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INVESTIGATING THE PATHOLOGICAL HEART AND ITS REGENERATIVE POTENTIAL

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On the cover: Immunostaining of isolated adult human cardiomyoctyes showing the cytoskeletal protein α-actinin in red.

INVESTIGATING THE PATHOLOGICAL HEART AND ITS REGENERATIVE POTENTIAL

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family and friends.

ABSTRACT

Cardiovascular disease is a leading contributor to mortality the world over, affecting millions of people each year. This, combined with its associated monetary and societal costs, has made the investigation of the etiology and pathophysiology of CVD a scientific priority. Researchers have developed numerous tools to aid in these studies, many of which have been successful in developing effective clinical therapies. However, while technical and medical advances have helped slow the spread of this burgeoning epidemic, CVD-related deaths are still on the rise globally. It is for this reason that the doctoral thesis presented here aims to develop a better understanding of the pathogenesis of CVD and to determine the magnitude of cardiac regenerative potential.

In **PAPER I** we present clear methods for the isolation, culture, and functional characterization of adult murine cardiomyocytes for *in vitro* studies. We show that our method results in a single-cell suspension of electrochemically active, rod-shaped adult cardiomyocytes that maintain calcium sensitivity and contractile function. We further present proper cell culture conditions, optimized methods for whole-cell patch clamp and adenovirus-mediated gene delivery, as well as a clear protocol for the collection and analysis of contractility traces and calcium transients.

In **PAPER II** we identify estrogen-related receptor β (ESRRβ) as a causative factor in the pathogenesis of dilated cardiomyopathy (DCM). We show that mice lacking ESRRβ develop DCM in mid-life and die prematurely. We further show that human myocardial tissue samples from DCM patients lack the expected nuclear ESRRβ localization, suggesting a role for ESRRβ also in human disease onset.

In **PAPER III** we describe the role of SGK1 in the development of the fatal ventricular arrhythmias commonly associated with heart failure. Using transgenic mice expressing either constitutively active or dominant negative forms of SGK1, we show that chronic SGK1 activation in disease results in fatal ventricular arrhythmias due to an increased persistent sodium current, I_{NaL}, leading to prolonged action potential duration. We further show a direct interaction between SGK1 and the primary cardiac voltage-gated sodium channel, $Na_v1.5$, and have identified putative phosphorylation sites contained in the *SCN5a* gene. Together, these findings suggest direct SGK1 regulation of sodium channel kinetics and gating.

In **PAPER IV** we compare transcriptional profiles from three zebrafish models of cardiac regeneration to determine common genetic drivers of the regenerative response. By comparing the transcriptional profile of our genetic cardiomyocyte ablation model to those of two apical resection models and one cryoablation model, we have identified 16 genes to be up-regulated in all screens and 1 gene down-regulated. Gene ontology analysis of this gene set reveals significant enrichment in biological processes associated with the cell cycle and mitosis, and pathway analysis has provided a list of putative upstream regulators of these genes. Further analysis of all transcription profiles also indicated a large number of model-specific genes, indicating that these disease models, and thus the regenerative responses activated

therein, should not be considered interchangeably, but should be regarded as genetically distinct models of cardiac regeneration.

Finally, in **PAPER V** we use ^{14}C dating to determine the magnitude and dynamics of human cardiac cell turnover in dilated cardiomyopathy. The amount of ^{14}C contained in postmortem cardiac tissue from patients diagnosed with DCM was compared to atmospheric levels at the time of the patient's birth to determine the rate of cardiac cell turnover. Our mathematical model predicts that cardiomyocyte turnover is at least maintained in DCM. Importantly, though we report a significant increase in cardiomyocyte ploidy with disease onset, this was not sufficient to entirely account for the increased ^{14}C concentration.

Together, this work provides valuable information regarding the pathogenesis of both DCM and arrhythmogenic heart failure, and contributes to the growing literature surrounding potential regenerative therapies for cardiovascular disease.

SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. Graham, E.L., Balla, C., Franchino, H., Melman, Y., del Monte, F., Das, S. Isolation, Culture, and Functional Characterization of Adult Mouse Cardiomyocytes. *J. Vis. Exp.* (79), (2013).

II. Rowe, G.C., Asimaki, A., Graham, E.L., Martin, K.D., Margulies, K.B., Das, S., Saffitz, J., Arany, Z. Development of dilated cardiomyopathy and impaired calcium homeostasis with cardiac-specific deletion of ESRRβ. *Am. J. Physiol. Heart Circ. Physiol.* (312), (2017).

III. Das, S., Aiba, T., Rosenberg, M., Hessler, K., Xiao, C., Quintero, P.A., Ottaviano, F.G., Knight, A.C., Graham, E.L., Boström P., Morissette, M.R., del Monte, F., Begley, M.J., Cantley, L.C., Ellinor, P.T., Tomaselli, G.F., Rosenzweig, A. Pathological Role of Serum- and Glucocorticoid-Regulated Kinase 1 in Adverse Ventricular Remodeling. *Circulation* (126), (2012).

IV. Graham, E.L., Baniol, M., Derks, W., Chi, N., Andersson, O., Bergmann, O. Exploring distinct gene expression profiles in zebrafish models of cardiac regeneration. *Manuscript.*

V. Bergmann, O., Collin, S., Graham, E.L., Jashari, R., Andrä, M., Salehpour, M., Alkass, K., Possnert, G., Druid, H., Bernard, S., Jovinge, S., Frisén, J. Regeneration of human cardiomyocytes in cardiomyopathy. *Manuscript.*

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Graham, E.L., Bergman, O. Dating the Heart: Exploring Cardiomyocyte Renewal in Humans. *Physiology (Bethesda).* 32(1), (2017)

II. Bosma, M., Gerling, M., Pasto, J., Georgiadi, A., Graham, E.L., Shilkova, O., Iwata, Y., Almer, S., Söderman, J., Toftgård, R., Wermeling, F., Boström, E.A., Boström, P.A. FNDC4 acts as an anti-inflammatory factor on macrophages and improves colitis in mice. *Nat Commun.* 12(7), (2016).

III. Boström, P.A., Graham, E.L., Georgiadi, A., Ma, X. Impact of exercise on muscle and nonmuscle organs. *IUBMB Life*. 65(10), (2013).

IV. Xu, J., Zhao, J., Graham, E.L., Xiao, C., Cheng, Y., Xiao, J. Circulating microRNAs: novel biomarkers for cardiovascular diseases. *J Mol Med (Berl).* 90(8), (2012).

LIST OF ABBREVIATIONS

^{14}C	Carbon-14
AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
AMS	Accelerator mass spectrometry
AMVM	Adult mouse ventricular cardiomyocyte
AP	Action potential
APD	Action potential duration
AR	Apical resection
ARVC	Arrythmogenic right ventricular cardiomyopathy
ARVM	Adult rat ventricular cardiomyocyte
AV node	Atrioventricular node
bpm	Beats per minute
CA	Cryoablation
CAD	Coronary artery disease
CICR	Calcium-induced calcium release
cmlc2	Cardiac myosin light chain 2
CVD	Cardiovascular disease
DCM	Dilated Cardiomyopathy
Dox	Doxycycline
dpa	Days post amputation
DTA	Diphtheria toxin chain A
ECC	Excitation-contraction coupling
ECM	Extracellular matrix
EDV	End diastolic volume
EF	Ejection Fraction
ESRRβ	Estrogen-related receptor β
ESV	End systolic volume
FS	Fractional Shortening
GA	Genetic ablation
GFP	Green fluorescent protein
GO	Gene ontology
HCM	Hypertrophic cardiomyopathy
hiPSC	Human induced pluripotent stem cell

HM	Heavy membrane
iCM	Ischemic cardiomyopathy
ICP	Isovolumic contractile period
iDCM	Idiopathic dilated cardiomyopathy
IR	Ischemia/Reperfusion
LA	Left Atrium
LAD	Left anterior descending artery
LTCC	L-type calcium channel
LV	Left Ventricle
LVNC	Left ventricular non-compaction cardiomyopathy
LVEDD	Left ventricular end diastolic dimension
LVESD	Left ventricular end systolic dimension
MI	Myocardial infarction
Mtz	Metronidazole
NCX	Sodium/Calcium exchanger
NRVM	Neonatal rat ventricular cardiomyocyte
NTR	Nitroreductase
PH3	Phospho-histone H3
PKA	Protein kinase A
PLB	Phospholamban
RA	Right Atrium
RV	Right Ventricle
RyR	Ryanodine receptor
SA node	Sinoatrial node
SERCA	SR Ca-ATPase
SES	Socioeconomic status
SGK1	Serum- and glucocorticoid regulated kinase 1
SR	Sarcoplasmic reticulum
SV	Stroke volume
TAC	Transverse aortic constriction
TF	Transcription factor
TnI, TnT	Troponin I, Troponin T
WT	Wild type
αMHC	α-Myosin heavy chain

CONTENTS

Cardiovascular Disease – An Epidemic

1

The epidemic of non-communicable diseases, in the midst of which we currently find ourselves, is the most widespread and longest-standing human epidemic the world has ever seen. It has been apparent to researchers and clinicians for some time that, of these diseases, the primary contributors to the global health burden are cardiovascular diseases (CVDs). However, it was not until 2011 that the global heads of state joined at the United Nations to discuss this issue with a focus on prevention. It was clear then, as it was in 2001 with the snowballing HIV/AIDS epidemic, that CVDs do not only present a substantial global health burden, but have also affected the global economy, and in many countries have contributed to the widening of socioeconomic gaps and the reinforcement of disparate socioeconomic classes.

In this chapter I will discuss the current state of cardiovascular disease across the globe and the measures being taken to decrease its prevalence. I will then discuss the economic burden of cardiovascular disease, and conclude with a discussion of cardiovascular disease from a sociological perspective.

1.1 Cardiovascular Disease as a Global Health Concern

In 2013 the UN announced targets for reducing mortality due to non-communicable disease by 2025. These targets involve a reduction of several behavioral and biological risk factors including: harmful alcohol use, physical inactivity, salt/sodium intake, tobacco use, high blood pressure, and obesity/diabetes. By reducing the prevalence of these risk factors they would like to achieve a 25% reduction of all-cause premature CVD mortality by 2025. It is estimated that if current trends in diabetes mellitus, tobacco use, changes in blood pressure, and obesity continue, the

number of premature CVD deaths will rise from 5.9 million (in 2013 when the study was conducted) to 7.8 million by 2025 [4]. For comparison consider that, according to the World Health Organization, in 2015 1.1 million people died from HIV/AIDS, 1.4 million people died from tuberculosis, 1.25 million people died on the world's roads, and approximately 800,000 deaths were self-inflicted. If unchecked, the premature CVD mortality in 2025 could be greater than all of these figures combined.

Of the targets studied, the prevalence of hypertension showed the greatest influence on the premature CVD mortalities in 15 and 16 of the 21 regions studied for men and women respectively, though it must be said that estimates differed strikingly with geographical region [4]. Notable exceptions were women in tropical Latin America, men in high-income Asia Pacific, and both men and women in Australasia and high-income North America, where halting obesity had the greatest impact. Additionally, smoking was the leading risk factor scenario for men in Central Asia, Central Sub-Saharan Africa, North Africa and the Middle East, and for women in high-income Asia Pacific and Western Europe. Interestingly, a reduction in obesity had the second largest impact on premature CVD mortality for women, while a reduction in tobacco smoking was the second-largest determinant for men [4].

Overall, the combined scenario in which all of the risk factor targets are achieved in 2025 does have the potential to decrease global CVD deaths to an estimated 5.7 million. However, perhaps understandably, the change in risk varies widely across regions. This is to say that while this global health crisis may be somewhat mitigated for certain privileged populations, the gains will not be distributed equally. For higher-income regions already experiencing rapid declines in CVD mortality, the current UN targets may not even be stringent enough to yield any measurable benefits, while in some middle- and lower-income regions a 25% reduction in CVD mortality is only achievable when all of the targets are reached. Thus, if this epidemic is to be curbed universally, it is clear that global healthcare disparities will need to be addressed, and investments in CVD prevention, treatment, research, and education will need to be expanded beyond western society.

1.2 Cardiovascular Disease as an Economic Burden

Cardiovascular disease is not only the world's most prevalent killer, but also the most costly. In a policy statement from the American Heart Association, researchers calculated projections for CVD prevalence between 2010 and 2030 and the resultant direct and indirect medical costs [5].

The primary cause for the increased prevalence of CVD in the United States is the aging population. People 65 years or older – especially those over 80 years of age – show a higher prevalence for all CVD, and with the near exponential advances in medical technology and end-of-life care, this population is expected to grow significantly over the next two decades. This growth translates to an additional 3 million people with heart failure, 8 million people with coronary heart disease, 4 million people with stroke, and a devastating 27 million people diagnosed with hypertension by 2030. Overall, the authors project that by 2030, 40.5% of the US population will have some form of CVD, and between 2010 and 2030 the direct

medical costs associated with these diseases are expected to triple, from $272.5 billion to $818.1 billion. Indirect costs are expected to increase by 61% between 2010 and 2030, from $171.7 billion to $275.8 billion. Together, these increases amount to a projected total cost of CVD exceeding $1 trillion by 2030 [5].

The projected economic burden associated with CVD does not however have to be as debilitating as the disease itself. Because CVD is largely preventable, decreasing its prevalence will in turn decrease its economic impact. The projections presented assume no change in preventative health-care measures and a linear increase in risk over time. It stands to reason that focusing on improving population-level risk factors will result in a significant decline in CVD prevalence and its economic impact in turn. Medical research is one clear way to help decrease the burden of CVD, by helping to develop more effective treatments for those afflicted, but widespread educational programs and equitable access to CVD specialists and services will also be necessary to ensure that everyone, regardless of socioeconomic status, receives the knowledge and healthcare they require and deserve.

1.3 The Socioeconomic Implications of Cardiovascular Disease

The inequalities currently associated with cardiovascular healthcare represent a persistent public health concern that is not exclusive to developing nations. Even in the most affluent nations the risk of developing coronary disease is unevenly stratified across socioeconomic classes, with socioeconomic depravation serving as a powerful independent predictor of HF development. As such, understanding the relationship between socioeconomic status (SES), the onset of CVD, and its eventual progression to heart failure may help policymakers develop more equitable and effective prevention, diagnosis, and treatment strategies.

Socioeconomic position can be assessed by numerous measures, the importance of each varying over the course of one's life. They may be characterized broadly as education, employment relations, occupation, income, social class, total assets, housing characteristics, area level measures, and composite indicators [6]. The most frequently applied composite area level indicator in HF research is the Carstairs deprivation index, which assigns individuals a score based on residence postcodes. Scores range from 1 (least deprived) to 5 (most deprived). There have been nine studies reporting an increased incidence of HF associated with lower SES. Two studies have compared high vs. low income. In Malmö, Sweden the risk of HF hospitalization is 45% lower for individuals with an annual income >250,000 Swedish Kronor as compared to individuals whose income was <50,000 Swedish Kronor [7]. Similarly, in Denmark the incidence of HF is 34% and 33% lower for high-income men and women respectively [8]. Similar results were attained when education level was substituted for income as the primary SES indicator and, perhaps most remarkably, education alone was independently associated with reduced ejection fraction and left ventricular dilation. The remaining seven reports compare levels of deprivation and illustrate, using different primary measures, that the adjusted risk of developing HF is ~30-50% higher with greater deprivation [9-15].

SES also associates with overall morbidity and mortality. Age-standardized rates of HF hospitalization climb incrementally across income deciles [6], and an investigation of the elderly participants in the Longitudinal Study on Aging shows that an eighth grade education or less is significantly associated with a primary discharge diagnosis of HF [16, 17]. Socioeconomically deprived patients hospitalized with heart failure are at higher risk for readmission, and low neighborhood median household income confers a greater risk of all-cause re-hospitalization. Furthermore, the relationship between SES and hospitalization is echoed in figures of mortality; the more deprived, the lower the survival. In the Medicare cohort, low and lower-middle class patients had higher 1-year mortality than higher-class subjects, and participants in the Atherosclerosis Risk in Communities study with a high co-morbidity burden, and living in low-income areas also experienced an increased risk of death [10]. It is interesting then that, despite the vast literature illustrating the variation in HF morbidity and mortality across socioeconomic strata, there is a paucity of evidence supporting equitable treatment.

It is, however, clear from these studies that socioeconomic deprivation is a powerful indicator of cardiac pathogenesis. The pathways mediating these inequalities can be modulated by both public health and clinical interventions, but only through concerted, interdisciplinary efforts will we be able to attenuate these unacceptable and unequivocal inequalities. By learning as much as we can about how to combat this growing epidemic, we will ensure that the proper care be administered to any and everyone who needs it.

2 The Healthy Heart

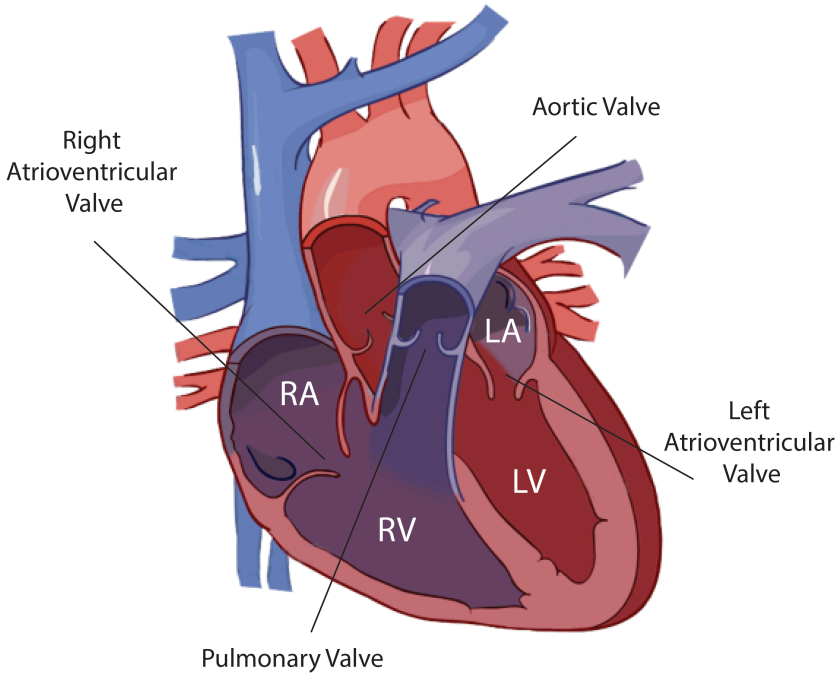

Figure 1: A schematic of the adult human heart. Blue and red areas represent the flow of deoxygenated and oxygenated blood, respectively. Image adapted from [18].

Cardiac dysfunction occurs when the heart, for any reason, is unable to meet the physical demands placed upon it and is therefore unable to supply oxygen to the systemic circuit or to itself. There are myriad known causes for heart disease, often with interrelated pathogeneses, but in order to fully characterize the pathological mechanisms governing these various conditions, and to determine salient therapeutic strategies, it is first necessary to understand normal heart structure and function.

In this chapter I will outline the basic structure and function of the normal human heart, define the common physiological and diagnostic parameters, and provide a brief summary of the regulation of normal human circulation.

