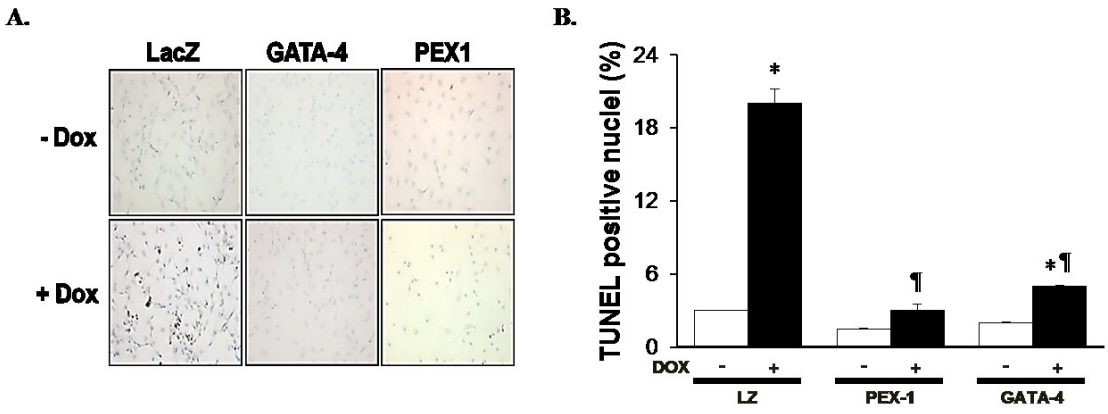


Figure 4.2. PEX-1 Protects Primary Cardiomyocytes Against DOX Toxicity. A. Representative TUNEL images from cardiomyocytes infected with either adeno-LacZ (LacZ), adeno-GATA-4 (GATA-4), or adeno-PEX-1 (PEX-1) and treated with vehicle or DOX. **B.** Graph representing the quantification of TUNEL assays preformed on treated cardiomyocytes. The data shown are the mean of 3 different experiments done with ten fields counted per experiment. ‘’*’’ indicates significance in comparison to the LacZ controls and ‘’¶’’ indicates statistical significance in comparison to the LacZ infected Dox-treated cells.

4.4.3. PEX-1 and GATA-4 Synergistically Activate BCL-X_L and BCL-2 Expression

GATA-4 prosurvival effects in DOX-induced cardiotoxicity were found to involve the upregulation of the antiapoptotic factors, BCL-X_L [117] and BCL-2 [118]. We therefore checked if PEX-1 upregulation can activate the expression of these antiapoptotic factors too. QPCR assays done on cardiomyocytes infected with adeno-LacZ, adeno-GATA4, or adeno-PEX-1 demonstrated the ability of PEX-1, like GATA-4, to upregulate the expression of both BCL-X_L (**Figure 4.3A**) and BCL-2 (**Figure 4.3D**). GATA-4 was found to regulate the expression of these genes by directly binding to the consensus GATA-binding sites on the BCL-X_L as well as BCL-2 promoters [117,212]. Based on the above results, PEX-1 possible regulation of the BCL-X_L and BCL-2 promoter regions was tested by performing promoter reporter assays on BCL-X_L-Luc and BCL-2-Luc reporter constructs. Both transcriptional factors were separately able to activate the BCL-X_L (**Figure 4.3B**) and the BCL-2 (**Figure 4.3E**) promoters in a dose-dependent manner reiterating their prosurvival roles in cardiomyocytes. Amazingly, PEX-1 and GATA-4 synergistically activated both promoters (**Figures 4.3C and Figures 4.3D**) hinting to their possible collaboration in common survival pathways.