2.1 Normal Cardiac Function

The adult human heart consists of four chambers whose primary function is to pump deoxygenated blood to the lungs and oxygenated blood throughout the rest of the body. The two atria, the right atrium (RA) and the left atrium (LA) are composed of two thin, orthogonal, overlaying muscular sheaths and serve both as reservoirs and pumps for deoxygenated (RA) and oxygenated (LA) blood. The two ventricles, the right ventricle (RV) and the left ventricle (LV), are composed of much thicker muscle walls and are tasked with pumping blood from the low-pressure venous systems to the higher-pressure arterial systems (**Figure 1**).

The right atrium is the first to receive the body's deoxygenated blood. Deoxygenated blood from the upper and lower limbs enters through the superior and inferior vena cava, respectively, and deoxygenated blood from the heart itself enters through the coronary sinus. The right ventricle then receives the deoxygenated blood from the right atrium through the right atrioventricular (tricuspid) valve at 0 pressure, and pumps the blood through the pulmonary artery to the lungs at a peak systolic pressure of ~25 mmHg. The blood is oxygenated in the lungs via diffusion and returns to the left atrium through the pulmonary veins. The left ventricle receives the newly oxygenated blood from the left atrium via the left atrioventricular (mitral) valve, also at 0 pressure, and pumps this blood into the aorta and systemic circuit at a peak pressure of ~120 mmHg. Both ventricles eject similar volumes of blood at the same rate (heart rate). Therefore, since the left ventricle has a much higher ejection pressure, it performs approximately 5 to 7 times more pressure work than the right ventricle. It is for this reason that most cardiomyopathies stem from left ventricular dysfunction, and why most therapies focus on increasing left ventricular functionality [19].

2.2 The Cardiac Cycle

At rest, the heart beats at a rate of approximately 70-75 beats per minute (bpm). Normal, non-stenotic valves offer zero resistance and open as a function of the pressure differential across the valve. At a heart rate of 75 bpm, a complete filling and emptying cycle takes 0.8 seconds and consists of two primary phases: systole and diastole [19]. The cardiac cycles are analogous between the two sides of the heart, the primary differences pertaining only to the lower pressures maintained in the right ventricle and pulmonary artery. For the sake of simplicity, the following discussion will use the left heart as an example.

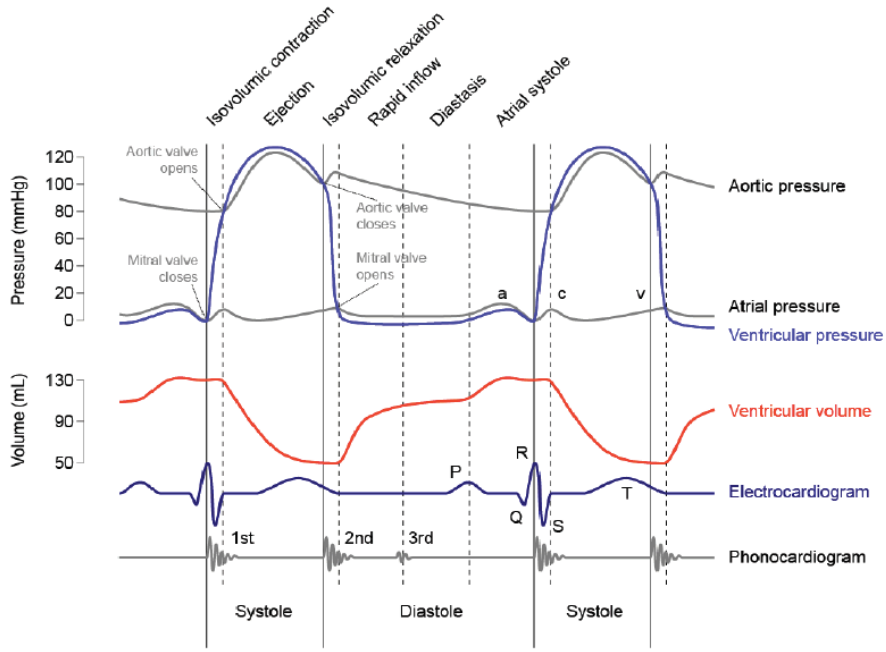

Figure 2: Pressure and volume curves depicting the cardiac cycle. Aortic and atrial pressures are superimposed on ventricular pressure curves. Ventricular volume is depicted in red, and below is a corresponding electrocardiogram. The phonocardiogram depicts the 1st, 2nd, and slight 3rd heart sounds during systole and diastole. Image adapted from [20]

Left ventricular systole is the contractile period of the heart cycle, which can be subdivided into three phases: the isovolumic contraction period (ICP), the rapid ejection phase, and the slower ejection phase. In the left ventricle the ICP begins with the closure of the mitral valve (the first heart sound) and ends with the opening of the aortic valve. It is during this phase that the maximum rate of ventricular pressure $\left(\frac{dP}{dt}\right)$ is reached, and this pressure work is used to pump blood to the aorta during rapid ejection. Approximately 2/3 of the ventricular blood is emptied into the aorta during the rapid ejection phase, resulting in slight aortic distension and a decrease in aortic $\frac{dP}{dt}$. The slower ejection period occurs at the peak of the aortic pressure curve as the ventricles begin to relax, and ventricular pressure decreases below aortic pressure. For a short period, even though the ventricular pressure is lower than the aortic pressure, blood is still able to flow into the aorta at a slow rate due to its inertia. However, as the pressure differential between the aorta and the left ventricle increases further, a small backflow of blood closes the aortic valve (the second heart sound) [21].

Left ventricular diastole is the relaxation and filling period of the heart cycle, consisting of two primary events: the isovolumic relaxation period, spanning the time between the closure of the aortic valve and the opening of the mitral valve, and the filling period, during which time blood flows into the ventricle. During the isovolumic phase the ventricle relaxes, but no filling can occur until the ventricular pressure is low enough to allow the mitral valve to open. Once the ventricular pressure is sufficiently decreased, approximately 2/3 of the ventricle is filled by passive blood flow from the atrium. This occupies the first 1/3 of diastole and is called passive

rapid-filling. The remaining third of the blood is then pumped into the heart by atrial contraction late in diastole and is called active rapid-filling. As in systole, there is a short period of slower, inertial filling between these two phases, and diastole ends with the closure of the mitral valve prompting a new ventricular contraction and a new cardiac cycle [21] (**Figure 2**).

Normal end diastolic volume (EDV) is ~120 mL, and normal end systolic volume (ESV) is ~40 mL. This equates to a normal stroke volume (SV) of ~80 mL (EDV-ESV). Stroke volume thus describes the total volume of blood pumped into the systemic vasculature during systolic contraction. This parameter, however, is most often expressed in relation to the total end diastolic volume, giving the fraction of blood ejected from the heart, or the ejection fraction (EF), according to the following equation:

$$\mathrm{EF} = \frac{SV}{EDV} \times 100.$$

A normal ejection fraction is thus ~67% (55% - 70%) with decreases likely indicating weakened myocardium and/or cardiac dysfunction. Similar calculations can be performed using left ventricular end systolic and diastolic dimensions (LVESD, LVEDD) to quantify changes in ventricular morphology. Fractional shortening (FS) is defined as the fraction of any diastolic dimension that is lost in systole, and is calculated as follows:

$$FS = \frac{(LVEDD - LVESD)}{LVEDD} \times 100.$$

Similar to EF, decreased FS often indicates dysfunction of the heart's mechanical capability. Normal FS values range between 25-45% while values <15% are considered to be severe.

Once oxygenated blood leaves the heart it enters the systemic vasculature through arteries. Arteries carry oxygenated blood away from the heart down to arterioles and further to capillaries where nutrient and oxygen transfer occurs with the systemic tissues. Arteries are necessarily thicker and stronger than veins, as they must withstand significantly higher pressures from left ventricular contractions. Deoxygenated blood from the capillaries then enters the veins, which carry blood back to the heart (**Figure 3**). Veins constitute a low-pressure, large volume system called the venous capacitance system, which can be used to regulate heart rate during physical activity.

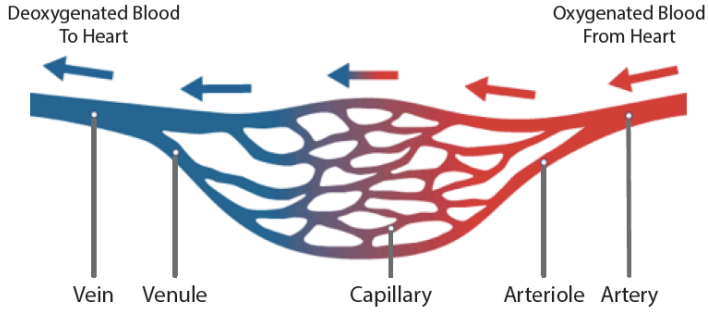

Figure 3: Schematic showing the flow of oxygenated blood from the heart through the venous capacitance system. Image adapted from [1].

During exercise blood is forced out of the system and returned to the right atrium by muscle contraction and various nervous influences. This so-called venous return results in increased heart rate as atrial filling stimulates contraction. The only exceptions to the artery/vein dichotomy are the pulmonary arteries and veins. While they still shunt blood away from and toward the heart, because they lead to the lungs, the pulmonary arteries actually carry deoxygenated blood, while the pulmonary veins carry oxygenated blood from the lungs to the heart's left atrium [19].

Arterioles are the small arteries providing the major arterial resistance against which the left ventricle must work. The regulation of this peripheral vascular resistance determines how much blood reaches the capillaries, the rate of oxygen and nutrient transfer, but perhaps most importantly, the arterioles serve as the main contributors to systemic blood pressure. Resistance hypertension can be defined thus as an excess of arteriolar constriction resulting in increased overall pressure of the arterial tree. Many treatments for hypertension, and indeed many forms of cardiomyopathy, are focused on decreasing this pressure.

2.3 Excitation-Contraction Coupling (ECC)

Though the heart is comprised of many different cell types, the cells primarily responsible for cardiac contractile function are (1) the cardiac pacemaker cells, which produce the electrical signal for contraction, and (2) the cardiomyocytes themselves, which receive the signal and perform the contraction. The cardiac pacemaker cells are a group of specialized cells comprising the sinoatrial (SA) node in the heart's right atrium, the atrioventricular (AV) node in the right ventricle, and the Bundle of His and Purkinje fibers spreading throughout the heart. These cells are capable of spontaneously generating action potentials (APs), and propagate the depolarization wave throughout the heart. In cardiac muscle, AP propagation occurs via intercellular ion "leakage" via gap junctions, which alters cardiomyocyte membrane potential and leads to cell contraction. This entire process is referred to as excitation-contraction coupling (ECC) [22].

In its broadest use, cardiac ECC refers to the entire process by which electrical activation of cardiomyocytes leads to the activation of a cardiomyocyte's contractile apparatus. This includes everything from the initial membrane depolarization via the AP, to the activation of the Ca^{2+} transient, to myofilament activation in response to increased intracellular Ca^{2+} levels ($[Ca^{2+}]_i$).

Though the process is continuous in the heart, it can be said to begin with the depolarization of the sarcolemma due to the propagation of the AP from the SA node. The AP causes depolarization of the cardiomyocyte's sarcolemma via a large influx of sodium ions, primarily through the most abundant cardiac voltage-gated sodium channel, $Na_v1.5$. The resultant increased membrane potential triggers the opening of the voltage-gated L-type calcium channels (LTCC) allowing an inward flux of calcium ions (I_{Ca}). The LTCCs, which span the t-tubular membrane, are localized in close proximity to the sarcoplasmic reticulum (SR), and specifically to the ryanodine

receptor (RyR). The RyR is a specialized Ca^{2+}-activated Ca^{2+} channel housed within the SR membrane. The Ca^{2+} that enters the small cleft between these two channels activates the RyR and allows for more Ca^{2+} to exit the SR into the cytosol. This process, termed calcium-induced calcium release (CICR), is essential to the proper regulation of the ECC mechanism [23, 24].

The rise in $[Ca^{2+}]_i$ causes sarcomeric contraction via myofilament activation, as Ca^{2+} ions bind to the troponin complex found on the actin filaments in complex with tropomyosin. The Ca^{2+} binding causes a conformational change in tropomyosin, exposing the myosin-binding site on the actin filament. Using the energy stored in the ADP and P_i molecules found on the myosin head, myosin binds to the actin filament, pulls it and creates the force for contraction. The return of ATP molecules to the myosin head allows for the dissociation of actin and myosin and results in relaxation (**Figure 4**). The sarcomeres are composed of two myosin heavy chain (MHC) isoforms, α and β. Their expression is exceptionally tightly regulated with deviations from this ratio often indicating pathology, as will be discussed later.

In the continued presence of calcium this process will repeat. In this way, relaxation ultimately depends on the decline of $[Ca^{2+}]_i$. Ca^{2+} is primarily removed from the cytosol by reuptake into the SR through the SR Ca-ATPase (SERCA), which is regulated by phospholamban (PLB), or by being transported out of the cell via the sarcolemmal Na/Ca exchanger (NCX). Mitochondrial uptake and transport out of the cell via the sarcolemmal Ca-ATPase also play a role [23, 24].

2.4 Nervous Regulation of Circulation

Though the heart is able to function without continuous nervous activity, the autonomic nervous system does play an important role in modulating contractile activity in times of stress or increased mechanical loading. Autonomic nervous control of the circulation occurs through the concerted efforts of the sympathetic and parasympathetic nervous system pathways. The sympathetic pathway is governed by adrenergic (catecholamine) signaling and, via interaction with α- and β-adrenergic receptors (α-AR and β-AR), generally results in increased heart rate, cardiac output, and EF. Conversely, the parasympathetic pathway is governed by cholinergic (acetylcholine) signaling, and generally leads to the opposite effect [25].

The primary effectors of adrenergic signaling in the heart are β_1- and β_2-ARs. It has been shown that α-ARs do also have positive inotropic activity, but their most prominent role is in regulating peripheral resistance via the venous capacitance system. In the heart both β_1- and B_2-AR are coupled to the stimulatory G protein, G_s. In the presence of β-AR agonists, conformational changes in the protein complex result in activation of adenylyl cyclases (AC), which convert ATP to the second messenger, cAMP. The increased level of cAMP results in the activation of protein kinase A (PKA), which phosphorylates many proteins involved in excitation-contraction coupling such as: LTCC, phospholamban (PLB), RyRs, and troponin I (TnI), resulting in increased contractility. β_1-AR appears to be coupled only to the stimulatory G_s pathway, while β_2-AR has also been shown to couple with the

inhibitory G_i, which limits cAMP formation. In this way β_2-AR can contribute to a negative feedback system ensuring that overstimulation does not occur [25] (**Figure 4**).

The discovery of the essential role that β-ARs play in the control of circulation has lead to the therapeutic approaches widely used for heart failure treatment today. The first β-AR therapies were based around the concept of increasing myocardial contractility via inotropic agents. The prevailing dogma was based on a simple line of reasoning: (1) heart failure is directly associated to poor contractility, (2) catecholamines are the best stimulators of myocardial contractility, and (3) catecholamines should thus be used to increase contractility in patients with heart failure. However, at the time, the concept of β-AR desensitization had not yet been established, and researchers did not appreciate the high oxygen cost of increasing heart rate, myocardial contractility, and LV wall stress. The β-agonist treatments eventually lead to fatal arrhythmias in many patients, and patients with coronary artery disease also showed increased incidence of myocardial ischemia. It was later determined that, while acute activation of the sympathetic system is required to maintain homeostasis in the healthy individual, in heart failure the sympathetic stimulation in the heart is already excessively elevated. Further sympathetic stimulation thus becomes deleterious, as the increased oxygen demand cannot be appropriately met by coronary blood flow. Furthermore, prolonged β-AR stimulation activates multiple cellular processes involved in adverse cardiac remodeling and, perhaps most importantly, as an adaptive response to increased catecholamine levels, β-ARs desensitize naturally through receptor down-regulation and decreased downstream signaling. Therefore, in an environment where β-ARs are already resistant to activation, β-AR agonist treatment is maladaptive [25].

These new discoveries provided the intellectual basis for β-AR blockade therapy in the early 1960s. In 1962, together with JS Stephenson, Sir James Black introduced a non-selective β-blocker, pronethalol, which showed strong antiarrhythmic effects in guinea pigs and dogs [26]. Similar effects were seen in the clinic, and from then the field grew as more β-blockers were developed with more selectivity, and less toxicity.

Though originally considered counterintuitive, today the administration of β-blockers for treating heart failure is almost taken for granted. Now often prescribed with angiotensin-converting enzyme (ACE) inhibitors, which decrease vasoconstriction [27], β-blockers work to effectively decrease the workload placed on the heart. However, the cellular and molecular mechanisms governing these improvements in cardiac function are still being elucidated. Mechanistic studies are further confounded by the fact that some of the later generations of β-blockers show only weak blockade and can have off-target effects, such as vasodilation, but still reach salutary endpoints. Future studies will need to focus on identifying other aspects of the β-AR cascade for potential therapeutic intervention, and on characterizing the etiology and pathogenesis of the various cardiomyopathies leading to heart failure to develop disease-appropriate therapies.

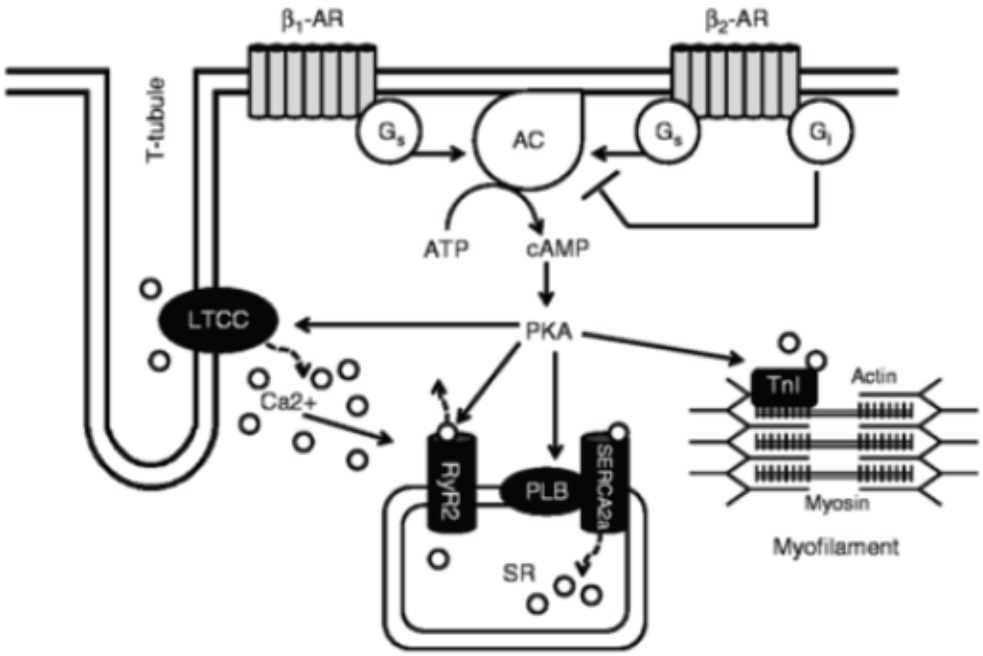

Figure 4: Schematic showing the key players in the ECC and β-adrenergic signaling pathways. Action potentials activate LTCC allowing Ca^{2+} to enter the cell. This binds and activates RyR_2 allowing for Ca^{2+} to leave the SR and enter the cytosol. The cytosolic Ca^{2+} activates the myofilament via binding to TnI, causing contraction. The β-adrenergic signaling can further enhance contraction via cAMP-mediated PKA signaling, which is able to modulate various aspects of the ECC mechanism to increase contractility. Image adapted from [25].