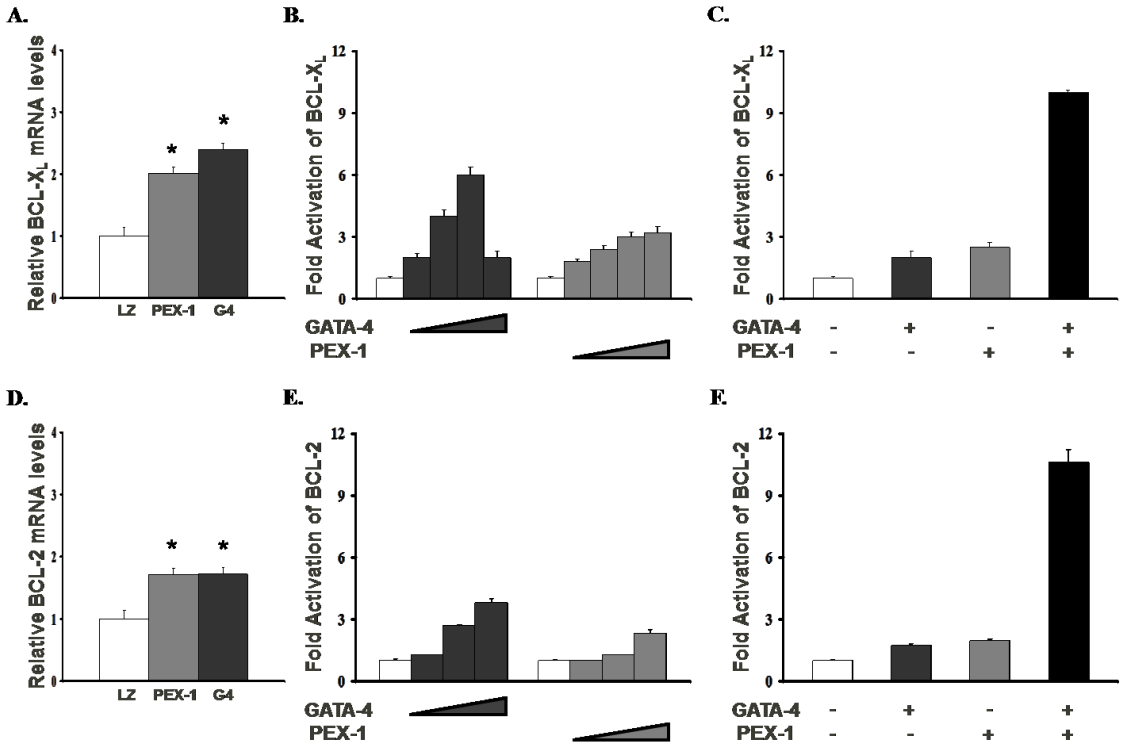

A.
B.
C.
Relative BCL-X$_L$ mRNA levels
Fold Activation of BCL-X$_L$
LZ
PEX-1
G4
GATA-4
PEX-1
D.
E.
F.
Relative BCL-2 mRNA levels
Fold Activation of BCL-2

Figure 4.3. PEX-1 Activates the Expression of Antiapoptotic Genes. A. Real-time PCR results showing the levels of mRNA of BCL-X_L in cultured cardiomyocytes infected with either LacZ, PEX-1, or GATA-4. The expression of these genes is normalized over the expression of the housekeeping gene GAPDH. **B.** Graph representing fold activation of a BCL-X_L-Luc reporter by increasing concentrations of either GATA-4 or PEX-1 (Dose response). **C.** Synergistic activation of the BCL-X_L promoter by GATA-4 and PEX-1. **D.** Q PCR results showing the levels of mRNA of BCL-2 in cultured cardiomyocytes infected with either LacZ, PEX-1, or GATA-4. The expression of these genes is normalized over mRNA levels of GAPDH. **E.** Graph representing fold activation of a BCL-2-Luc reporter by increasing concentrations of either GATA-4 or PEX-1 (Dose response). **F.** Synergistic activation of the BCL-2 promoter by GATA-4 and PEX-1.

4.5. Discussion

In this chapter, we provide evidence that PEX-1 is implicated in cardiomyocyte survival, through its partnership with GATA-4 and its role in counterbalancing DOX-induced cardiomyocyte death. The present work also shows that PEX-1 activates and upregulates pro-survival genes BCL-2 and BCL-X_L. Postnatal cardiomyocytes have a limited regenerative capacity; and their loss due to stress is capable of causing heart failure [115,116]. Investigating the poorly understood mechanisms governing myocyte survival and proliferation could pave the way for the discovery of new therapeutic approaches based on promoting cardiac myocyte survival and repair.

Previous work on anthracycline-induced cardiotoxicity showed that DOX induces a depletion of GATA-4 [117,118]. GATA-4, a critical regulator of heart development, was recently identified as being a prosurvival factor in adult cardiac myocytes [34,35,117-119]. For instance, restoring GATA-4 levels through α1-adrenergic signaling or virus mediated gene transfer has protected against DOX-induced cardiotoxicity [117]. ZFP260 (PEX-1) is a novel transcription regulator in the heart and a nuclear effector of both α1-adrenergic and endothelin signaling hypertrophic pathways [85,211]. PEX-1 was found to physically interact with GATA-4 and functionally cooperate to activate the transcription of key cardiac markers, such as ANF [211]. Therefore, we investigated the possibility of PEX-1 being a GATA-4 collaborator in cardiac prosurvival pathways.

Transgenic mice hearts with cardiomyocyte-specific PEX-1 overexpression were resistant to DOX-induced decrease in cardiac function. In addition, the well-established cell loss and fibrosis associated with DOX were greatly attenuated in this PEX-1 overexpressing mouse model. These *in vivo* results confirm PEX-1, for the first time, as a key cardiac survival factor capable of counterbalancing cardiotoxicity induced by antineoplastic drugs

like doxorubicin. At the molecular level, Cardiomyocyte apoptosis plays a major role in DOX-induced cardiotoxicity [229]. Interestingly, we found that PEX-1 upregulation, similar to GATA-4, inhibits apoptotic cell death triggered by DOX. These results clearly prove that PEX-1 is a prosurvival factor playing a similar role as GATA-4, both *in vivo* and *ex vivo*.

Finally and based on the very similar roles played by PEX-1 and GATA-4, we searched for common antiapoptotic targets. BCL-2 and BCL-X_L are two antiapoptotic factors known to be key regulators of the intrinsic signaling pathway of apoptosis and are thus considered as essential mediators of prosurvival signals [117-119]. DOX ability to induce cardiomyocyte apoptosis was found to be mediated by the downregulation of both BCL-X_L and BCL-2 expression levels [117-119]. Our QPCR results and *Ex vivo* reporter assays confirm PEX-1 as a regulator of both BCL-X_L and BCL-2. More importantly, PEX-1 and GATA4 were found to synergistically activate the promoters of these antiapoptotic genes. In essence, these findings confirm PEX-1 as a prosurvival factor, which might be part of the same antiapoptotic pathways regulated by its collaborator, GATA-4. This could be of great importance and may pave the way for potential pharmacological PEX-1 inducers that can promote cardiomyocyte survival and alleviate the side effects of many anticancer drugs.

5. General Discussion

In this thesis, we confirmed that Imatinib is cardiotoxic in mice and in primary cultures. Interestingly, we found that the cardiotoxicity of two cancer drugs converge on GATA-4, which seems to play a central role in cardiomyocyte survival. Mechanistically, we uncovered a novel AKT/GATA-4 prosurvival pathway, in which AKT binds and phosphorylates GATA-4 enhancing its nuclear localization and transcriptional activity. Finally, we discovered a role for PEX-1 in survival, collaborating with GATA-4 on promoters of antiapoptotic genes.

5.1. Imatinib Cardiotoxicity Is Rapid and Age-dependent

Targeted chemotherapy is one of the most promising fields of cancer treatment. It encompasses a group of antibodies and small-molecule kinase inhibitors designed to specifically target proteins involved in growth and proliferation pathways. The FDA has so far approved 11 agents while many more are awaiting approval. The high mutation rate affecting proto-oncogenic protein kinases in cancers triggered the search for their specific inhibitors, and nowadays, targeted drugs, like Imatinib mesylate (Gleevec), have transformed the way solid tumors and many blood malignancies are treated [176]. Unfortunately, unexpected cardiac toxicity of these new targeted "designer" therapeutics has recently emerged [187,188,196-198,217]. Imatinib mesylate was the first "smart" anticancer drug to be approved by the FDA after results from the IRIS clinical study confirmed its efficacy against chronic myelogenous leukemia (CML) with nominal side effects and an estimated 1% CHF incidence [199-201]. The controversy surrounding Imatinib-induced cardiotoxicity could be attributed to the modest monitoring of cardiac function and the relative short term nature of clinical studies.