3 The Pathological Heart

Given the delicate complexity of the circulatory system, it is no surprise that there exists a large array of potential confounders. Cardiovascular disease, or "heart disease", is an umbrella term describing the wide range of conditions that can deleteriously affect cardiac function. Diseases under this umbrella include, but are not limited to:

- Congenital heart defects (birth defects), which restrict blood flow to, from, and within the heart
- Acquired heart defects, such as stenosis of heart valves, arteries, or veins
- Blood vessel diseases, such as coronary artery disease (CAD) or stroke, which can limit the amount of oxygen reaching the heart muscle itself
- (Silent) Cardiac ischemia, which is a form of CAD producing few symptoms but resulting from insufficient oxygen delivery to the heart
- Myocardial infarction (heart attack), which occurs when part of the heart muscle is damaged due to lack of nutrients or oxygen, usually due to advanced CAD
- Heart rhythm dysfunction, such as ventricular arrhythmias, which disrupt the heart's electrical signaling
- Angina, which is transient discomfort from limited oxygen and nutrient delivery to the heart
- Heart infections, such as myo- and endocarditis, which occurs when bacteria, fungi, or other pathogens infect the endocardium
- Cardiomyopathy, which is a family of heart diseases affecting cardiac cells themselves, often with genetic etiology

- Hypertensive heart disease, which includes heart diseases stemming from high blood pressure
- Heart failure, which can occur as a result of diabetes, lung disease, or any of the aforementioned cardiac conditions, and is defined as the inability of the heart to pump enough blood to meet the body's needs

This investigation is primarily focused on the pathogenesis and pathophysiology of cardiomyopathy and heart failure, so in this chapter I will provide a brief description of the pathogenesis of systolic and diastolic heart failure, with specific focus on the contributions from dilated cardiomyopathy.

3.1 Heart Failure

The heart muscle can fail because it is too weak to carry out normal functions or because it is too stiff to act as an efficient pump. It is important to note that heart failure is not simply one disease, but a chronic and progressive condition that can arise from a number of structural and functional cardiac disorders, from diabetes, or from lung diseases such as emphysema. When heart failure syndrome presents with an EF less than 40 percent, the dysfunction is most often referred to as "systolic heart failure" because the ventricles are unable to perform an effective systole. Conversely, "diastolic heart failure" occurs with preserved EF, but overly stiff ventricular walls result in inefficient diastolic filling. In this case, while the heart muscle maintains the strength to pump the blood to the body, there is simply not enough blood collected in the left ventricle at the end of diastole.

The primary symptoms of heart failure include peripheral fluid retention, fatigue, and dyspnea (labored breathing). The retention of excess fluid increases pressures in the heart, lungs, and the rest of the body, which is called "congestion". Congestive heart failure on the left side of the heart can lead to shortness of breath after modest activity or even at rest. On the right side of the heart the elevated venous pressures can lead to the peripheral edema and abdominal swelling [28].

3.1.1 Systolic Heart Failure

Systolic heart failure is the most common type of heart failure in patients younger than 65 years. The most common causes of systolic heart failure are cardiomyopathy and coronary artery disease, the latter often resulting in myocardial infarction. Systolic heart failure is also often associated with enlargement of the heart, likely due to hypertrophic or dilated cardiomyopathies.

The decreased EF related to systolic heart failure can lead to decreased blood flow during periods of increased physical activity, causing fatigue and exhaustion. In advanced cases, cardiac output can even be lower than normal at rest. If the reduction in cardiac output is life threatening it is called cardiogenic shock [28].

3.1.2 Diastolic Heart Failure

While heart failure is most commonly associated with systolic dysfunction, as many as 30-40% of heart failure patients have normal or only slightly reduced EF [29]. In these cases diastolic dysfunction is implicated as the major contributor. If the left ventricle is stiff, it relaxes too slowly in early diastole and offers greater resistance to filling. This stiffness is often caused by the presence of fibrotic tissue, which can arise from cardiac injury or as a result of adverse ventricular remodeling. The increased resistance thus increases diastolic pressures and results in significantly decreased cardiac output. As in systolic heart failure, the low cardiac output is manifest as fatigue and shortness of breath. However, unlike in systolic heart failure, the increased end diastolic pressure is transmitted backwards to the pulmonary venous system, resulting in dyspnea. This is particularly evident in times of physical exertion.

The diagnosis of diastolic heart failure depends on the simultaneous satisfaction of three conditions: (1) presence of the signs and symptoms associated with heart failure; (2) presence of normal or only slightly decreased EF; and (3) increased diastolic pressure or impaired filling [29]. Primary diastolic dysfunction is typically seen in patients with hypertension, restrictive, or hypertrophic cardiomyopathy and has a disproportionately high prevalence in the elderly.

3.2 Cardiomyopathy

As opposed to other heart diseases, which primarily affect cardiac oxygen and nutrient consumption via blood flow, cardiomyopathies form a family of heart diseases affecting the heart muscle itself. The cardiomyopathy family can be further subdivided into hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy (ARVC), left ventricular non-compaction cardiomyopathy (LVNC), and dilated cardiomyopathy (DCM). Though most of these diseases have genetic origins, the discovery of various acquired cardiomyopathies, as well as overlapping symptoms, often complicate diagnosis. Interested readers are directed to the Cardiomyopathy Compendium recently published in *Circulation Research* for a more in-depth review of each cardiomyopathy [30]. As the current investigation is primarily focused on dilated cardiomyopathy, here I will present a brief overview of its known genetic determinants and mechanisms, estimations of its prevalence, and common treatments.

3.2.1 Dilated Cardiomyopathy

DCM is characterized, generally, by left ventricular enlargement concomitant with systolic dysfunction (**Figure 5**). This manifests clinically as (1) FS < 25% or EF < 45%, and (2) as an LVEDD > 117% [31]. Confusion often arises when diagnosing DCM due to the fact that its most common cause is in fact ischemic injury. However, because DCM is a condition with multiple etiologies, it must be subdivided further into ischemic and non-ischemic forms. Approximately 50% of all DCM cases are ischemic in origin, but after identifying left ventricular dilatation and systolic dysfunction, an

etiological assessment must be performed. If no cause other than genetic can be identified, a clinical diagnosis of 'idiopathic' DCM (iDCM) is assigned. These are the cases that comprise the "non-ischemic DCM" category. The prevalence of DCM is estimated to be between 1:400 and 1:250 individuals in the United states [31], but without a formal, population-based epidemiological study, this estimate cannot be confirmed.

Oftentimes the genetic variations leading to DCM are found to be heritable, and are grouped accordingly as familial DCM. It is estimated that between 20-35% of identified DCM cases are familial, but the classification of familial DCM is assigned according only to phenotype and pedigree assessments, not molecular genetic data. Familial DCM can therefore be easily misdiagnosed or missed completely due to incomplete family history or simple professional oversight.

While it may still be difficult to assess the true prevalence of DCM, a multitude of causal genes have been reported from the identified cases to date [32]. Mutations in more than 30 causal genes have been identified and, in contrast to the genes associated with other cardiomyopathies, have quite diverse ontologies. Many are sarcomeric genes, the most common of which is *TTN* encoding the large sarcomeric protein titin [33], but there is also a large proportion encoding Z-disk proteins, ion channel proteins, proteins encoding the nuclear envelope, heat-shock chaperones, and mitochondrial proteins. The majority of DCM-associated mutations are rare, or even unique to a family. The 30+ causal genes that have been discovered to date are estimated only to account for approximately 40% of the genetic cause of DCM [31].

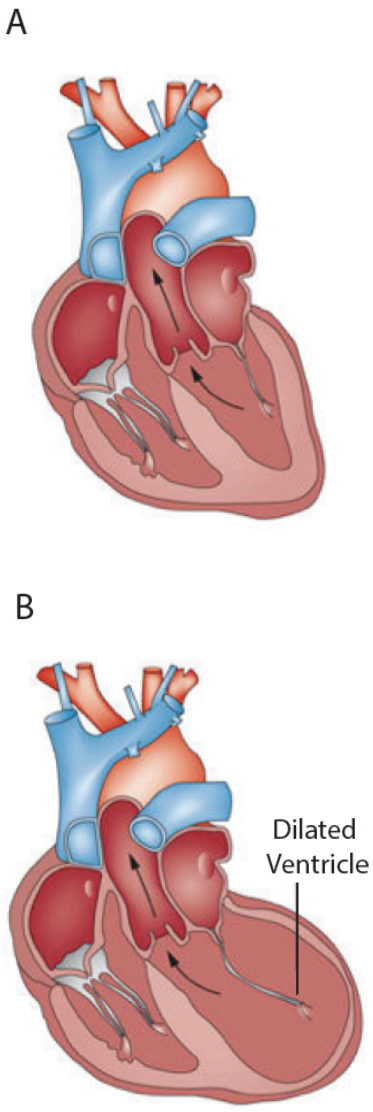

Figure 5: (A) Normal vs. (B) DCM heart. Image adapted from [3].

As with most cardiomyopathies, the current treatments for DCM patients are primarily focused on symptom management. DCM treatment is focused on arrhythmia surveillance and treatment, managing LV dimension and function, and dealing with any apparent congestive symptoms [34]. Today, symptomatic DCM with HF and depressed EF is treated with ACE or angiotensin receptor inhibitors, along with β-blockers, aldosterone antagonists, and in some cases, general vasodilators. These medications exploit the various cellular mechanisms described above to reduce blood pressure and decrease the overall workload placed on the heart. Arrhythmogenic DCM patients are treated according to the standard guidelines for sudden cardiac death management and implantable cardioverter defibrillator implantation. Those patients presenting with a disease-causing genotype but no evidence of pathology are simply monitored yearly and sometimes advised against

strenuous physical activity. Perhaps a better understanding of the molecular genetics of these disease-causing genes would help physicians to reach a consensus on the proper medical management of this last group, but it is clear that uncovering the other genetic mutations leading to DCM will broaden our understanding of its pathophysiology, help increase the efficacy of diagnosis, and of course aid in the development of much more effective and comprehensive therapies.

3.2.2 Cellular and Molecular Basis of Cardiomyopathy

Despite the many different classifications of cardiomyopathy, there is a marked overlap in associated symptoms, emphasizing the relatively limited range of cellular responses within the cardiomyocyte. With the exception of DCM, the changes in cellular morphology, gene expression patterns, and metabolism associated with cardiomyopathies are initially compensatory, but prove insufficient or even maladaptive in the long term. In DCM patients, the dilated phenotype decompensates rapidly after the appearance of symptoms, with no observable compensatory mechanism [35].

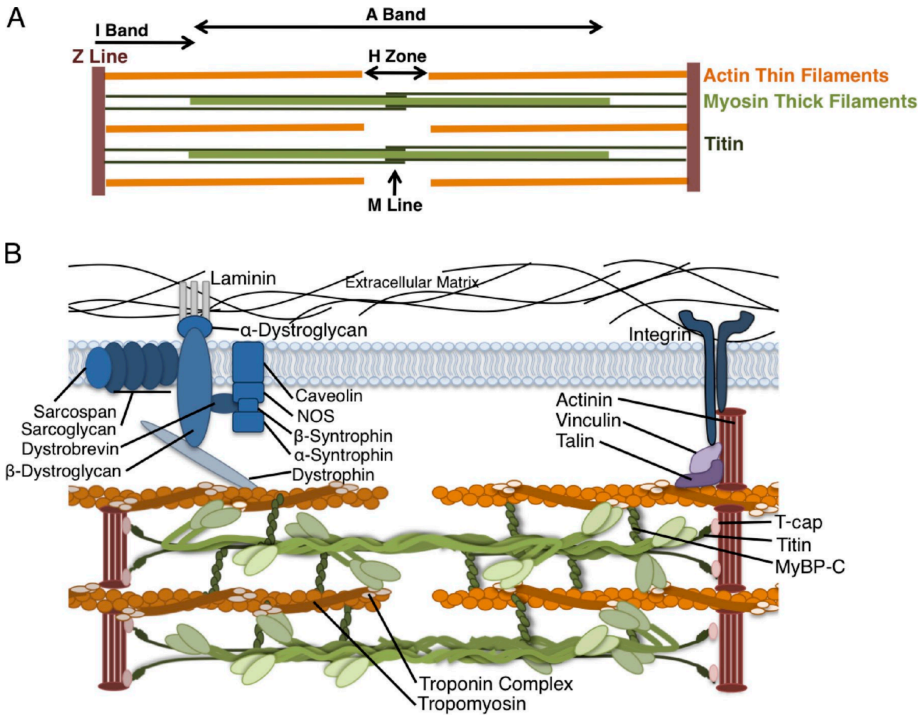

Figure 6: Molecular schematic of a cardiac sarcomere and associated transmembrane and ECM proteins. (A) Overview image of primary sarcomere components. (B) Detailed representation of functional players in mechanotransduction, calcium sensing, and contraction. Image adapted from [35].

In an attempt to relieve the mechanical strain placed on the heart, ventricular cardiomyocytes enlarge resulting in cardiac hypertrophy. This response is considered to be initially compensatory as it normalizes ventricular wall stress and increases the heart's contractile capacity. Cardiac hypertrophy is due to the addition of sarcomeres either in series (eccentric) or in parallel (concentric) within the cardiomyocytes themselves, thus increasing cardiomyocyte length or width, respectively [36]. Concentric hypertrophy increases force generation in stressed cells, but also causes

misalignment of the cardiomyocytes, which can negatively affect contractility [37]. Furthermore, persistent hypertrophy of either type correlates with HF onset. Further addition of sarcomeres after the initial compensatory period results in increased cardiomyocyte length/width ratios, which actually reduces force generation. Additionally, eccentric hypertrophy is associated with ventricular dilation in DCM and HF, and demonstrates limited compensatory function [38].

On the genetic level, cardiomyopathy results in the induction of a gene program typically associated with embryogenesis. The expression of natriuretic factors – atrial natriuretic factor (ANF) in the atria, and brain natriuretic peptide (BNP) in the ventricles – induce diuresis and vasodilation to reduce blood pressure in response to increased wall stress. The expression of these genes appears to be a cardioprotective mechanism, and there is evidence to suggest an anti-hypertrophic effect [39, 40]. Increased expression of the β-MHC isoform also occurs with pathology. The expression of the two myosin heavy chain isoforms, α- and β-MHC, occur in such exceptionally regulated ratios that an increase in β-MHC with concomitant decrease in α-MHC is a sensitive marker for early cardiomyopathy. This response may also be initially adaptive, as β-MHC demonstrates improved ATP usage due to lower ATPase activity. However, myofilaments with high β-MHC levels are also slower to contract, which may contribute to overall dysfunction [41-43].

Fibrosis is a well-known hallmark of cardiomyopathy, and fibrotic tissue is composed primarily of collagens type I and III. Fibrosis occurs in the early stages of cardiomyopathy but increases with disease progression. Eventually the fibrotic tissue disrupts ECC interactions between the cardiomyocytes and causes increased myocardial stiffness resulting in decreased contractility. The collagen is typically produced by activated resident fibroblasts, but there is also evidence supporting collagen production by stressed cardiomyocytes [44].

The increased demand for ATP during pathology progression results in decreased availability of energy-rich resources, such as phosphocreatine. In the initial stages of cardiomyopathy, the heart's metabolism adapts to utilize more free fatty acids, which is slightly less efficient. In DCM and end-stage HF, energy substrate utilization shifts from fatty acid utilization to the even less efficient glucose metabolism. This shift is thought to occur as a consequence of increased cardiac growth with insufficient angiogenesis, thus decreasing the oxygen available for efficient oxidative metabolism [45]. This quasi-hypoxic state causes the expression of genes facilitating anaerobic glucose metabolism [46, 47].

These cellular responses are primarily mediated by ECM-sarcomere connections (**Figure 6**), and aim to maintain contractile force and steer metabolism towards efficient substrate utilization. These responses are achieved through various signaling pathways including, mechanotransduction signaling [48], Akt and PI3K signaling [49], G-protein signaling [35], Ca^{2+} regulation and calcineurin signaling [50], and even apoptotic signaling pathways [51, 52]. As with their physiological sequelae, the activation of these pathways is initially cardioprotective, but chronic activation is often associated with decompensation and heart failure.

Modeling Cardiomyopathy and Heart Failure

4

In order to better understand the mechanisms governing the onset of cardiomyopathy and heart failure, researchers have developed numerous tools to model various cardiac pathologies. In general terms, a biological disease model can be defined as an animal or cell population that displays all or some of the pathological processes that are observed in the actual animal or human disease. These model systems help us to simplify complex biological processes, to glean valuable information regarding the development of the disease, and to test potential treatment approaches.

The complexity of the cardiovascular system in particular requires models that are exceptionally precise and robust. Furthermore, as most models have clinical translation as the ultimate endpoint, it is especially important for heart disease models to be physiologically relevant. Cardiovascular disease paradigms have been developed in small mammals such as the rat, rabbit, and cat; in large mammals such as the non-human primate, dog, sheep, and pig; and even in non-mammalian vertebrates such as newts and zebrafish. However, due to its high genetic similarity to the human, its short gestation period, and the relative ease of genetic manipulation, the common house mouse, *mus musculus*, has become the organism of choice for modeling cardiovascular disease *in vivo*. *In vitro* studies have made large use of isolated neonatal rat ventricular cardiomyocytes (NRVMs), though isolated adult mouse ventricular cardiomyocytes (AMVMs) also offer unique benefits.

In this chapter I will introduce cell-based cardiac disease models as well as the most commonly used whole animal-based cardiac injury model systems. I will provide an overview of the currently accepted techniques for neonatal and adult cardiomyocyte isolation, culture, and functional characterization, and provide an introduction to mouse transgenesis.

4.1 *In vitro* Models of Cardiac Disease

The versatility, cost-effectiveness, and convenience of cell-based disease models has made *in vitro* modeling attractive in the field of cardiovascular research. The large amount of material obtained from a single preparation of cells allows for more biologically comparable experiments and even provides the potential for high-throughput drug screening for the eventual discovery of novel therapeutics. Additionally, cell-based modeling also affords the unique opportunity to investigate cell-autonomous responses to cardiac insult and injury such as hypertrophy or cell cycle activation. Preparations of heart cells have been reported for numerous species, with the neonatal rat and the adult mouse being the two most common sources for cardiomyocytes [53].

4.1.1 Neonatal Rat Ventricular Cardiomyocyte Isolation, Culture and Functional Characterization

Like the mouse, the rat shares a high level of genetic and genomic similarity to the human, making it a suitable model system for translational experimentation. However, its advantages over the mouse for *in vitro* cardiac research stem primarily from technical and economic considerations. In the case of neonatal cardiomyocytes, mice and rats exhibit similar cytoskeletal structures, metabolic properties, and protein signaling, making them relatively equal models systems [53]. However, hearts from newborn rats are larger in size, enabling a much larger yield of cardiomyocytes from a single preparation.

The relative ease of isolation, culture, and genetic manipulation also makes NRVMs an attractive model system. Because rat pups are larger than mouse pups, the actual dissection of the heart from the neonatal rat is much easier and, in comparison to adult cardiomyocytes (from either rats or mice), the cardiomyocyte isolation procedure is markedly less complex. After removal of the hearts from the rat pups, they are minced and subject to a series of enzymatic digestions. Two to three rounds of digestion are typically carried out using an enzyme buffer containing common salts, glucose, and a mixture of pancreatin and Collagenase Type II (Collagenase B) [54]. The result of the digestion step is a single-cell suspension of cardiomyocytes that are then plated in a mammalian cell culture media. Once plated, the cells can be cultured for weeks and used for subsequent experimentation.