First, we focused on validating previous reports on the impact of Imatinib treatment on cardiac function and cardiomyocytes. Our results confirmed the reported Imatinib-induced cardiotoxicity in both mice hearts and primary cardiomyocytes [188]. This toxicity was rapid and Imatinib-dose dependent in primary cardiomyocytes, while it was mild in young adult mice, which suffered from diastolic dysfunction and reduced left ventricular mass accompanied by cell loss. The observed increase in apoptotic cell death is usually associated with a dysregulated mitochondrial function, known to worsen with age [202]. Therefore, we investigated the effect of age on the severity of Imatinib cardiotoxicity and we found that older mice are highly sensitive to cardiac stress triggered by this anticancer drug. Heart function and structure were severely impaired, where cardiac contractility was greatly compromised and the amount of fibrosis was obvious in heart sections from treated older mice. Interestingly, important prosurvival and mitochondrial integrity genes were downregulated consistent with an activated stress response that involves an impaired mitochondrial function. In our *ex vivo* model simulating hypoxic aging heart cells, H_2O_2-treated cardiomyocytes were less resistant to Imatinib toxic effects. Collectively, these results prove that Imatinib activates mitochondrial stress pathways, leading to the impairment of mitochondrial function and increased apoptotic cell death. The latter toxicity is austere in the already stressed aging myocytes (**Figure 5.1**). This surely offers a decent explanation to the current controversy surrounding Imatinib and suggests a closer cardiac monitoring of older patients on therapy.

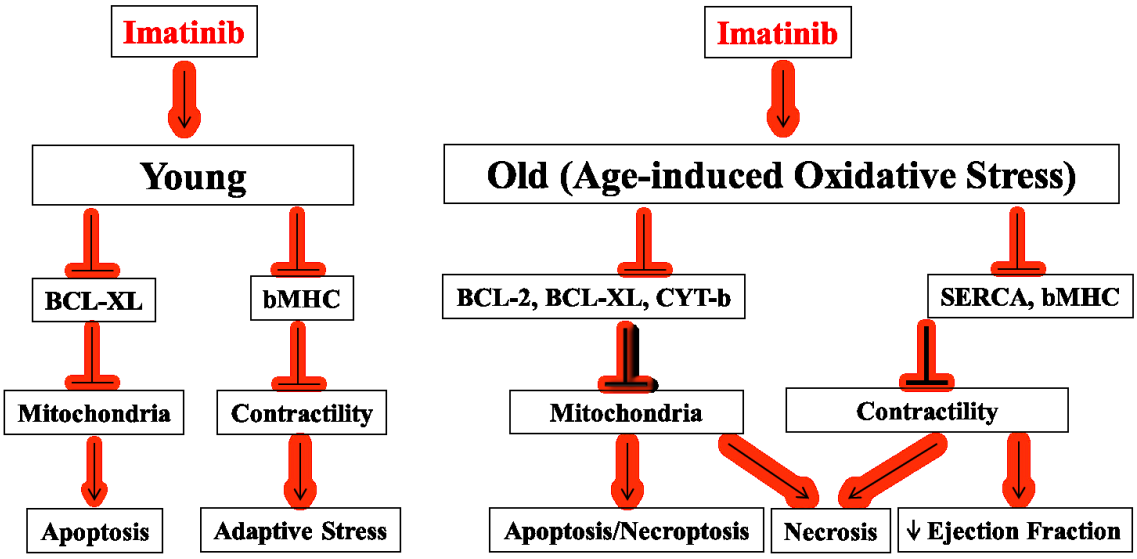


Figure 5.1. Age-dependent Imatinib Cardiotoxicity.

5.2. Identification of GATA-4 as a Common Target

Heart failure caused by antineoplastic drug-induced dysregulated mitochondrial biogenesis and cell death is also a common feature of anthracycline cardiotoxicity. For instance, we and others previously reported that Doxorubicin, the most commonly used anthracycline, induces cardiomyocyte loss triggered by the dysregulation of prosurvival factors regulating mitochondrial integrity and cellular homeostasis [117-119,181,183,184]. Transcription factor GATA-4, which is a known regulator of heart development, has recently emerged as an indispensable factor in the adaptive response of the adult heart as well as cardiomyocyte survival [117]. Specifically, GATA-4 was found to be rapidly depleted in doxorubicin-treated hearts and primary cardiomyocytes. Conversely, its upregulation counterbalanced this cardiotoxicity, in part by inducing the expression of antiapoptotic proteins, such as BCL-2 and BCL-X_L [117-119]. In this thesis, we report a similar prosurvival role played by GATA-4 in Imatinib-induced cardiac toxicity. Imatinib significantly reduced GATA-4 nuclear protein levels as well as transcriptional activity in mice hearts and cultured

cardiomyocytes. Au contraire, the upregulation of GATA-4 to physiological levels was efficient in reversing Imatinib induced myocyte death. Therefore, it is noteworthy that two chemically distinct antineoplastic drugs induce cardiotoxicity in part by targeting GATA-4 prosurvival activity in adult cardiomyocytes. Essentially, this raises the enthralling possibility that cancer drugs may be negatively affecting the same prosurvival pathway(s), in which GATA-4 is a critical component.

5.3. GATA-4 a Central Regulator of Cardiomyocyte Survival

GATA-4 activity is usually regulated either at the level of gene expression or posttranslationally. Complex mechanisms of gene regulation control these elaborate expression patterns. Although poorly studied, the GATA-4 promoter region is now known to contain two different transcription initiation sites as well as many distally located enhancer regions that modulate and direct the spatio-temporal expression of GATA-4 [63].

Posttranslational modifications on GATA4 by upstream regulators have been extensively studied in normal and disease states. GATA-4 phosphorylation at Thr100 and Ser105 within its N-terminal transactivation domain by ERK and p38 MAPK are two known examples [24]. The Ser105 phosphorylation of GATA-4 enhances its DNA binding and transactivational activity, and plays an indispensable role in cardiac myocytes growth and survival responses [24,64]. GATA-4 activity has also been reported to be enhanced upon phosphorylation by important protein kinases, like PKC and PKA [65,66]. These positive modifications usually facilitate GATA-4 nuclear translocation, interaction with collaborators, and DNA binding. Conversely, the activity of GATA-4 can be negatively regulated via phosphorylation. GSK-3β, an antigrowth factor usually inhibited by AKT, phosphorylates and inhibits GATA-4 nuclear transportation [67].

5.3.1. Identification of Growth Factors and the AKT Pathway as Upstream Regulators

Understanding the molecular basis of this cardiotoxicity and deciphering the upstream GATA-4 regulators targeted by Imatinib are critical for developing preventive/protective approaches. Previous studies reported that Imatinib-induced cardiotoxicity is partly due to its partially rescue [188]. We were able to replicate these results and confirm that c-ABL is targeted by Imatinib in cardiomyocytes. c-ABL, a cytoplasmic tyrosine kinase, is known to activate phosphoinositol 3-kinase (PI3K) [189], which in turn triggers the plasma membrane translocation and phosphorylation of AKT [131,150,151]. The latter is a key player that regulates pathways implicated in a many cellular processes ranging from metabolism to survival [131]. In cell survival, AKT aids in the preservation of mitochondrial integrity and prevention of cell death by counteracting proapoptotic BCL-2 family proteins [155]. Activated AKT phosphorylates an array of targets [131,151], or translocates to other subcellular compartments like the nucleus and mitochondria [153]**.** Recently, several studies reported an Imatinib-induced decrease in AKT activity in a variety of cell types [222,223].