Isolated NRVMs have been used extensively to model various disease paradigms via genetic manipulation. NRVMs lend themselves to genetic manipulation due to the relative ease and efficiency of nucleic acid delivery. Numerous studies have reported very efficient gene knockdown with siRNAs [55-57], effective overexpression has been achieved using adenoviral DNA delivery [58-60], and miRNAs have recently proven to be influential regulators of cardiac development and regeneration [61-65]. Unlike with *in vivo* experimentation, this scale allows investigators to study the effects of a single gene on any aspect of the cellular machinery – from the level of protein-DNA interactions to changes in overall cellular morphology – with little to no background from other cell types. Additionally, this format lends itself well to high-throughput

screening platforms [66], the likes of which have been used to discover novel small-molecule inhibitors of cardiac hypertrophy [67] and potential miRNA drivers of cardiomyocyte proliferation [68].

However, while many of the signaling pathways and protein expression patterns in NRVMs are similar to adult cardiomyocytes, this model system has garnered significant critique due to its structural disparities. The lack of clearly defined sarcomeres in neonatal cardiomyocytes weakens studies of cardiomyocyte structural and contractile changes, and the lack of t-tubules complicates ECC investigations (**Figure 7A**). It is for this reason that many researchers have turned to isolated adult cardiomyocytes for their *in vitro* studies. Though they are much more difficult to isolate and culture, these cells have clearly defined cytoskeletal structures that more directly mimic human cardiomyocyte morphology (**Figure 7B**).

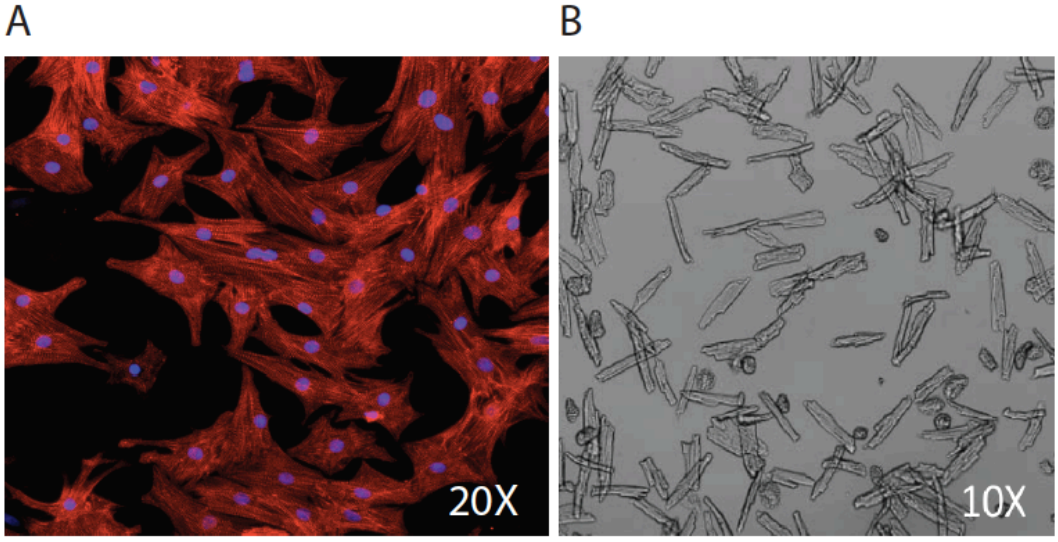

Figure 7: (A) Isolated neonatal rat ventricular cardiomyocytes in culture. The cytoskeletal protein alpha-actinin is stained in red. Nuclei are stained blue with DAPI. (B) Brightfield image of isolated adult mouse ventricular cardiomyocytes in culture showing clear rod-shaped morphology as opposed to the less-uniform, immature morphology of the neonatal cardiomyocytes. Image of AMVMs adapted from [69].

4.1.2 Adult Mouse Ventricular Cardiomyocyte Isolation, Culture, and Functional Characterization

The early studies using adult ventricular myocytes saw them isolated from relatively large animals, such as cats, dogs, and rabbits, thus yielding large amounts of cells per isolation [70-72]. Cardiomyocytes have also been successfully isolated from guinea pigs [73], but because of the relative ease and cost-effectiveness of isolation, the adult rat has become the most commonly used animal model to date. Though the adult rat ventricular cardiomyocytes (ARVMs) have been used as an *in vitro* model for over 40 years [74], no clear universal isolation protocol has been established. Researchers typically follow protocols that maintain the same basic principle of retrograde perfusion of the heart with an enzyme solution, but vary significantly in key details such as enzyme concentration, perfusion rate, apparatus, and dissociation

methods. These disparities, while seemingly minor, can affect the reproducibility of results obtained from ARVM experiments.

The isolation procedure of adult mouse ventricular cardiomyocytes (AMVMs) is much the same as that for rats [75], with the added complications that mouse hearts are smaller, more difficult to manipulate, and yield less cells. However, for many researchers, the opportunity to study the effects of transgenic manipulation in adult mice on the cellular level offsets the decreased isolation efficiency. Using this system, researchers are able to study the cellular effects of long-term cardiac challenges such as altered gene expression, fibrosis, or tissue remodeling. In some cases, for studying acute effects, it is more advantageous to treat WT adult cardiomyocytes with hypertrophic agonists [76], but many sound conclusions regarding altered cardiac function in disease have been drawn from studies using isolated AMVMs from transgenic animals [76-78]. Furthermore, as AMVMs have become more popular as a model system, isolation and culture procedures have become more widespread and standardized [79, 80]. However, because certain physiological characteristics of isolated adult ventricular cardiomyocytes can change over time in culture, most experiments with these cells must be performed within 24 – 48 hours to maintain the morphological and behavioral similarities to cells in intact tissue.

Indeed, the greatest advantage of using adult ventricular cardiomyocytes as a model system for cardiac disease is their similarity to cells in intact tissue. Cardiomyocytes isolated from adult mammals are rod-shaped, are often binucleated, have a well-organized sarcomeric structure, and exhibit contractile function when paced with an electrical stimulus. The ability to control the pacing frequency permits another level of experimental control and has been suggested to create a more physiological environment for the cells [81]. This unique characteristic of isolated adult cardiomyocytes also presents the possibility to employ cell-based electrophysiological methods, such as a patch clamp and calcium transient analysis, in a much more physiologically relevant setting.

4.1.2.1 Measuring Ion Channel Dynamics Using Patch Clamp

The patch clamp technique is an innovation of the traditional voltage-clamp recording techniques pioneered by Kenneth Cole, Alan Hodgkin, and Andrew Huxley. The aim of this technique is to measure and characterize the currents through the ion channels of excitable (and non-excitable) cells.

The first step of all patch-clamp recordings is to establish the high-resistance (≥1-10 GΩ) seal between the patch pipette and the cell membrane. When the patch pipette is close to the cell membrane, the application of suction will create a tight seal allowing for direct current measurement. If the suction is not too great as to break the cell membrane, the result is a “cell-attached” configuration, allowing the most physiological readings, as the contents of the cytosol remain undisturbed. However, the membrane potential of the membrane cannot be quantified using this configuration. By applying a stronger negative suction or a high-frequency voltage pulse across the membrane, a rupture can be formed creating a low-resistance

pathway between the cell's cytoplasm and the ionic solution inside the pipette. This "whole-cell" configuration allows for control of the composition of both the intracellular and extracellular compartments, permitting the measurement of voltage potential changes as well as macroscopic current changes across the entire cell membrane. The whole-cell patch clamp technique is routinely used for investigating gross changes in cardiomyocyte electrophysiology due to disease or injury, but studies focused on small molecule validation would most likely use the "inside-out" configuration. This is achieved by pulling back the patch pipette after a gigaseal has been created in the cell-attached configuration. This allows researchers to test the effects of small molecules or proteins on specific ion channels, by adding them directly to the pipette solution. The final common configuration is the "outside-out" configuration, which is obtained from the whole-cell configuration by pulling back the patch pipette. If the gigaseal is stable enough, the two parts of the membrane will anneal allowing for voltage and current measurements. This configuration allows for the investigation of the effects of extracellular proteins on ion channel function, with single-channel resolution [82].

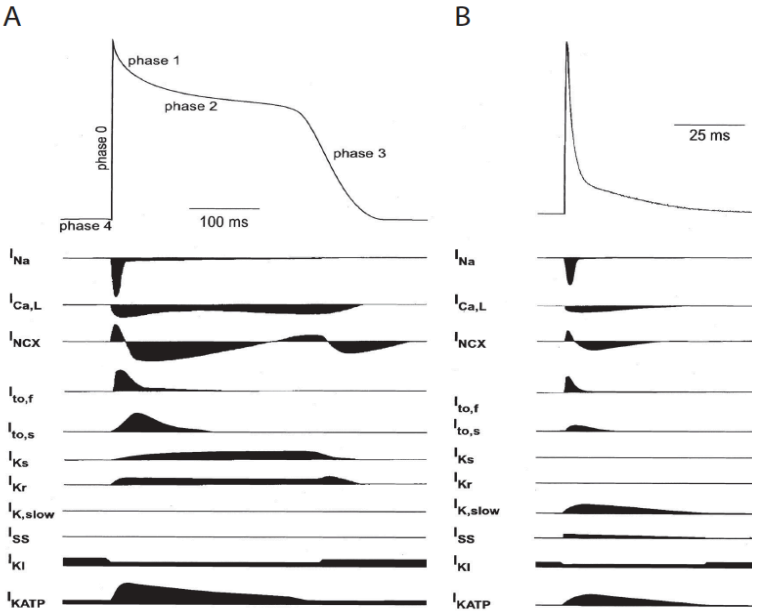

Figure 8: Representative contributions of common ion channel currents in (A) human and (B) mouse ventricular cardiomyocyte action potential generation. In both cases the depolarization due to the cardiac sodium current is a key action potential initiator. Image adapted from [83].

The whole-cell patch technique has been very successful in describing the properties of membrane currents in cardiomyocytes. Most approaches make use of pharmacological inhibitors, often included in the patch pipette solution, to isolate particular currents of interest for analysis. Currents are sometimes measured before and after blockage and subtracted to yield the "blocker-sensitive" currents. Tetrodotoxin (TTX) and Cd^{2+} ($CdCl_2$) are commonly used for blocking inward Na^+ and Ca^{2+} currents, respectively. Addition of Cs^+ and tetrathylammonium (TEA) to the pipette solution is often used to block outward K^+ currents. Additionally, some more specific voltage-clamp protocols use step depolarizations or hyperpolarizations of fixed duration (sometimes also in combination with blockers) to target specific current components [82] (**Figure 8**).

4.1.2.2 Quantifying Calcium Dynamics and Contractility Using IonOptix

Because of the large role that calcium plays in the molecular function of the heart, understanding Ca^{2+} dynamics is of upmost importance when investigating cardiac dysfunction. The well-defined sarcomeric structures of isolated adult cardiomyocytes allow for direct contractility measurements using light-phase microscopy upon electrical field stimulation, and numerous calcium-activated fluorescent dyes have been developed to quantify the resultant localized changes in $[Ca^{2+}]_i$, often referred to as calcium "sparks". The summation of these calcium sparks over the entire cell represents the transient increase in $[Ca^{2+}]_i$ during each contraction, and is thus termed the calcium transient.

The first reports of measurable calcium transients during cardiac EC coupling were made in 1978 by David G. Allen who quantified aequorin luminescence in frog cardiac muscle [84], and by Gil Wier in 1980 who injected photoproteins into canine Purkinje fibers [85]. Since then, innovations of calcium-activated fluorescent dyes have improved their sensitivity and precision through the addition of an acetoxymethyl (AM) ester group, enabling cell permeability, and by engineering a shift in the maximum excitation wavelength upon Ca^{2+} binding, allowing for ratiometric quantification of $[Ca^{2+}]_i$. Today, advanced digital imaging techniques, such as laser scanning confocal microscopy, have allowed researchers to visualize contraction events and trace calcium transients in isolated adult cardiomyocytes in real time. Various companies, such as IonOptix, have also now developed commercial hardware to record these data as well as multivariate analysis software for subsequent data analysis.

The IonOptix system is able to combine brightfield imaging with sarcomere and cell-length detection algorithms to translate image data from recordings of single cells to quantifiable displacement transients describing contractility in real time. Monotonic transient analysis is then often used to report various parameters describing the characteristics of the transient, which can be used as markers of physiological function. Peak contractile shortening/re-lengthening velocities and percent sarcomere/whole cell shortening are often reported for contractile studies; the latter often being considered analogous to fractional shorting in whole heart studies. In the case of calcium transients, the IonOptix system records the fluorescence of intracellular Ca^{2+} indicators in real-time to create plots depicting the change in $[Ca^{2+}]_i$ with time. The same monotonic transient analysis methods can be applied to these transients to quantify rates of calcium release and reuptake, peak $[Ca^{2+}]_i$, and time to recovery of baseline Ca^{2+} levels.

The unidirectional flow setup of the IonOptix system also lends itself well to testing the effect of proteins or pharmaceuticals on cardiomyocyte contractility and calcium handling. Because of the in-line profusion system, the drug of interest can simply be added to the perfusion buffer for a given time, and the effect can be recorded. This technique has been used extensively to investigate various pharmaceutical modulators of contractile function and calcium handling [86-88].

4.2 *In vivo* Models of Cardiac Disease

While much can be gained from *in vitro* cardiovascular studies, because a single cell cannot accurately represent the functionality of an entire tissue or organ system, cell-based experimentation also has many disadvantages. *In vitro* studies are well suited for investigating the cell-autonomous consequences of genetic manipulations or pharmaceutical treatments, but it is often very difficult to fully recapitulate the effects of gross cardiovascular injury, such as myocardial infarction or cardiac ischemia. *In vivo* studies however offer researchers the opportunity to investigate the effects of injury or genetic manipulation at the whole-organ and whole-organism level. This is especially important for regenerative studies, as multiple cell types and organ systems are involved in the regenerative response, as well as for preclinical testing, in which off-target effects can prove dire. Furthermore, as most *in vitro* studies are conducted using homogenous cell populations, the effects of intercellular signaling cannot be examined. By studying the entire organism, researchers are also able to uncover endocrine and paracrine signaling pathways, the drivers of which could form the basis for novel therapeutics.

4.2.1 Commonly Used Disease Paradigms for Cardiac Injury

The need for animal-based models of cardiovascular disease stems primarily from the need for a suitable system for preclinical testing of potential pharmaceuticals or treatment strategies. The ideal model system for such studies would be donated human hearts themselves, but the paucity of material, the inherent variability of human samples, as well as the priority given to transplant patients makes this avenue impractical. Both large- and small-animal models of cardiac injury have been developed to this end [89, 90], but for the purposes of this report, only mouse models of cardiovascular disease will be discussed.

4.2.1.1 Ligation-induced Models of Myocardial Infarction (MI) and Ischemia Reperfusion (IR)

The adult mouse model of MI is one of the most commonly used models of cardiovascular disease, as it has been shown to recapitulate the human response to ischemic injury accurately enough to yield physiologically relevant results [91, 92]. To achieve the infarction, the mice are first anesthetized and intubated for ventilation, then subject to a left-sided thoracotomy to expose the left anterior descending (LAD) coronary artery for permanent ligation. The ligation of the LAD blocks the flow of blood to the heart, resulting in an ischemia-induced MI. The chest and skin are then closed, and the mice are allowed to recover. Upon injury, inflammatory cells and fibroblasts infiltrate the infarct area, scar tissue replaces the necrotic myocardium and, as in humans, the inflexible scar tissue persists and results in markedly decreased cardiac functionality [93]. If MI is not the desired experimental endpoint, the ligation can be removed allowing for reperfusion of the myocardium [94]. In this way, a defined period of ischemia can be achieved, and the tissue damage associated with the reperfusion of ischemic myocardium can also be studied.

4.2.1.2 Pressure Overload Model of Heart Failure Using Transverse Aortic Constriction (TAC)

Pressure overload of the cardiac system leads to pathological hypertrophy, as the heart struggles to adapt to the increased mechanical load, and ultimately results in congestive heart failure. In humans, this is typically caused by stenosis or blockage of the great vessels, disallowing adequate outflow from the heart chambers and causing the internal chamber pressure to rise. To model this in mice, a suture is placed around the aorta distal to the brachiocephalic artery. The suture can be tightened around a blunt needle, with the gauge of the needle determining the severity of the constriction. A 27-gauge needle is commonly used. The needle is then removed and the chest and overlaying skin are closed. The TAC procedure results in a significantly increased heart-weight-to-body-weight ratio, indicative of cardiac hypertrophy, and significantly decreased cardiac functionality [95].

One major drawback of these types of *in vivo* injury models is the non-standardized experimental procedures. Because the surgeries can be technically challenging, it is also difficult to ensure comparable levels of injury severity, making the translation of results between investigations difficult. Furthermore, as rodents and humans are phylogenetically distant, some of the pathophysiological features of disease may not be reliable predictors of human physiology. However, both the LAD ligation and TAC techniques have given researchers a much broader understanding of the pathogenesis of MI and HF, and employing these techniques in transgenic backgrounds has provided invaluable insights into the effects of specific genes on disease progression.

4.2.2 Transgenic Mouse Models of Cardiomyopathy

Perhaps the foremost advantage of using the house mouse, *mus musculus*, for biological experimentation is the relative ease of genetic manipulation. Most transgenic mouse models of CVD are focused on modeling cardiomyopathies through gene knockout, overexpression, or mutation.

The primary goal of gene knockout or mutation is to isolate the effects of the target gene by ablating its expression or modifying its activity, and assessing the morphological and/or functional consequences. This is typically accomplished by replacing the endogenous gene with in an engineered sequence encoding an inoperable gene product. The construct containing the mutated gene is usually introduced into cultured mouse embryonic stem cells via electroporation, where it will be naturally integrated into the genome of some of the cells. The cells containing the gene are isolated and inserted into the blastocyst of another mouse. The blastocyst is allowed to develop in the uterus of a female mouse. The resulting chimera is mated with a wild-type mouse, and after two generations, a mouse homozygous for the gene knockout can be obtained. However, loss of genes that are critical for development can lead to embryonic lethality. As mice containing embryonic lethal constructs do not come to term, the use of these model systems is limited to studies of the embryonic fibroblasts [96, 97].

The technique for gene overexpression is somewhat less complex. In this case, the construct containing the gene or mutation to be overexpressed can be injected into a singe cell of the mouse embryo. The construct is then randomly incorporated into the genome, and mice expressing the construct will begin to express the protein at higher levels depending on the integration locus and the number of copies integrated. One particular drawback of these types of overexpression systems is the propensity for artificially high protein overexpression [98]. In these cases, the high overexpression can lead to non-physiological responses that do not recapitulate human disease and therefore cannot provide translatable results.

To circumvent some of these difficulties, scientists have made use of tissue-specific and inducible gene expression systems. By expressing the gene of interest under the control of a tissue-specific promoter, researchers can restrict the expression of their target gene to a single tissue or cell type. In cardiac research, the α-myosin heavy chain (αMHC) promoter has been used extensively to restrict gene expression to cardiomyocytes [43]. In so doing, genes that may have been embryonic lethal when overexpressed or knocked out ubiquitously can be assessed for potentially nonfatal, cardiac-specific phenotypes.