Data obtained from cardiomyocytes treated with Imatinib confirmed the previous finding and reinforced the role of AKT as a central regulator of heart homeostasis. Conversely, AKT upregulation efficiently counterbalanced the effects of Imatinib on GATA-4 localization and cardiomyocyte survival, ascertaining its role in cardiomyocyte survival. The fact that GATA-4 localization was rescued and that its knockdown abolished AKT protective effects, triggered us to investigate whether the former is a direct target of the latter in this prosurvival pathway. In this work, we reported and for the first time that prosurvival AKT binds and phosphorylates GATA-4, essentially potentiating its transcriptional activity. Finally, insulin-like growth factor 1 (IGF-1), a known prosurvival growth factor and upstream regulator of AKT, was found to be GATA-4 dependent (**Figure 5.2**). The fact that

Imatinib targets this prosurvival pathway has immense potentials and showcases GATA-4 as an interesting pharmacologic target for cardioprotection against tyrosine kinase inhibitors.

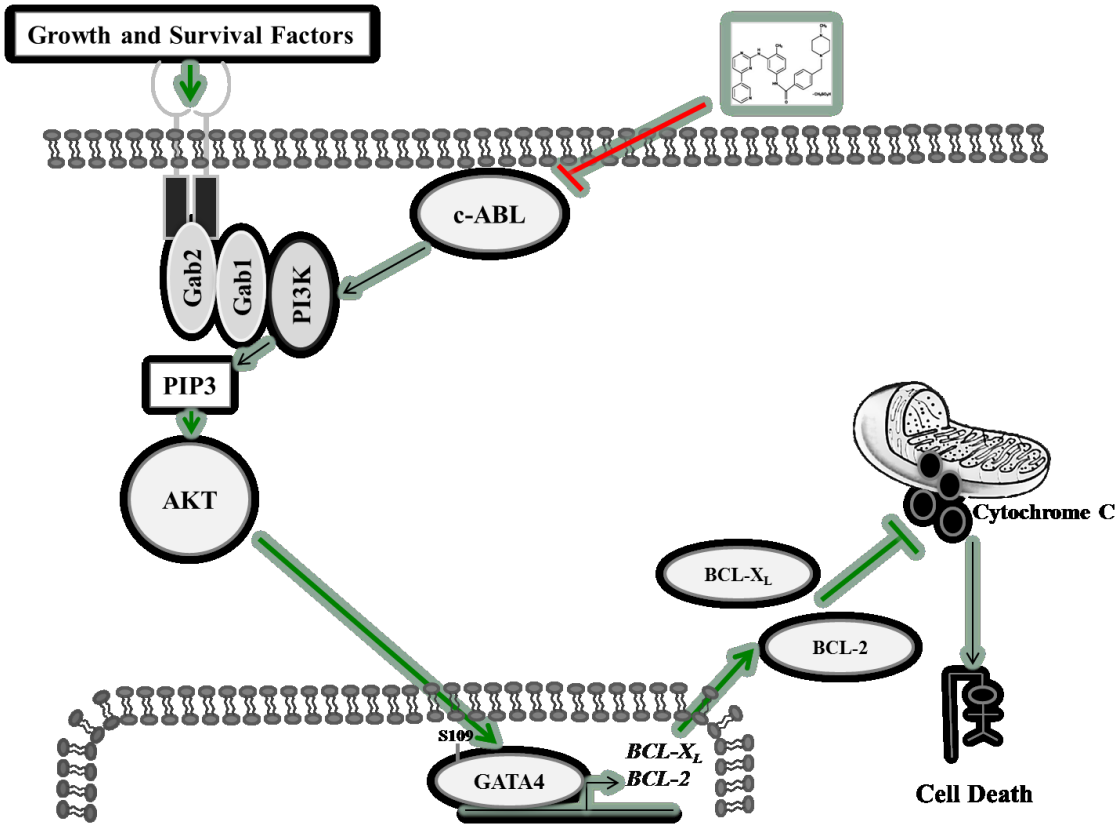


Figure 5.2. Schematic of AKT/GATA-4 Survival Pathway.

5.3.2. Identification of PEX-1 as a Novel GATA-4 Partner Involved in Cardiac Survival

Complex interactions between various transcription factors orchestrate gene expression to insure proper heart function. GATA-4, a key player in cardiac homeostasis, is known to partner with various transcription factors with either a net positive or a net negative effect on their transcriptional activation strength. GATA-4 physically interacts with NKX-2.5, MEF-2 [44,45], SRF [56], NFAT-3 [57], d-HAND [58], and many more.

We recently identified the zinc finger ZFP260 as a new GATA-4 collaborator. ZFP260, or phenylephrine-induced complex-1 (PEX-1), is a 13 zinc finger transcription factor. PEX-1 binds the consensus GGGGAGGGG, known as the PERE element. It is highly

expressed in the embryonic heart but is downregulated postnatally. We earlier found that PEX-1 plays a key role in relaying α_1-adrenergic as well as endothelin-1 signaling in the heart [85,211]. Moreover, the upregulation of PEX-1 in murine hearts induces genetic and phenotypic features of adaptive cardiac hypertrophy [85]. Finally, we found that PEX-1 physically and functionally interacts with GATA-4 on promoters of several genes implicated in the heart hypertrophic response. Therefore we hypothesized that PEX-1 is a collaborating partner of GATA-4 in prosurvival pathways. We verified this hypothesis by investigating the role of PEX-1 in DOX-induced cardiotoxicity. In the past few decades, research on DOX-induced cardiotoxicity generated a wealth of data regarding the mechanisms underlying this toxicity. Most of these studies confirmed the key role played by oxidative stress due to the inherent ability of DOX to generate free radicals during its metabolism. At the mitochondrial level, DOX-induced DNA damage was found to cause respiratory chain failure and a further increase in ROS production [229]. Moreover, DOX cardiotoxicity is aided by an array of contributors including, calcium handling dysregulation, adrenergic dysfunction, and inhibition of cardiomyocyte specific genes expression, like GATA-4, which is rapidly depleted in response to DOX treatment [117]. In chapter three, we proved that PEX-1 is a prosurvival factor and a GATA-4 collaborator. *In vivo*, cardiomyocyte-specific PEX-1 overexpression prevented the negative effects exerted by DOX on cardiac function and structure. In primary cardiomyocytes, PEX-1 upregulation counterbalanced DOX-induced cell death, through its inhibitory effects on proapoptotic genes and its induction of prosurvival genes like BCL-2 and BCL-X_L. Moreover, PEX-1 and GATA-4 were found to synergize on the promoters of BCL-2 and BCL-X_L, uncovering a collaborative interaction between these two transcription factors in prosurvival pathways. This possibility will be

further investigated by gene manipulation and complementation studies to check if these factors are downstream each other or just converge on common targets.

This work helped establish a plausible mechanism for Imatinib cardiotoxicity and provided answers on the pathways involved. Moreover, findings aided in our understanding of the role of GATA-4, AKT, and PEX-1 in cardiomyocyte survival (**Figure 5.3**).