Temporal regulation of gene expression can be achieved by using inducible constructs. Various methods have been developed to this end, the most common of which are the Cre/Lox and tetracycline methods. In the case of the Cre/Lox system, when mice overexpressing Cre-recombinase are mated to mice with a gene cassette containing the gene of interest downstream of a stop sequence flanked by two LoxP sites, the Cre-recombinase recognizes the LoxP sites and excises the "floxed" stop sequence allowing for expression of the gene of interest. This method can also be used to for gene knockout by placing the LoxP sites on either end of the gene of interest directly [99]. If a tissue-specific promoter drives the expression of the Cre-recombinase, the overexpression of the gene of interest will also be spatially regulated.

However, in many cases, it is beneficial for the regulation of a certain gene to be reversible. In these cases, the use of Tet-On and Tet-Off systems are highly beneficial [100]. The basis of tetracycline-mediated gene expression is the presence of a gene cassette containing an upstream tet-responsive element that regulates target gene expression depending on the presence or absence of a specific antibiotic, typically tetracycline itself or one of its derivatives (e.g. doxycycline (Dox)). Tet-On systems activate gene expression in the presence of Dox, while Tet-Off systems activate gene expression in the absence of Dox. Simply by removing or adding the antibiotic, it is possible to temporally regulate gene expression. Furthermore, utilizing this technology in a tissue-specific Cre-mediated background allows researchers to reversibly modulate gene expression both spatially and temporally.

In sum, the vast array of experimental techniques for studying the cardiovascular system that has been developed in the recent decades has afforded researchers the opportunity to discover many of the molecular drivers of cardiac development and to begin to determine the molecular drivers of pathology. While these insights have

aided in the development of novel pharmaceutical therapies, complete functional recovery remains elusive. Today's therapies function to decrease the symptoms of cardiac dysfunction, slow disease progression, and can indeed prolong life, but they fail to address the problem of cardiomyocyte loss directly. The next paradigm of cardiovascular medicine will undoubtedly be characterized by therapies that enhance cardiomyocyte proliferation and result in complete cardiac regeneration.

5 Cardiac Regeneration

Recent advances in cardiovascular science and the development of various animal models of cardiac regeneration have given researchers a new insight into the natural biology of cardiac development. We have uncovered an innate regenerative capacity of the mammalian heart and in so doing have elucidated a novel prospect for direct therapeutic intervention. It is therefore essential to understand the magnitude, dynamics, and mechanisms governing this regenerative response, whether or not it can be enriched, and whether or not enrichment is clinically beneficial.

In this chapter I will introduce the concept of cardiac regeneration beginning from the early discoveries in non-mammalian vertebrates. I will then discuss modeling cardiac regeneration in neonatal mice, and conclude with a summary of the recent reports describing cardiac cell turnover in humans.

5.1 Cardiomyocyte Renewal in Mammalian and Non-Mammalian Vertebrates

Despite the seemingly insurmountable insult to the organism arising from cardiomyopathy, many species have developed methods to overcome cardiovascular injury and completely regenerate the heart muscle. Studies regarding the regenerative capacity of amphibians date back to the mid 18th century when Spallanzani described limb regeneration. Since then scientific curiosity surrounding this remarkable ability has grown significantly, and resulted in a deeper physiological, cellular, and molecular understanding of the regenerative process. It is no surprise then that the first evidence of cardiac regeneration was observed in early amphibian studies. Ventricular injury resulted in DNA synthesis concomitant with karyokinesis in

newts, frogs, and axolotls, and cardiac regeneration after cardiac resection was reported for the first time in 1974 [101-103]. Most of the cardiac regeneration studies in amphibians showed reactivation of cardiomyocyte proliferation, but incomplete restoration of cardiac function. However in 2011, a smaller injury performed by puncturing the heart wall with a hypodermic needle (as opposed to the earlier amputation studies) showed complete recovery [104]. A large-scale, systematic study of the effects of injury severity on regenerative capacity in amphibians will be necessary to discern whether or not these discrepancies in regenerative capacity are merely due to the degree of injury. However, despite these slight regenerative limitations, it is clear that the capacity for self-renewal in amphibians far outreaches that of humans. These first newt studies successfully illustrated the phenomenon of endogenous cardiac regeneration, and prompted scientists to investigate the conservation of this regenerative capacity in non-mammalian and mammalian species alike. Today, the use of amphibious species for cardiac research is often limited by the availability of genetic and molecular tools. It is likely for this reason that researchers have turned to more genetically malleable species such as the mouse and zebrafish.

5.1.1 Cardiac Regeneration in Zebrafish

The zebrafish heart is only comprised of one atrium and one ventricle making it a much simpler system than the mammalian heart. It is approximately 1 mm^3 and blood-flow is unidirectional (**Figure 9A**). However, its histological composition is similar to mammals, making it a suitable model organism for translational cardiac research (**Figure 9B**). Similar to amphibian species, adult zebrafish have a remarkable capacity to regenerate many organs through various, organ-specific mechanisms [105]. For example, fin regeneration depends on the formation of a blastema at the injury site composed of highly proliferative, pluripotent, dedifferentiated cells, while telencephalon regeneration in the brain is blastema-independent and instead relies on the mobilization of the resident progenitor cell population via Notch signaling [106]. Studies in the teleost fish *Danio rerio* have proven that zebrafish are able to completely regenerate their hearts after cardiac injury [107], and recent studies have shown that regeneration is driven primarily by parenchymal cell dedifferentiation and proliferation [108, 109]. Interestingly, not all teleost species maintain this regenerative capacity [110], but in adult zebrafish (aged 1 to 2 years), 20% of the

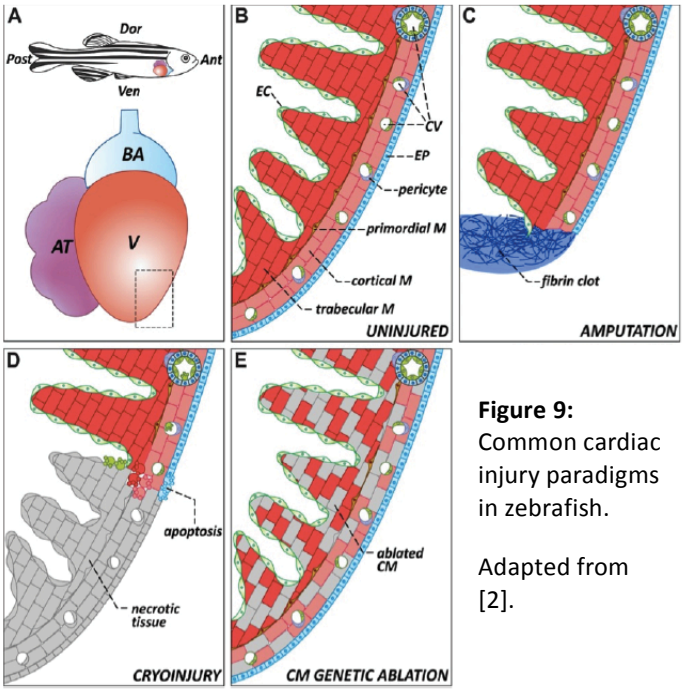

Figure 9: Common cardiac injury paradigms in zebrafish.

Adapted from [2].

ventricular myocardium can be surgically removed from the heart's apex with no discernable cardiac dysfunction after 60 days of recovery [107]. Today numerous studies have been performed in attempts to determine the molecular mechanisms governing the regenerative events in the zebrafish heart, and various cardiac injury model systems have been designed to that end.

5.1.1.1 Apical Resection Model

In 2002 Poss and Keating first illustrated the ability of the adult teleost *Danio rerio* to completely regenerate its heart by surgically removing ~20% of the heart apex and allowing the fish to recover naturally [107]. Profuse bleeding occurred directly upon resection, but was quickly quelled by the formation of a large fibrin clot after a few seconds (**Figure 9C**). In as little as 7 days post-amputation (dpa), zebrafish behavior was indistinguishable from control animals. The fibrin clot was replaced by new myocardium in the first weeks after amputation, and there was a significant increase in ventricular surface area between 0 and 30 dpa [107]. Increased BrdU incorporation suggests that this regenerative response is, at least in part, due to cardiomyocyte hyperplasia, and genetic fate-mapping [109] studies have since verified that the primary effectors in this recovery are the remaining cardiomyocytes themselves via a Gata4-dependent proliferative mechanism. The most remarkable characteristic of heart regeneration in the zebrafish using this injury model is its lack of scar formation. It has been suggested however that, as ventricular resection is based on tissue removal rather than cell death, scarring does not occur because there is no cell debris to be removed [2].

5.1.1.2 Cryoablation Model

Poss and Keating's ventricular resection model quickly became the gold standard for zebrafish cardiac regeneration studies, and is still used by many researchers today. It was not until 2011 that the first alternative approach to apical resection was attempted to further challenge the regenerative capacity of the zebrafish heart. Chablais et. al. among others employed cryoinjury to study the regenerative response after a more severe insult to the cardiac system [111-113]. In this disease paradigm, a precooled metal filament is used to freeze a portion of the ventricular surface, resulting in necrosis of the contacted cells and subsequent apoptosis of the cells surrounding the necrotic area (**Figure 9D**). This injury model is more reminiscent of human MI, and the apoptotic response affects all cell types, including the epicardium, endocardium, and the coronary vasculature.

As opposed to the apical resection model, which shows no evidence of scar formation, cryoinjury results in transient scarring. Shortly after injury, inflammatory cells infiltrate the necrotic area, and the presence of activated myofibroblasts contributes to the deposition of fibrotic tissue. However, as opposed to the permanent scarring observed in mammalian cardiac injury, the fibrotic scar in the zebrafish is cleared within 3 to 4 months post injury [2]. The concomitant recovery of the epicardium and endocardium creates a suitable microenvironment for cardiomyocyte proliferation and vasculogenesis. This suggests that in zebrafish, while some ventricular remodeling may occur, ventricular scarring is not an insurmountable hindrance to the restoration of cardiac functionality.

5.1.1.3 Genetic Cardiomyocyte Ablation Model

Recently, more non-invasive cardiac injury strategies have been devised involving inducible genetic systems to ablate cardiomyocytes via cardiomyocyte-specific expression of enzymes or cytotoxins. One such strategy exploits the catalytic activity of the bacterial nitroreductase (NTR). Curado and colleagues expressed NTR specifically in cardiomyoctyes by placing it downstream of the cardiomyocyte light chain (*cmlc2*) promoter [114, 115]. NTR is not toxic in itself, but it is able to catalyze the pro-drug metronidazole (Mtz) to a cytotoxic metabolite. Thus, when Mtz is added to the system water, it is taken up by the fish and reduced to a cytotoxic agent specifically in the cardiomyocytes of the fish expressing NTR. In this way, only cardiomyocytes are ablated while surrounding cells remain unharmed (**Figure 9E**). Despite the insult the cardiac system, there is little evidence of scarring. This system offers temporal control of cardiomyocyte ablation, as the Mtz can be added and removed from the water at will. This method is especially beneficial for studies of cardiac regeneration during zebrafish development, as the embryonic zebrafish heart is considerably smaller rendering mechanical manipulations impractical.

Another genetic ablation model, more targeted towards adult zebrafish, makes use of cardiomyocyte-specific expression of diphtheria toxin chain A (DTA) [116]. DTA expression results in diffuse ablation of ~60% of all cardiomyocytes. The fish are able to tolerate this loss of cardiomyocytes, but they develop signs of heart failure. Remarkably, the injured hearts are completely regenerated after ~30 days with no apparent scarring.

Because the genetic ablation models affect cardiomyocytes specifically, these disease paradigms are often considered to be more reminiscent of advanced cardiomyopathy than an MI. Additionally, because the injuries are chemically based, the severity of the injury can be more easily titrated and the injury itself is much more reproducible in large animal cohorts.

5.1.2 Cardiac Regeneration in Small Mammals

Both mature non-mammalian vertebrate hearts and embryonic mammalian hearts exhibit a two-chambered organization with single-circulation and contain small cardiomyocytes, which are primarily mononucleated [117]. The implications of these similarities were highlighted in 2008 when Cox and colleagues described the embryonic heart's ability to compensate for a 50% reduction of tissue via hyperproliferation of healthy cardiac cells [118]. This finding not only elegantly illustrated the regenerative capacity of the fetal heart, but also indicated that the factors conducive to cardiomyocyte cell-cycle reentry may exhibit differential expression with age. It has been suggested that these cardiogenic factors comprise a fetal genetic program for cardiogenesis that can be reactivated upon cardiac injury [119].

However, the mammalian regenerative response is not completely lost after birth. Neonatal mice possess a 7-day window for postnatal cardiac regeneration [120]. After 15% resection of the left ventricular apex of 1-day-old mice, progressive regeneration results in restoration of the resected myocardium within 21 days, with normal

ejection fraction and systolic function present after 2 months [120]. However, this regenerative capacity is only maintained up to postnatal day 7, and survival rates are decreased in older mice. Ligation of the LAD [121, 122] and cryoinjury [123] in neonatal mice also result in myocardiogenesis, although only nontransmural cryoinjury results in complete myocardial repair [124]. Additionally, Senyo and colleagues have shown that cardiomyocyte renewal exists at a low rate even in adult mice. By administering the non-toxic thymidine analog ^{15}N-thymidine, and using multi-isotope imaging mass spectrometry, they show that cardiomyocytes are exchanged at a rate of 0.76% per year in young adult mice and at lower rates in older animals. Of note, cardiomyocyte renewal was increased after MI, suggesting activation of regenerative pathways upon injury [125].

5.2 Cardiomyocyte Renewal in Humans

Cardiomyocyte renewal in humans is a more challenging phenomenon to investigate. Studies in animal model systems are able to use radiolabeled nucleotide analogs and various genetic modifications that cannot be readily adapted for human studies [126]. Early evidence for postnatal cardiomyocyte cycling in humans came from observed instances of mitotic segregation in postmortem studies of children with diphtheria in the 1920s and 30s [127, 128]. We now know that the mere appearance of mitotic events does not necessarily preclude cardiomyocyte division [129-132], but this finding suggested an innate cell-cycle activity that wanes, or is completely lost, later in life. Later studies have suggested that cardiomyocyte proliferation is the primary contributor to heart growth in infants and adolescents [133], and studies using the thymidine analog iododeoxyuridine (IdU) have suggested that this proliferative capacity is maintained in adulthood [134]. Thus, an understanding of the magnitude and mechanism of this phenomenon could help develop future therapeutics.

However, as in mice, the controversy surrounding the magnitude and dynamics of cardiomyocyte turnover in the human heart has been entertaining the scientific community for over a decade [135-137]. Likely due to difficulties differentiating cardiomyocyte proliferation events from multinucleation or polyploidization, studies of cardiac cell-cycle dynamics have yielded markedly inconsistent results. To provide more concrete evidence supporting the existence of cardiomyocyte renewal in humans, researchers have exploited the presence of radioactive carbon isotopes (^{14}C) in nuclear DNA. By adapting conventional radiocarbon dating techniques, cardiomyocyte turnover has been verified in adult humans. This technique has also been used to determine the dynamics of cardiomyocyte and non-cardiomyocyte renewal [138].

Radiocarbon Dating

6

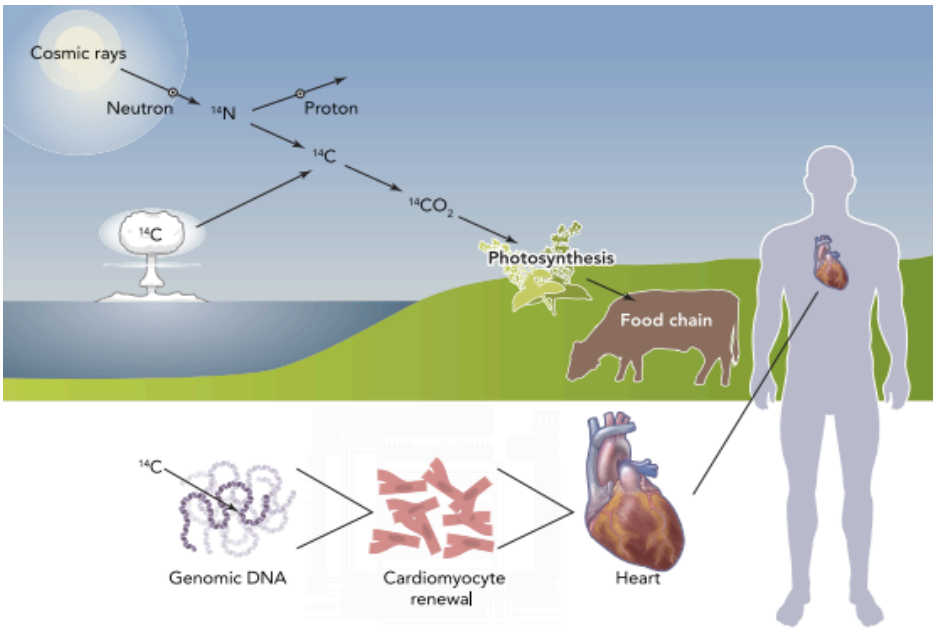

Figure 10: The carbon cycle depicting the contribution of ^{14}C from above ground nuclear bomb testing. The atmospheric ^{14}C is incorporated into genomic DNA during cell renewal. Image adapted from [139]

The use of the radioactive carbon isotope ^{14}C to determine the age of biological specimens is not a new concept. Willard Libby first described the technique in the late 1940s as a method to determine the age of biological samples by determining the level of ^{14}C radioactive decay [140]. Since then this technique has become the gold standard for dating ancient biological samples, and recent innovations have extended the use of this technique to tissue specimens and even cell populations.

In this chapter I will introduce the concept of ^{14}C dating and describe how it has been combined with accelerator mass spectrometry to quantify cell turnover in postmortem human heart tissue samples. I will then provide a brief summary of the magnitude and dynamics of cardiomyocyte and non-cardiomyocyte cell turnover in humans.

6.1 The Carbon Cycle

The utility of radiocarbon dating stems from the incorporation of atmospheric ^{14}C into the carbon cycle. The ^{14}C isotope is formed when cosmic rays, composed primarily of heavy atomic nuclei and high-energy protons, bombard the atmosphere's diatomic nitrogen ($^{14}N_2$). This liberates a proton and creates ^{14}C according the following (simplified) equation:

$$\mathbf{^{14}_{7}N + ^{1}_{0}n \rightarrow ^{14}_{6}C + ^{1}_{1}p}$$

Where *n* represents an unstable free neutron contained in the cosmic ray, and *p* the resulting proton. Although ^{14}C is an unstable carbon isotope, it is still able to interact with atmospheric oxygen (O_2) to form radioactive carbon dioxide ($^{14}CO_2$). The resulting $^{14}CO_2$ is incorporated into the biosphere via photosynthetic plants, and bioamplification of the radioactive element occurs through consumption. The ^{14}C an organism consumes reaches a steady state with the atmospheric ^{14}C until the organism dies and ceases exchanging carbon with its environment. As the remaining ^{14}C decays the sample's age can be determined by measuring the amount of ^{14}C in a biological sample, relating it to the level of atmospheric ^{14}C and adjusting for the ^{14}C rate of decay (**Figure 10**).

6.2 Retrospective Birth Dating Using Above-Ground Nuclear Bomb Tests

The long half-life of ^{14}C (5,740 years) typically does not allow birth dating of tissue samples, but the dramatic increase of atmospheric ^{14}C levels from above-ground nuclear weapons testing during the 1950s and 1960s afforded researchers a new level of sensitivity, since a small time differential corresponded to a large change in ^{14}C concentration [141, 142]. One of the first applications of the radiocarbon bomb pulse for medical research purposes was performed by Mok et. al. in 1986 using gallstones collected from human subjects [143]. In their study they were able to deduce the birth dates of each sample by comparing the ^{14}C concentration of the samples to the ^{14}C bomb curve. However, since this first application, both the carbon isolation and isotope counting techniques have improved dramatically. Decades of advances in DNA isolation techniques and the recent adaptation of accelerator mass spectrometry (AMS) technologies have decreased the required amount of starting material by 1 million-fold, from the order of grams to the order of micrograms [144].

To understand the utility of radiocarbon dating of biological material one must be acquainted with the fundamentals of the carbon cycle. The exchange of carbon between an organism and the atmosphere extends even to the organism's DNA and the atoms contained therein. During each cell division, new carbon atoms are incorporated into the cell's DNA. The ^{14}C integrated into the genomic DNA reflects the level of ^{14}C in the troposphere at the time of that cell's formation. Therefore, analysis of the ^{14}C content in the nuclear DNA of a cell population can give a precise date for the genesis of that population [145-147]. Carbon exchange within the genomic DNA ceases when the cell stops dividing. It is this crucial consideration that allows for retrospective birth dating of human cells. When a cell ceases to divide, and no more

carbon exchange occurs, the ^{14}C contained therein begins to decay and the genesis of that cell can be determined.

6.3 Evidence for Cardiomyocyte Renewal in Humans

To date, the radiocarbon dating technique has been used to determine the extent of postnatal DNA synthesis in numerous cell types [148, 149] including in the myocardial cells of the left ventricle, and specifically in cardiomyocytes [136, 138]. In heart tissue from subjects born up to 22 years before the onset of the nuclear bomb tests, the elevated "post-bomb" ^{14}C concentrations illustrated the turnover of cardiac cells [136]. However, as cardiomyocytes constitute only 20% of all cells in the human myocardium, markers against the nuclear localized cardiac troponins (TnI, TnT) [136] or pericentriolar material 1 (PCM-1), a perinuclear myocyte marker [150], were used to specifically identify cardiomyocyte nuclei [151-154]. The study showed that the cardiomyocyte DNA was indeed younger than the individual. Furthermore, since the proportion of multinucleated cardiomyocytes remains at a constant 25% throughout life [138] and the isolated diploid cardiomyocyte nucleus population was also younger than the individual, *de novo* nucleus formation was the only plausible explanation for the changes in ^{14}C concentration.

6.4 Dynamics of Cardiac Cell Turnover in the Healthy Human Heart

The radiocarbon dating technique has also been used in conjunction with design-based stereology and flow cytometry techniques to develop a comprehensive characterization of the natural history of cardiomyocyte generation and turnover [138]. Stereology techniques have shown that cardiomyocyte nuclear density decreases 15-fold shortly after birth and have corroborated the previous finding that the nucleation ratio does not change substantially during heart growth or aging. Interestingly, the final number of cardiomyocytes (3.2 ± 0.75 billion cells) is reached already 1 month after birth. These data are in accordance with early stereological studies showing no further increase in cardiomyocyte cell number after the perinatal period [155]. The radiocarbon data revealed that human cardiomyocytes are on average 7 years younger than the individual.

Fitting the radiocarbon data to mathematical models describing turnover rates can be used to establish the dynamics of cell generation and renewal [148, 149]. The model giving the best fit to the cardiomyocyte data from healthy individuals allowed for turnover rates to change with time in an inverse linear declining manner. The model predicts a renewal rate of 1% per year at age 25, which declines to < 0.5% by 70 years of age. Interestingly, in adult humans, 40% of the cardiomyocytes present in the left ventricle have actually been generated after birth, but only 3% of these were generated after the age of 10. Although the birth rates of cardiomyocytes are highest during the perinatal period and in pediatric hearts, ^{14}C-based turnover data support our stereological analysis, suggesting a constant number of cardiomyocytes from birth to death [138].

7 Present Investigation

The primary aims of the present investigation were to develop a better understanding of the mechanisms governing cardiac dysfunction, specifically those pertaining to dilated cardiomyopathy and heart failure. We also aimed to investigate the regenerative capacity of the pathological heart, in an attempt to uncover novel regenerative pathways.

Secondary aims included optimizing cardiomyocyte isolation, culture, and functional characterization techniques, to be able to develop *in vitro* systems for hypothesis testing, and to determine key players in cardiac pathogenesis and the cardiac regenerative response.

7.1 PAPER I – Isolation, Culture, and Functional Characterization of Adult Mouse Cardiomyocytes

7.1.1 Introduction

The lack of adequate cell lines reflecting the structure and function of adult cardiomyocytes has been a significant limitation in the development of *in vitro* modeling systems for cardiovascular disease. Primary neonatal cardiomyocytes have been used successfully to illustrate vital signaling pathways and to test the effects of genetic manipulation, but their immature cytoskeletal structure and markedly different subcellular organization from adult cardiomyocytes renders them inadequate for the investigation of ECC or ion fluxes. It is for this reason that researchers have turned to isolated adult cardiomyocytes, as these cells maintain the complex cytoskeletal structure seen in intact tissue samples and are electrically excitable, allowing for much more physiologically relevant studies of electrochemical dynamics, excitation-contraction coupling, and molecular signaling cascades.

Adult rat ventricular cardiomyocytes have been used extensively to this end, but because of the relative ease of transgenic manipulation, the adult mouse has gained much more popularity. However, the isolation of AMVMs has proven problematic, with non-standardized isolation methods yielding widely differing levels of cell purity and stability. Furthermore, few studies have reported clear cell culture or gene delivery protocols for AMVMs, or provided full methods for the functional characterization of ion fluxes and calcium transients. Therefore, in **PAPER I** we present detailed methodologies for the isolation, culture, adenoviral transfection, and functional analysis of adult mouse ventricular cardiomyocytes.

7.1.2 Summary of Results

The methods presented in this report result in Ca^{2+}-tolerant, excitable adult ventricular cardiomyocytes. The isolated cells maintain the expected rod shape and are amenable to electrical field stimulation. We show that we are able to culture them for up to 72 hours and transiently transfect them using an adenovirus containing a GFP construct. We further provide detailed methods for contractile and Ca^{2+} transient analysis, whole-cell patch clamp, and present representative graphs depicting a typical AMVM AP, calcium transient, and contractility trace.

The basis for this procedure is retrograde perfusion of the adult murine heart *ex vivo* with a specialized enzyme buffer containing collagenases B and D, and protease XIV from *Streptomyces griseus*. After the mouse is anesthetized, the heart is extracted ensuring that the aorta remains visible. The heart is then mounted onto a Langendorff Apparatus by pulling the aorta around a non-hypodermic, blunt end needle. After securing the heart to the needle with a small length of suturing silk, the heart is lowered into a temperature-regulated conical glass and perfused first with a isotonic perfusion buffer and then with an enzyme buffer. The heart is perfused for 5 min at a rate of 1mL/min with perfusion buffer, and then the glass chamber is blocked to allow enzyme perfusate to envelop the heart. As the enzyme perfuses the heart it

digests the connective tissue and results in a paler tissue color and decreased tissue integrity. After sufficient enzyme perfusion (~7-10 min), the ventricles are cut away from the hanging heart, minced, filtered to remove large tissue remnants, and subject to calcium equilibration.

The resultant cell suspension can be cultured on glass coverslips that have been pre-coated with natural mouse laminin. The desired number of cells are resuspended in plating warm media whose dissolved CO_2 has been equilibrated to 2%. The cells are allowed to attach for at least 30-60 minutes in an incubator set to 37^{o}C and 2% CO_2. If adenovirus is to be transfected, the appropriate amount can be added directly to the plating media and incubated with the cells for 2 hours. The plating media is then removed and replaced with culture media. If the media is changed each day the cells can be cultured for up to 72 hours using this protocol.

If the aim is to record contractile and calcium transient measurements, we have provided a protocol for the use of the MMSYS system. After proper start-up of the system hardware, the system should be primed with calcium buffer. A coverslip is placed in the flow chamber, and this is where the cells are visualized. Fura2-AM is applied to a 500µL aliquot of cell mixture and allowed to incubate at room temperature for 5-7 minutes. An appropriate number of cells are then dropped onto the center of the chamber and allowed to settle. Once settled, the calcium buffer flow is initiated and the contractility and calcium transient measurements can be taken using the MMSYS software.

Patch clamp recordings are performed using a fire-polished pipette made of borosilicate glass. Cardiomyocytes should be cultured on glass coverslips for this procedure, but attachment to collagen- or laminin-coated coverslips will also allow the use of fresh cells. In either case, coverslips are washed with PBS and placed in the perfusion chamber of the inverted microscope. The patch pipette is back-filled with intra-cellular buffer and attached to the pipette holder. The pipette is lowered into the cell bath and placed in close proximity to the AMVM. Mouth pressure is used to establish negative pressure on the cell surface to obtain a gigaseal. Once the gigaseal is obtained, the resting membrane potential can be measured using current clamp with I=0 pA. Once whole-cell patch mode is established, 15 minutes of equilibration is required before beginning voltage or current clamp protocols.

7.1.3 Discussion

Though protocols for AMVM isolation have been previously reported, and there exist numerous studies using isolated AMVMs as a model system, in many cases the technical minutia of the isolation procedure have been left out of the methods section. It is thus of utmost importance that researchers are provided with a complete and comprehensive protocol for AMVM isolation, so that healthy cells can be obtained reproducibly and to ensure high quality experimentation. The aim of our report was to be as thorough as possible in the description of our isolation, culture, and functional characterization methods, to provide investigators with a clear procedure that can be followed step-by-step. We were also interested in describing

potential pitfalls of the procedure, in offering cautionary instructions, in providing helpful tips based on experience, and in giving examples of common mistakes. We believe that this level of detail should always be provided in any methods paper, to ensure that results are reproducible, and to make scientific experimentation more effective and efficient.

7.2 PAPER II – Development of dilated cardiomyopathy and impaired calcium homeostasis with cardiac-specific deletion of ESRRβ

7.2.1 Introduction

While many genetic mutations, frequently occurring in the large sarcomeric protein titin, have been linked to the development of idiopathic DCM (iDCM), the molecular triggers of DCM pathogenesis are still largely unknown. One important hallmark of DCM and heart failure is metabolic dysfunction. Notably, mutations in vital components of mitochondrial function lead to DCM [156-158], highlighting the importance of cardiac oxidative metabolism in disease progression.

In **PAPER II** we performed a gene expression screen in various oxidative tissues and cell populations to identify novel transcriptional regulators of cardiac oxidative metabolism. We have found estrogen-related receptor β (ESRRβ) to be highly expressed in conditions of enhanced metabolic activity, and shown that mice lacking cardiac ESRRβ develop DCM in mid-life. We further show that human heart tissue samples from iDCM patients lack nuclear ESRRβ expression, as opposed to samples from patients with other forms of cardiomyopathy, suggesting a role for human ESRRβ specifically in DCM pathogenesis.

7.2.2 Summary of Results

Using a high-throughput qPCR screen containing ~2000 transcription factors (TFs), we compared the expression of TFs in: (1) slow twitch soleus muscle vs. fast twitch quadriceps muscle, (2) quadriceps vs. cardiac muscle, (3) cultured PGC-1α overexpressing myotubes vs. GFP-only expressing myotubes, and (4) quadriceps muscle from transgenic PGC-1α overexpressing mice vs. littermate controls. Five TFs were significantly enhanced in the more oxidative alternative in all four cases: PGC-1α itself, PPARα, FHL2, HMG20b, and ESRRβ. ESRRβ was the most highly regulated TF after PGC-1α, as confirmed by qPCR, and the heart showed the highest ESRRβ expression.

A cardiac-specific knockout mouse was generated to determine the role of ESRRβ in the heart. Floxed ESRRβ mice were mated to mice containing an α-MHC driven Cre-recombinase to create the cardiac-specific ESRRβ knockout mice (MHC-ERRB KO). The MHC-ERRB KO mice showed a 95% reduction in cardiac ESRRβ with no loss in the skeletal muscle or in the kidneys. They were born at expected Mendelian ratios, exhibited no gross congenital defects, but had poor survival rates after 9-10 months of age. Postmortem analysis of the 9-10 month old hearts revealed significant enlargement with increased fibrosis, and qPCR analysis showed an increase in genetic markers of heart failure. Noninvasive echocardiography (ECHO) revealed significant

DCM as determined by increased anterior wall thickness, increased LVESD and LVEDD, and a 60% decrease in FS. No evidence of overall cardiac dysfunction was observed at earlier time points, but disruptions in contraction occurred at 6 months and, irregular calcium handling occurred already at 4 months.

Finally, to test whether or not the subcellular localization of ESRRβ is altered in human disease, human heart tissue samples were obtained from patients diagnosed with iCM, HCM, iDCM, and ARVC. Interestingly, nuclear ESRRβ expression was completely absent only from the samples from the iDCM patients. We did not, however, observe any significant decrease in ESRRβ mRNA expression, suggesting posttranscriptional regulation.

7.2.3 Discussion

Aside from the electrical and mechanical remodeling that occurs upon the onset of heart failure, late-stage heart failure also presents with defects in oxidative metabolism. Because the heart is such a highly metabolic organ, disruption of any aspect of the oxidative phosphorylation machinery can lead to cardiomyopathy. In fact, mutations in many vital components of mitochondrial function have been shown to lead to DCM [156-158]. In this report we have identified ESRRβ as one such vital component, whose absence in the heart leads to late-onset DCM.

Cardiac metabolic function is essential for maintaining the proper contraction of the heart. PGC-1s and many of their downstream effectors, including members of the estrogen-related receptor (ESRR) family of orphan receptors, have emerged as vital regulators of cardiac metabolism. The three ESRRs (α, β, and γ) bear significant homology to the estrogen receptor, but do not bind estrogen themselves. While little is known about the role of ESRRβ in the heart, ESRRα is considered to be a key regulator of mitochondrial biogenesis [159], and germline deletion of ESRRγ results in postnatal lethality due to cardiac dysfunction [160]. Germline deletion of ESRRβ is also lethal, but lethality occurs already at the embryonic stage due to abnormal placental formation [161]. The data presented in this report suggest a role for ESRRβ in calcium homeostasis preceding disease onset, but more comprehensive studies will be necessary to divine the mechanism by which ESRRβ loss leads to impaired calcium handling.

7.3 PAPER III – Pathological Role of Serum- and Glucocorticoid-Regulated Kinase 1 in Adverse Ventricular Remodeling

7.3.1 Introduction

Heart failure is associated with altered cardiomyocyte electrical characteristics, including the prolongation of the action potential duration (APD), which can exacerbate the symptoms of adverse mechanical remodeling and even lead to fatal arrhythmias. It has been shown that the persistent (late) sodium current (I_{Na} or I_{NaL}) can contribute to APD prolongation [162], but the mechanisms governing this activity are unclear. It will thus be necessary to fill this knowledge gap to develop a more

comprehensive understanding of the electrical and mechanical remodeling concomitant with heart failure.

In this context, serum- and glucocorticoid-regulated kinase 1 (SGK1) is of particular interest. SGK1 is a member of the serine/threonine family of kinases, is involved in the phosphatidylinositol 3-kinase (PI3K) signaling pathway, and has been shown to regulate sodium ion transport in the kidney [163, 164] and in other heterologous expression systems [165]. The PI3K signaling pathway has been shown to promote cardiomyocyte survival, but proximal PI3K signaling is also enhanced in patients with cardiac dysfunction and heart failure. This paradoxical activity of the PI3K signaling pathway has raised the possibility that, while its acute activation is initially cardioprotective, prolonged activation may become maladaptive and contribute to the adverse ventricular remodeling commonly associated with heart failure. Acute activation of SGK1 has been shown to promote cardiomyocyte survival after TAC in mice. However, in **PAPER III** we have demonstrated that SGK1 is also persistently activated after TAC as well as in human heart disease. We show that chronic activation induces hallmarks of both adverse mechanical and electrical remodeling, echoing previous reports describing cardiac dysfunction upon sustained PI3K signaling. Based on these findings, we expect that inhibition of chronic SGK1 activation may provide a novel avenue for heart failure treatment, especially in patients with a history of cardiac arrhythmia. Indeed, aspects of adverse remodeling could be reversed with treatment of the late sodium current inhibitor, ranolazine.

7.3.2 Summary of Results

In this report we show that cardiac SGK1 remains elevated in TAC heart failure models. We did not, however, observe any changes in phosphorylated (pSGK1) or total SGK1 levels in an exercise model of physiological hypertrophy, suggesting that the observed elevation of SGK1 levels corresponds to pathological remodeling. Total SGK1 levels were also elevated in human heart tissue samples from patients with hypertensive heart disease, and pSGK1 levels were increased in DCM samples with no change in total SGK1.

The functional role of SGK1 in the heart was tested using transgenic mouse lines with cardiac-specific expression of either a constitutively active or dominant negative form of SGK1 (SGK1-CA, SGK1-DN). The baseline SGK1 kinase activity of the SGK1-CA mice was comparable to that seen in the hearts of mice with TAC-induced heart failure (TAC-HF), and the baseline kinase activity in the SGK-DN mice was no different than that seen in WT littermates. However, after TAC, the rise in SGK1 activity seen in the WT mice was absent in the SGK1-DN mice. No gross changes in cardiac structure were observed in young adult mice (3-6 months) of either genotype, but SGK1-CA mice presented with mild, but significant, systolic and diastolic dysfunction.

When these mice were subjected to TAC, there was a significant increase in the heart-weight-to-body-weight ratio (HW/BW) in both the SGK1-CA mice and WT littermates, but the SGK1-CA hearts were dilated, had thinner walls, and exhibited reduced cardiac functionality. The SGK1-DN mice tolerated the TAC to a similar extent as the WT mice, though the HW/BW was less pronounced in the SGK1-DN seven weeks after

TAC. Furthermore, also after seven weeks, ECHO revealed decreased FS and increased LV dilation in the WT but not in the SGK1-DN hearts. SGK1-DN mice also showed less fibrosis than WT littermates.

Because SGK1 is known to interact with ion channels, we next investigated the electrophysiological effects of chronic SGK1 activation. SGK1-CA mice showed increased R-wave amplitude, QRS duration, and QT_c interval, with no change in RR or PR intervals. Ventricular tachycardia was also reported at 12-months. SGK1-DN ECGs were no different from WT mice, and no ventricular arrhythmias were observed. Whole-cell patch clamp of SGK1-CA cardiomyocytes from young adult mice showed AP prolongation as compared to WT littermates. Early and delayed after-depolarizations were also more frequent in SGK1-CA mice.

Based on the reasoning that prolonged APD is likely secondary to alterations in the peak calcium, potassium, or sodium currents (I_{Ca}, I_{to}, I_{K1}, or I_{Na}) we next performed whole-cell patch clamp on isolated cardiomyocytes from SGK1-CA mice or WT littermates to investigate ion current densities. We found no changes in I_{to}, I_{K1}, I_{Ca}, but observed substantial changes in I_{Na}. SGK1-CA mice exhibited a 3.6-fold increase in the persistent sodium current I_{NaL} compared to WT littermates, which is comparable to that seen in arrhythmogenic *SCN5a* mutations or in acquired HF [166, 167]. Blocking I_{NaL} with low concentrations of ranolazine normalized APD in SGK1-CA without affecting WT APD, and reduced the number of SGK1-CA cardiomyocytes with after-depolarizations. Furthermore, *in vivo* ranolazine treatment of SGK1-CA mice reduced the incidence of lethal ventricular arrhythmias after I/R injury, and showed a trend towards improved FS, with no difference in placebo-treated animals.