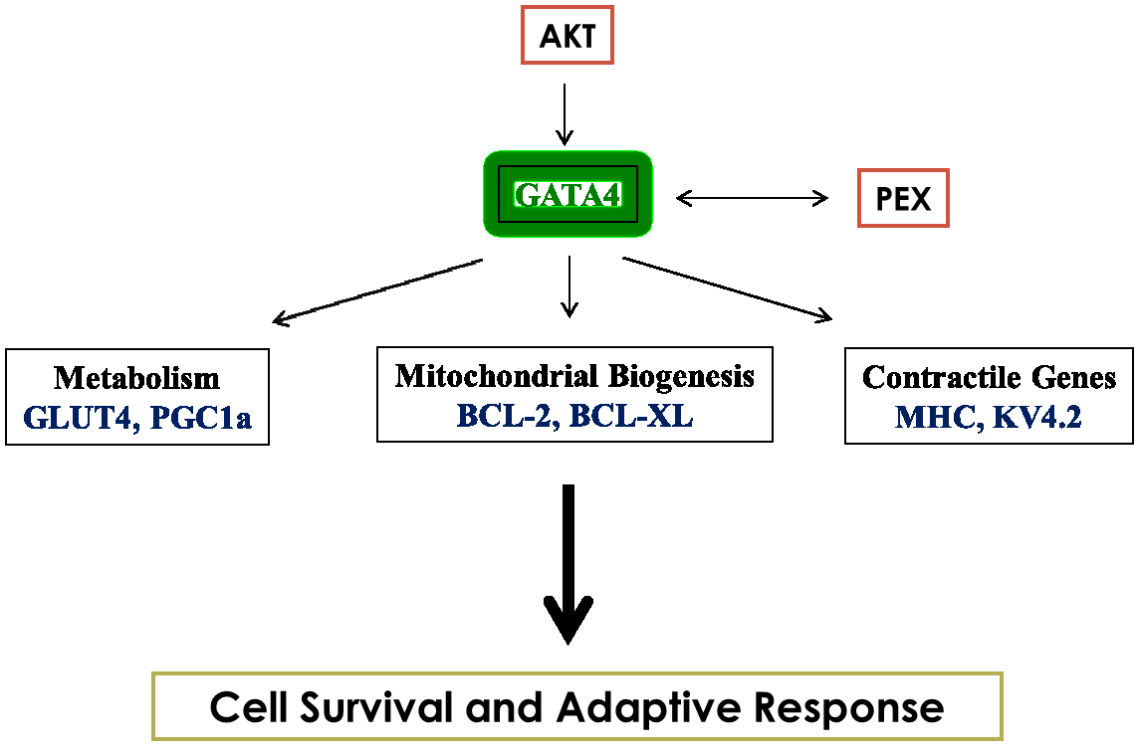


Figure 5.3. Schematic Showing the Relation Between AKT, GATA-4, and PEX-1.

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Education	2000-2004 Faculty of Sciences-**Lebanese University** Hadath-Lebanon **Degree: "Maitrisse es Sciences Naturelles"** awarded on July 2004. Major: Biology **Emphasis:** Animal Biology 2004-2006 Faculty of Medicine-**AUB** Beirut-Lebanon **Degree:** "**MS in Physiology**" awarded on June 24, 2006 2007- 2012 Faculty of Medicine- University of Ottawa Ottawa-Canada **PhD in Biochemistry**, Successfully defended on September 27, 2012 2008 Faculty of Medicine- Experimental Medicine Montreal-Canada Visiting Student, Mcgill University
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- Age dependent Imatinib mesylate induced cardiac toxicity.
Maharsy W, Aries A, Mansour O, Nemer M.
Ready for submission.

- Imatinib-induced cardiotoxicity: role of a novel AKT/GATA-4 survival pathway.
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Final stages before submission.

- Preventing Doxorubicin-induced cardiotoxicity by a novel GATA-4 partner, ZFP-260 transcription factor.
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*The Open Drug Delivery Journa*l. **2007**; 1: 1-6

- **Abstracts:**

Maharsy W, Nemer M. Searching for new approaches to prevent chemotherapy induced cardiotoxicity. BMI Poster Day. **2007** May.

Maharsy W, Nemer M. New Insights into Chemotherapy Induced Cardiotoxicity. Research Symposium, Biochemistry Department, University of Ottawa. **2008** February.

Maharsy W, Nemer M. Nouvelles Perspectives sur la Cardiotoxicité Induite par Chimiothérapie. ACFAS. **2009** April.

Maharsy W, Nemer M. Protective role of GATA4 in drug induced cardiotoxicity. BMI Poster Day. **2009** May.

Maharsy W, Nemer M. Enhancing cardiomyocyte survival in drug induced cardiac injury. Research Symposium, Biochemistry Department, University of Ottawa. **2010** February.

Maharsy W, Aries A, Komati H, Nemer M. GATA4, an integrator of myocyte survival and death signals. Keystone Symposium. **2011** Februaury.

- Awards and Talks

- International Student Admission Scholarship 2008 – 2011

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  Basic Science of Imatinib Cardiotoxicity. June 21, 2012.

Computer Skills

- MS Word
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Personal Skills

- Good team player with good communication and interpersonal skills.
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Languages

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Arabic	Excellent	Excellent	Excellent
English	Excellent	Excellent	Excellent
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Reference

Available upon request