In order to understand the molecular basis for the changes in I_{Na}, we next explored the effects of SGK1-CA on $Na_v1.5$, the primary pore-forming subunit of the cardiac voltage-gated sodium channel complex. While there were no changes in total $Na_v1.5$ protein expression in the SGK1-CA or SGK1-DN mice, the subcellular localization of $Na_v1.5$ was altered in both TAC-HF as well as in SGK1-CA mice, but not in sham-operated WT or TAC'd SGK1-DN mice. In sham-operated WT mice most of the Nav1.5 was localized to lipid rafts, with small amounts localized to the heavy membrane (HM) fraction. Conversely, TAC-HF and SGK1-CA hearts both showed increases in $Na_v1.5$ in the HM fraction. Of note, the amount of HM $Na_v1.5$ was markedly decreased in SGK1-DN mice following TAC as compared to TAC'd WT littermates. We also found that the increased HM $Na_v1.5$ was consistent with increased membrane availability due to decreased binding to Nedd4-2. Nedd4-2 is a ubiquitin ligase that has been shown to bind and decrease the surface expression of the epithelial sodium channel E_{NaC} in renal tubular cells in response to SGK1 activation [163]. Nedd4-2 binding was preserved in SGK1-DN mice after TAC.

The altered binding to Nedd4-2 could potentially explain the global increases in I_{Na}, but not the changes in channel gating and kinetics. To investigate alternative contributors, we also tested whether or not SGK1 interacts directly with $Na_v1.5$. Immunoprecipitation assays revealed that endogenous SGK1 does bind to cardiac sodium channels, but perhaps not only to $Na_v1.5$. However, using a peptide library,

we determined the preferred SGK1 phosphorylation target and have identified putative consensus sites on the *SCN5a* gene, suggesting a physical role for SGK1 in $Na_v1.5$ regulation.

7.3.3 Discussion

It has been established that proximal PI3K activation is maladaptive in heart failure, and here we present chronic activation of SGK1 as a vital player in the associated electrical remodeling. By demonstrating its effect on the subcellular localization of the primary cardiac voltage-gated sodium channel, $Na_v1.5$, we have shown that SGK1 activation is necessary for at least some of the aspects of adverse electrical remodeling. Other kinases are also potent modulators of $Na_v1.5$ [168-170], so the singular inhibition of SGK1 may not be sufficient to fully protect against heart failure phenotypes. We were, however, able to block the biochemical changes caused by constitutive SGK1 activation by using a dominant negative SGK1 mouse model and also by abrogating I_{NaL} with ranolazine. Further studies will be necessary to determine to what extent SGK1 contributes to electrical remodeling when considering other $Na_v1.5$ regulatory elements, and a deeper understanding of the physiological degradation of SGK1 via the 26S proteasome [171] in the cardiac milieu may also elucidate novel therapeutic strategies.

Our report of putative SGK1 phosphorylation sites on *SCN5a* also begs further investigation. Previous studies have shown that mutations of human *SCN5a* can lead to both arrhythmia and cardiomyopathy, so the direct $Na_v1.5$ phosphorylation by SGK1 could provide a mechanistic link between the PI3K signaling pathway, heart failure, and ventricular arrhythmia, especially considering that one of the candidate SGK1 phosphorylation targets resides in a region known to be important in channel inactivation gating [172].

In sum, this study highlights the importance of SGK1 activation in heart failure pathogenesis and concomitant arrhythmogenesis. It cannot be concluded that SGK1 is the sole player in the observed adverse electrical remodeling, but inhibition of chronic SGK1 in heart failure could prove salutary and certainly warrants further investigation.

7.4 PAPER IV – Exploring distinct gene expression profiles in zebrafish models of cardiac regeneration

7.4.1 Introduction

In mammals, the cardiomyocytes lost as a result of myocardial infarction are replaced by inflexible scar tissue, severely hampering cardiac functionality. Cardiac regeneration has been observed in neonatal mammals and in various non-mammalian vertebrates [101, 102, 120, 173], but a comprehensive understanding of the molecular drivers of this regenerative capacity is still lacking. For example, the neonatal mouse has demonstrated a seven-day postnatal regenerative window [120], urodele amphibians have shown complete cardiac regeneration after both apical and basal resection [104], and approximately 20% of the ventricular myocardium can be

removed from the apex of the adult zebrafish heart with no discernable dysfunction after 60 days of recovery [107].

In order to understand the mechanisms governing the initiation of this remarkable regenerative capacity in the zebrafish, in **PAPER IV** we have employed a genetic cardiomyocyte ablation (GA) model of cardiac regeneration in conjunction with microarray analysis to identify key regulatory signaling pathways and molecular drivers of cardiomyocyte proliferation. We compared the transcription profile from our genetic injury model to models of apical resection and cryoinjury in an attempt to identify a core regulatory gene set and to determine the level of genetic discrepancy between the three different models of zebrafish cardiac regeneration.

7.4.2 Summary of Results

To elicit a regenerative response, we made use of the NTR/MTZ system. In the absence of NTR, the MTZ is a benign substance. When exposed to NTR, MTZ is reduced to a DNA chelating agent, causing apoptosis of the cell expressing NTR [174]. Zebrafish expressing an mCherry-NTR fusion protein specifically in cardiomyocytes were exposed to 5mM MTZ for 2 hours resulting in a significant increase in cardiomyocyte proliferation, as measured by phospho-histone H3 (PH3) positivity, after 4 days of recovery.

Total mRNA from whole hearts of MTZ treated fish was then analyzed by microarray, and clustering analysis showed an intense and consistent gene activation profile. In all, 135 genes were found to be significantly up-regulated after injury and 46 genes were significantly down-regulated. Gene ontology (GO) analysis indicated an enrichment in biological pathways associated with cell-cycle and mitosis, amino acid biosynthesis and metabolism, folate metabolism, organic molecule ion transport, and cytoskeletal organization. Protein modification/ubiquitination, external stimulus response, and cell signaling were the most significantly enriched biological processes associated with the down-regulated genes.

When the transcriptional profile from our screen was compared with two models of apical resection (AR) and one model of cryoablation (CA), we found 16 genes to be commonly up-regulated in all screens, and one gene commonly down-regulated. A subset of these genes have been directly associated with the cell cycle, supporting the current hypothesis that zebrafish heart regeneration is driven primarily by cardiomyocyte proliferation. Furthermore, GO analysis of the 16 up-regulated genes revealed mitotic cell cycle activity as the most significant biological process associated with this gene set. The common down-regulated gene, *Cited4a*, has not been explored in the context of zebrafish regeneration, but has been shown to be cardioprotective in mice after ischemic injury [175].

We were also interested in understanding the interactions between the commonly regulated genes. To that end, we used the Ingenuity Pathway Analysis software to explore previously described interrelations and cellular localizations. Interestingly, the genes primarily associated with the cell cycle all coded for nuclear proteins, and were predicted to create an interconnected network therein. We were also able to

use this tool to predict potential transcriptional and miRNA regulators, most of which have been reported previously to interact with the cell cycle-related proteins.

Interestingly, analysis of the comparisons also revealed large discrepancies in gene expression between the various models. The two AR screens and the CA screen showed 59, 222, and 1315 model-specific up-regulated genes, respectively, while 82 genes were specifically up-regulated in our GA model. When considering the down-regulated genes, 74, 257, and 464 genes were observed respectively in the two AR screens and the CA screens. Thirty-four genes were down-regulated in the our GA model. To determine the specific biological processes associated with each model system, we also performed GO analysis on the model-specific up- and down-regulated genes and observed distinct ontology for each injury model. This suggests that the regenerative responses resulting from the disease paradigms may also be genetically distinct.

7.4.3 Discussion

In the current study we have identified 17 commonly regulated genes in four different transcriptional studies of zebrafish heart regeneration comprising three different injury model systems. We suggest that these genes comprise, at least in part, a core gene set necessary for complete cardiac regeneration regardless of injury methodology. We further present potential upstream regulators of these genes, so as to build a more comprehensive overview of the regenerative pathways at play after cardiac injury in the zebrafish. Future studies should focus on the validation of these potential signaling pathways, and on investigating the commonly up- and down-regulated genes in gain- and loss-of-function studies.

Our report of the model-specific regulated genes also begs further investigation. It is perhaps not surprising that different injury models lead to different regenerative responses, but the data presented here suggest that the regenerative response is not simply a function of injury severity, but that different injury paradigms may lead to genetically distinct regenerative avenues. In this way, these disease paradigms should not be used as interchangeable models of cardiac regeneration, but should be individually characterized to determine their complete pathophysiology, as the regenerative responses may be tailored accordingly.

7.5 PAPER V – Regeneration of human cardiomyocytes in cardiomyopathy

7.5.1 Introduction

There is an emerging consensus that human cardiomyocytes do have a limited proliferative capacity that extends even into adulthood. We have previously shown that cardiomyocytes in the healthy human heart are able to renew to a limited extent for the lifetime of the individual [136, 138, 176]. In fact, we have found that almost 40% of the ventricular myocytes present at birth will be exchanged during a normal lifetime. However, whether or not this innate regenerative capacity is utilized after cardiac injury is still controversial. Previous studies using mitotic markers have

reported cardiomyocyte division in end stage heart failure, but these studies may be confounded by the propensity of cardiomyocytes to polyploidize and/or multinucleate as a result of disease onset [126].

In order to provide a more holistic view of the magnitude and dynamics of cardiac cell turnover in the pathological human heart in **PAPER V** we have made use of ^{14}C-dating of postmortem cardiac tissue from patients diagnosed with dilated cardiomyopathy.

7.5.2 Summary of Results

In this report we analyzed heart samples from 15 DCM patients aged 17 to 65 years using retrospective ^{14}C birth dating to establish the age and turnover dynamics of cardiomyoctyes in dilated cardiomyopathy. Disease onset was assumed to be consistent with the time of diagnosis. Cardiomyocyte nuclear ^{14}C levels were measured using accelerator mass spectrometry, and cardiomyocyte nuclei were identified and isolated based on positive immunostaining for the perinuclear protein PCM-1. Ploidy levels were determined by nuclear DNA staining and fluorescence activated cell sorting.

Ploidy analysis revealed higher ploidy levels in DCM as compared to healthy control samples. This increased ploidy resulted in a total increase in cardiomyocyte DNA from 180.2% ± 13.1% in healthy subjects to 337.9% ± 108.7% in DCM (2n baseline = 100%). However, we did not detect a significant increase in the number of binucleated cardiomyocytes, suggesting that the increase in DNA content with disease is not due to binucleation.

The ^{14}C data obtained from the cardiomyocytes of DCM patients followed the atmospheric bomb curve to a less extent than the data from healthy samples, indicating increased DNA synthesis in pathological cardiomyocytes. While ploidy does explain a large portion of the ^{14}C integration in cardiomyocyte nuclear DNA of DCM patients, it could not explain the ^{14}C data entirely. To develop a model fitting the observed data, we tested three different polyploidization scenarios. The ploidy levels of both the healthy and DCM samples showed significant differences from the measured ^{14}C values in all cases, and none could be explained by polyploidization alone. Our mathematical model predicts that cardiomyocyte turnover is at least maintained in DCM, and that the turnover rate might be increased in DCM as compared with healthy individuals.

From these findings, we were able to continue data analysis under the condition of non-zero turnover with pathology. To address the question of renewal after disease onset, we assumed a constant rate of cardiomyocyte turnover before disease onset, and a different (constant) rate of renewal after onset. Comparing this model to the restricted, no-renewal model, we found that the data were best described by constant renewal after disease onset. This model suggests that the decreased cardiomyocyte turnover occurring before disease onset is compensated afterwards by higher turnover. However, increased post-onset turnover was not able to explain the data better than the restricted model, holding a constant, lifelong turnover rate.

Using similar ^{14}C dating methods we were also able to determine the turnover rates of non-cardiomyocyte populations in healthy and DCM subjects. Our model predicted a turnover rate of 5.9% per year in healthy subjects and 11.1% per year in DCM. This seemingly marked increase did not reach statistical significance, but we did detect an unexpected population of non-cardiomyocytes (~10% of the total complement) exhibiting no or low turnover rates. Further studies will be necessary to determine the identity of this cell population and its role in cardiac pathophysiology.

7.5.3 Discussion

In this investigation, we have used ^{14}C dating to determine the magnitude and dynamics of cardiomyocyte and non-cardiomyocyte renewal in dilated cardiomyopathy. While non-cardiomyocyte renewal rates were not significantly different between healthy and diseased individuals, the rate of cardiomyocyte renewal increased after the onset of DCM. Based on our model, we have found that cardiomyocytes corresponding to 2.3 – 3.3% of the full complement are generated per year after diagnosis. That is to say that in a patient with a ten-year history of DCM, ~28% of their existing cardiomyocytes will have been exchanged. However, though the rate of cardiomyocyte renewal is significantly increased after the onset of disease, using our model, we are not able to determine whether or not the increased cardiomyocyte generation is a result of an increased turnover rate after disease onset, or if it is due to a constant, lifelong turnover. In any case, all the DCM patients studied had clear evidence of reduced cardiac function, indicating that the augmented renewal was not sufficient to completely compensate for the pathological changes. It cannot be discounted that the new cardiomyocytes contributed to patient survivability, but further studies will be necessary to determine whether the prolonged disease duration resulted in increased cardiomyocyte renewal or if the increased cardiomyocyte renewal resulted in longer life.

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REFERENCES

[1] J. Furst, What are the three types of blood vessels and their functions?, First Aid for free, http://www.firstaidforfree.com/what-are-the-three-types-of-blood-vessels-and-their-functions/, 2015, Vol. 2018.

[2] J.M. González-Rosa, C.E. Burns, C.G. Burns, Zebrafish heart regeneration: 15 years of discoveries, Regeneration (Oxf) 4 (2017) 105-123.

[3] A.A. Wilde, E.R. Behr, Genetic testing for inherited cardiac disease, Nat Rev Cardiol 10 (2013) 571-583.

[4] G.A. Roth, G. Nguyen, M.H. Forouzanfar, A.H. Mokdad, M. Naghavi, C.J. Murray, Estimates of global and regional premature cardiovascular mortality in 2025, Circulation 132 (2015) 1270-1282.

[5] P.A. Heidenreich, J.G. Trogdon, O.A. Khavjou, J. Butler, K. Dracup, M.D. Ezekowitz, E.A. Finkelstein, Y. Hong, S.C. Johnston, A. Khera, D.M. Lloyd-Jones, S.A. Nelson, G. Nichol, D. Orenstein, P.W. Wilson, Y.J. Woo, A.H.A.A.C. Committee, S. Council, C.o.C.R.a. Intervention, C.o.C. Cardiology, C.o.E.a. Prevention, C.o. Arteriosclerosis, T.a.V. Biology, C.o. Cardiopulmonary, C. Care, P.a. Resuscitation, C.o.C. Nursing, C.o.t.K.i.C. Disease, a.I.C.o.Q.o.C.a.O.R. Council on Cardiovascular Surgery and Anesthesia, Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association, Circulation 123 (2011) 933-944.

[6] N.M. Hawkins, P.S. Jhund, J.J. McMurray, S. Capewell, Heart failure and socioeconomic status: accumulating evidence of inequality, Eur J Heart Fail 14 (2012) 138-146.

[7] Y. Borné, G. Engström, B. Essén, J. Sundquist, B. Hedblad, Country of birth and risk of hospitalization due to heart failure: a Swedish population-based cohort study, Eur J Epidemiol 26 (2011) 275-283.

[8] S. Christensen, R. Mogelvang, M. Heitmann, E. Prescott, Level of education and risk of heart failure: a prospective cohort study with echocardiography evaluation, Eur Heart J 32 (2011) 450-458.

[9] F.A. McAlister, N.F. Murphy, C.R. Simpson, S. Stewart, K. MacIntyre, M. Kirkpatrick, J. Chalmers, A. Redpath, S. Capewell, J.J. McMurray, Influence of socioeconomic deprivation on the primary care burden and treatment of patients with a diagnosis of heart failure in general practice in Scotland: population based study, BMJ 328 (2004) 1110.

[10] C.B. Roberts, D.J. Couper, P.P. Chang, S.A. James, W.D. Rosamond, G. Heiss, Influence of life-course socioeconomic position on incident heart failure in blacks and whites: the Atherosclerosis Risk in Communities Study, Am J Epidemiol 172 (2010) 717-727.

[11] S. Stewart, N.F. Murphy, J.J. McMurray, P. Jhund, C.L. Hart, D. Hole, Effect of socioeconomic deprivation on the population risk of incident heart failure hospitalisation: an analysis of the Renfrew/Paisley Study, Eur J Heart Fail 8 (2006) 856-863.

[12] J.S. Gottdiener, A.M. Arnold, G.P. Aurigemma, J.F. Polak, R.P. Tracy, D.W. Kitzman, J.M. Gardin, J.E. Rutledge, R.C. Boineau, Predictors of congestive heart failure in the elderly: the Cardiovascular Health Study, J Am Coll Cardiol 35 (2000) 1628-1637.

[13] E. Ingelsson, L. Lind, J. Arnlöv, J. Sundström, Socioeconomic factors as predictors of incident heart failure, J Card Fail 12 (2006) 540-545.
[14] M. Schaufelberger, A. Rosengren, Heart failure in different occupational classes in Sweden, Eur Heart J 28 (2007) 212-218.
[15] J. He, L.G. Ogden, L.A. Bazzano, S. Vupputuri, C. Loria, P.K. Whelton, Risk factors for congestive heart failure in US men and women: NHANES I epidemiologic follow-up study, Arch Intern Med 161 (2001) 996-1002.
[16] F.D. Wolinsky, J.M. Overhage, T.E. Stump, R.M. Lubitz, D.M. Smith, The risk of hospitalization for congestive heart failure among older adults, Med Care 35 (1997) 1031-1043.
[17] F.D. Wolinsky, D.M. Smith, T.E. Stump, J.M. Overhage, R.M. Lubitz, The sequelae of hospitalization for congestive heart failure among older adults, J Am Geriatr Soc 45 (1997) 558-563.
[18] S.L. Hub, Label the Heart - Science Learning Hub, Referencing Hub Media, https://www.sciencelearn.org.nz/labelling_interactives/1-label-the-heart, 2017, Vol. 2018.
[19] L.H. Opie, The Heart: Physiology, from Cell to Circulation, Lippincott-Raven Publishers, Philadelphia, PA, 1998.
[20] P. Gillam, Cardiac cycle and the Human Heart: A* understanding for iGCSE Biology 2.63 2.64, IGCSE Biology posts, https://pmgbiology.com/2015/02/17/cardiac-cycle-and-the-human-heart-a-understanding-for-igcse-biology/, 2015, Vol. 2018.
[21] J.J. Smith, Circulatory Physiology - the essentials, Williams and Wilkins, Baltimore, MD, 1990.
[22] J.R. Levick, An Introduction to Cardiovascular Physiology, Arnold, New York, 2000.
[23] D.A. Eisner, J.L. Caldwell, K. Kistamás, A.W. Trafford, Calcium and Excitation-Contraction Coupling in the Heart, Circ Res 121 (2017) 181-195.
[24] D.M. Bers, Cardiac excitation-contraction coupling, Nature 415 (2002) 198-205.
[25] B.I. Jugdutt, D.S. Naranjan, Cardiac Remodeling: Molecular Mechanisms, Springer Science+Business Media, New York, 2013.
[26] J.W. BLACK, J.S. STEPHENSON, Pharmacology of a new adrenergic beta-receptor-blocking compound (Nethalide), Lancet 2 (1962) 311-314.
[27] M.P. Love, W.G. Haynes, G.A. Gray, D.J. Webb, J.J. McMurray, Vasodilator effects of endothelin-converting enzyme inhibition and endothelin ETA receptor blockade in chronic heart failure patients treated with ACE inhibitors, Circulation 94 (1996) 2131-2137.
[28] Cardiovascular Disability: Updating the Social Security Listings, in: I.o.M.U.C.o.S.S.C.D. Criteria (Ed.), Heart Failure, Cardiomyopathy, and Right Heart Failure, National Academies Press (US), https://www.ncbi.nlm.nih.gov/books/NBK209980/, 2010, Vol. 2018.
[29] F. Aziz, L.A. Tk, C. Enweluzo, S. Dutta, M. Zaeem, Diastolic heart failure: a concise review, J Clin Med Res 5 (2013) 327-334.
[30] E. Braunwald, Cardiomyopathies: An Overview, Circ Res 121 (2017) 711-721.
[31] E.M. McNally, L. Mestroni, Dilated Cardiomyopathy: Genetic Determinants and Mechanisms, Circ Res 121 (2017) 731-748.

[32] R.E. Hershberger, D.J. Hedges, A. Morales, Dilated cardiomyopathy: the complexity of a diverse genetic architecture, Nat Rev Cardiol 10 (2013) 531-547.
[33] D.S. Herman, L. Lam, M.R. Taylor, L. Wang, P. Teekakirikul, D. Christodoulou, L. Conner, S.R. DePalma, B. McDonough, E. Sparks, D.L. Teodorescu, A.L. Cirino, N.R. Banner, D.J. Pennell, S. Graw, M. Merlo, A. Di Lenarda, G. Sinagra, J.M. Bos, M.J. Ackerman, R.N. Mitchell, C.E. Murry, N.K. Lakdawala, C.Y. Ho, P.J. Barton, S.A. Cook, L. Mestroni, J.G. Seidman, C.E. Seidman, Truncations of titin causing dilated cardiomyopathy, N Engl J Med 366 (2012) 619-628.
[34] B. Bozkurt, M. Colvin, J. Cook, L.T. Cooper, A. Deswal, G.C. Fonarow, G.S. Francis, D. Lenihan, E.F. Lewis, D.M. McNamara, E. Pahl, R.S. Vasan, K. Ramasubbu, K. Rasmusson, J.A. Towbin, C. Yancy, A.H.A.C.o.H.F.a.T.o.t.C.o.C. Cardiology, C.o.C.D.i.t. Young, C.o.C.a.S. Nursing, C.o.E.a. Prevention, a.C.o.Q.o.C.a.O. Research, Current Diagnostic and Treatment Strategies for Specific Dilated Cardiomyopathies: A Scientific Statement From the American Heart Association, Circulation 134 (2016) e579-e646.
[35] P.A. Harvey, L.A. Leinwand, The cell biology of disease: cellular mechanisms of cardiomyopathy, J Cell Biol 194 (2011) 355-365.
[36] I. Kehat, J. Davis, M. Tiburcy, F. Accornero, M.K. Saba-El-Leil, M. Maillet, A.J. York, J.N. Lorenz, W.H. Zimmermann, S. Meloche, J.D. Molkentin, Extracellular signal-regulated kinases 1 and 2 regulate the balance between eccentric and concentric cardiac growth, Circ Res 108 (2011) 176-183.
[37] J.G. Seidman, C. Seidman, The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms, Cell 104 (2001) 557-567.
[38] J. Heineke, J.D. Molkentin, Regulation of cardiac hypertrophy by intracellular signalling pathways, Nat Rev Mol Cell Biol 7 (2006) 589-600.
[39] K. Kuwahara, Y. Saito, M. Takano, Y. Arai, S. Yasuno, Y. Nakagawa, N. Takahashi, Y. Adachi, G. Takemura, M. Horie, Y. Miyamoto, T. Morisaki, S. Kuratomi, A. Noma, H. Fujiwara, Y. Yoshimasa, H. Kinoshita, R. Kawakami, I. Kishimoto, M. Nakanishi, S. Usami, M. Harada, K. Nakao, NRSF regulates the fetal cardiac gene program and maintains normal cardiac structure and function, EMBO J 22 (2003) 6310-6321.
[40] K. Kuwahara, T. Nishikimi, K. Nakao, Transcriptional regulation of the fetal cardiac gene program, J Pharmacol Sci 119 (2012) 198-203.
[41] B.D. Lowes, W. Minobe, W.T. Abraham, M.N. Rizeq, T.J. Bohlmeyer, R.A. Quaife, R.L. Roden, D.L. Dutcher, A.D. Robertson, N.F. Voelkel, D.B. Badesch, B.M. Groves, E.M. Gilbert, M.R. Bristow, Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium, J Clin Invest 100 (1997) 2315-2324.
[42] S. Miyata, W. Minobe, M.R. Bristow, L.A. Leinwand, Myosin heavy chain isoform expression in the failing and nonfailing human heart, Circ Res 86 (2000) 386-390.
[43] J.D. Molkentin, S.M. Jobe, B.E. Markham, Alpha-myosin heavy chain gene regulation: delineation and characterization of the cardiac muscle-specific

enhancer and muscle-specific promoter, J Mol Cell Cardiol 28 (1996) 1211-1225.
[44] C.Y. Ho, B. López, O.R. Coelho-Filho, N.K. Lakdawala, A.L. Cirino, P. Jarolim, R. Kwong, A. González, S.D. Colan, J.G. Seidman, J. Díez, C.E. Seidman, Myocardial fibrosis as an early manifestation of hypertrophic cardiomyopathy, N Engl J Med 363 (2010) 552-563.
[45] S. Neubauer, The failing heart--an engine out of fuel, N Engl J Med 356 (2007) 1140-1151.
[46] M. Rajabi, C. Kassiotis, P. Razeghi, H. Taegtmeyer, Return to the fetal gene program protects the stressed heart: a strong hypothesis, Heart Fail Rev 12 (2007) 331-343.
[47] H. Taegtmeyer, S. Sen, D. Vela, Return to the fetal gene program: a suggested metabolic link to gene expression in the heart, Ann N Y Acad Sci 1188 (2010) 191-198.
[48] D.E. Jaalouk, J. Lammerding, Mechanotransduction gone awry, Nat Rev Mol Cell Biol 10 (2009) 63-73.
[49] T. Aoyagi, T. Matsui, Phosphoinositide-3 kinase signaling in cardiac hypertrophy and heart failure, Curr Pharm Des 17 (2011) 1818-1824.
[50] J.D. Molkentin, Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs, Cardiovasc Res 63 (2004) 467-475.
[51] B.K. Hong, H.M. Kwon, K.H. Byun, D. Kim, E.Y. Choi, T.S. Kang, S.M. Kang, K.J. Chun, Y. Jang, H.S. Kim, M. Kim, Apoptosis in dilated cardiomyopathy, Korean J Intern Med 15 (2000) 56-64.
[52] J. Narula, F.D. Kolodgie, R. Virmani, Apoptosis and cardiomyopathy, Curr Opin Cardiol 15 (2000) 183-188.
[53] P. Camacho, H. Fan, Z. Liu, J.Q. He, Small mammalian animal models of heart disease, Am J Cardiovasc Dis 6 (2016) 70-80.
[54] S. Chlopcíková, J. Psotová, P. Miketová, Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart, Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 145 (2001) 49-55.
[55] G. Gong, Y. Li, X. Yang, H. Geng, X. Lu, L. Wang, Z. Yang, Protective effects of IL28RA siRNA on cardiomyocytes in hypoxia/reoxygenation injury, Anatol J Cardiol 18 (2017) 168-174.
[56] G.K. Goncalves, S. Scalzo, A.P. Alves, U. Agero, S. Guatimosim, A.M. Reis, Neonatal cardiomyocyte hypertrophy induced by endothelin-1 is blocked by estradiol acting on GPER, Am J Physiol Cell Physiol 314 (2018) C310-C322.
[57] X. Li, L. Yu, J. Gao, X. Bi, J. Zhang, S. Xu, M. Wang, M. Chen, F. Qiu, G. Fu, Apelin Ameliorates High Glucose-Induced Downregulation of Connexin 43 via AMPK-Dependent Pathway in Neonatal Rat Cardiomyocytes, Aging Dis 9 (2018) 66-76.
[58] W.G. Thomas, Y. Brandenburger, D.J. Autelitano, T. Pham, H. Qian, R.D. Hannan, Adenoviral-directed expression of the type 1A angiotensin receptor promotes cardiomyocyte hypertrophy via transactivation of the epidermal growth factor receptor, Circ Res 90 (2002) 135-142.

[59] J.C. Braz, O.F. Bueno, L.J. De Windt, J.D. Molkentin, PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2), J Cell Biol 156 (2002) 905-919.
[60] M. Xin, Y. Kim, L.B. Sutherland, X. Qi, J. McAnally, R.J. Schwartz, J.A. Richardson, R. Bassel-Duby, E.N. Olson, Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size, Sci Signal 4 (2011) ra70.
[61] T.M. Jayawardena, B. Egemnazarov, E.A. Finch, L. Zhang, J.A. Payne, K. Pandya, Z. Zhang, P. Rosenberg, M. Mirotsou, V.J. Dzau, MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes, Circ Res 110 (2012) 1465-1473.
[62] E.R. Porrello, microRNAs in cardiac development and regeneration, Clin Sci (Lond) 125 (2013) 151-166.
[63] R. Pandey, S. Velasquez, S. Durrani, M. Jiang, M. Neiman, J.S. Crocker, J.B. Benoit, J. Rubinstein, A. Paul, R.P. Ahmed, MicroRNA-1825 induces proliferation of adult cardiomyocytes and promotes cardiac regeneration post ischemic injury, Am J Transl Res 9 (2017) 3120-3137.
[64] Y. Tian, Y. Liu, T. Wang, N. Zhou, J. Kong, L. Chen, M. Snitow, M. Morley, D. Li, N. Petrenko, S. Zhou, M. Lu, E. Gao, W.J. Koch, K.M. Stewart, E.E. Morrisey, A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice, Sci Transl Med 7 (2015) 279ra238.
[65] A.M. Fuller, L. Qian, MiRiad Roles for MicroRNAs in Cardiac Development and Regeneration, Cells 3 (2014) 724-750.
[66] J.M. Churko, G.L. Mantalas, M.P. Snyder, J.C. Wu, Overview of high throughput sequencing technologies to elucidate molecular pathways in cardiovascular diseases, Circ Res 112 (2013) 1613-1623.
[67] B.G. Reid, M.S. Stratton, S. Bowers, M.A. Cavasin, K.M. Demos-Davies, I. Susano, T.A. McKinsey, Discovery of novel small molecule inhibitors of cardiac hypertrophy using high throughput, high content imaging, J Mol Cell Cardiol 97 (2016) 106-113.
[68] A. Eulalio, M. Mano, M. Dal Ferro, L. Zentilin, G. Sinagra, S. Zacchigna, M. Giacca, Functional screening identifies miRNAs inducing cardiac regeneration, Nature 492 (2012) 376-381.
[69] Z. Jian, Y.J. Chen, R. Shimkunas, Y. Jian, M. Jaradeh, K. Chavez, N. Chiamvimonvat, J.C. Tardiff, L.T. Izu, R.S. Ross, Y. Chen-Izu, In Vivo Cannulation Methods for Cardiomyocytes Isolation from Heart Disease Models, PLoS One 11 (2016) e0160605.
[70] S.L. Jacobson, H.M. Piper, Cell cultures of adult cardiomyocytes as models of the myocardium, J Mol Cell Cardiol 18 (1986) 661-678.
[71] V.A. Maltsev, J.W. Kyle, S. Mishra, A. Undrovinas, Molecular identity of the late sodium current in adult dog cardiomyocytes identified by Nav1.5 antisense inhibition, Am J Physiol Heart Circ Physiol 295 (2008) H667-676.
[72] J.S. Mitcheson, J.C. Hancox, A.J. Levi, Cultured adult cardiac myocytes: future applications, culture methods, morphological and electrophysiological properties, Cardiovasc Res 39 (1998) 280-300.
[73] M. Horackova, R.P. Croll, D.A. Hopkins, A.M. Losier, J.A. Armour, Morphological and immunohistochemical properties of primary long-

term cultures of adult guinea-pig ventricular cardiomyocytes with peripheral cardiac neurons, Tissue Cell 28 (1996) 411-425.
[74] T. Powell, V.W. Twist, A rapid technique for the isolation and purification of adult cardiac muscle cells having respiratory control and a tolerance to calcium, Biochem Biophys Res Commun 72 (1976) 327-333.
[75] T.D. O'Connell, M.C. Rodrigo, P.C. Simpson, Isolation and culture of adult mouse cardiac myocytes, Methods Mol Biol 357 (2007) 271-296.
[76] A.K. Peter, M.A. Bjerke, L.A. Leinwand, Biology of the cardiac myocyte in heart disease, Mol Biol Cell 27 (2016) 2149-2160.
[77] M. Zheng, S.J. Zhang, W.Z. Zhu, B. Ziman, B.K. Kobilka, R.P. Xiao, beta 2-adrenergic receptor-induced p38 MAPK activation is mediated by protein kinase A rather than by Gi or gbeta gamma in adult mouse cardiomyocytes, J Biol Chem 275 (2000) 40635-40640.
[78] A. Nakai, O. Yamaguchi, T. Takeda, Y. Higuchi, S. Hikoso, M. Taniike, S. Omiya, I. Mizote, Y. Matsumura, M. Asahi, K. Nishida, M. Hori, N. Mizushima, K. Otsu, The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress, Nat Med 13 (2007) 619-624.
[79] E.L. Graham, C. Balla, H. Franchino, Y. Melman, F. del Monte, S. Das, Isolation, culture, and functional characterization of adult mouse cardiomyoctyes, J Vis Exp (2013) e50289.
[80] R. Liao, M. Jain, Isolation, culture, and functional analysis of adult mouse cardiomyocytes, Methods Mol Med 139 (2007) 251-262.
[81] H.J. Berger, S.K. Prasad, A.J. Davidoff, D. Pimental, O. Ellingsen, J.D. Marsh, T.W. Smith, R.A. Kelly, Continual electric field stimulation preserves contractile function of adult ventricular myocytes in primary culture, Am J Physiol 266 (1994) H341-349.
[82] K.-C. Yang, W. W, N.J. M, Manual of Research Techniques in Cardiovascular Medicine, John Wiley and Sons, Ltd., Saint Louis, MO, 2014.
[83] J.M. Nerbonne, C.G. Nichols, T.L. Schwarz, D. Escande, Genetic manipulation of cardiac K(+) channel function in mice: what have we learned, and where do we go from here?, Circ Res 89 (2001) 944-956.
[84] D.G. Allen, J.R. Blinks, Calcium transients in aequorin-injected frog cardiac muscle, Nature 273 (1978) 509-513.
[85] W.G. Wier, Calcium transients during excitation-contraction coupling in mammalian heart: aequorin signals of canine Purkinje fibers, Science 207 (1980) 1085-1087.
[86] K.K. Hintz, J. Ren, Tetramethylpyrazine elicits disparate responses in cardiac contraction and intracellular Ca(2+) transients in isolated adult rat ventricular myocytes, Vascul Pharmacol 40 (2003) 213-217.
[87] L.E. Wold, J. Ren, Streptozotocin directly impairs cardiac contractile function in isolated ventricular myocytes via a p38 map kinase-dependent oxidative stress mechanism, Biochem Biophys Res Commun 318 (2004) 1066-1071.
[88] L. Pentassuglia, M. Graf, H. Lane, Y. Kuramochi, G. Cote, F. Timolati, D.B. Sawyer, C. Zuppinger, T.M. Suter, Inhibition of ErbB2 by receptor tyrosine kinase inhibitors causes myofibrillar structural damage without cell death in adult rat cardiomyocytes, Exp Cell Res 315 (2009) 1302-1312.

[89] J.C. Russell, S.D. Proctor, Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis, Cardiovasc Pathol 15 (2006) 318-330.
[90] H.G. Tsang, N.A. Rashdan, C.B. Whitelaw, B.M. Corcoran, K.M. Summers, V.E. MacRae, Large animal models of cardiovascular disease, Cell Biochem Funct 34 (2016) 113-132.
[91] M.V. Kolk, D. Meyberg, T. Deuse, K.R. Tang-Quan, R.C. Robbins, H. Reichenspurner, S. Schrepfer, LAD-ligation: a murine model of myocardial infarction, J Vis Exp (2009).
[92] Y. Kusakari, C.Y. Xiao, N. Himes, S.D. Kinsella, M. Takahashi, A. Rosenzweig, T. Matsui, Myocyte injury along myofibers in left ventricular remodeling after myocardial infarction, Interact Cardiovasc Thorac Surg 9 (2009) 951-955.
[93] F. Yang, Y.H. Liu, X.P. Yang, J. Xu, A. Kapke, O.A. Carretero, Myocardial infarction and cardiac remodelling in mice, Exp Physiol 87 (2002) 547-555.
[94] Z. Xu, J. Alloush, E. Beck, N. Weisleder, A murine model of myocardial ischemia-reperfusion injury through ligation of the left anterior descending artery, J Vis Exp (2014).
[95] A.C. deAlmeida, R.J. van Oort, X.H. Wehrens, Transverse aortic constriction in mice, J Vis Exp (2010).
[96] C. Tong, P. Li, N.L. Wu, Y. Yan, Q.L. Ying, Production of p53 gene knockout rats by homologous recombination in embryonic stem cells, Nature 467 (2010) 211-213.
[97] C. Tong, G. Huang, C. Ashton, P. Li, Q.L. Ying, Generating gene knockout rats by homologous recombination in embryonic stem cells, Nat Protoc 6 (2011) 827-844.
[98] M.S. Marber, R. Mestril, S.H. Chi, M.R. Sayen, D.M. Yellon, W.H. Dillmann, Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury, J Clin Invest 95 (1995) 1446-1456.
[99] B. Sauer, Inducible gene targeting in mice using the Cre/lox system, Methods 14 (1998) 381-392.
[100] Z.M. Guo, K. Xu, Y. Yue, B. Huang, X.Y. Deng, N.Q. Zhong, X. Hong, X.G. Chen, D. Xiao, Temporal control of Cre recombinase-mediated in vitro DNA recombination by Tet-on gene expression system, Acta Biochim Biophys Sin (Shanghai) 37 (2005) 133-138.
[101] J.O. Oberpriller, J.C. Oberpriller, Response of the adult newt ventricle to injury, J Exp Zool 187 (1974) 249-253.
[102] T. Piatkowski, C. Mühlfeld, T. Borchardt, T. Braun, Reconstitution of the myocardium in regenerating newt hearts is preceded by transient deposition of extracellular matrix components, Stem Cells Dev 22 (2013) 1921-1931.
[103] P.P. Rumyantsev, Post-injury DNA synthesis, mitosis and ultrastructural reorganization of adult frog cardiac myocytes. An electron microscopic-autoradiographic study, Z Zellforsch Mikrosk Anat 139 (1973) 431-450.
[104] N. Witman, B. Murtuza, B. Davis, A. Arner, J.I. Morrison, Recapitulation of developmental cardiogenesis governs the morphological and functional

regeneration of adult newt hearts following injury, Dev Biol 354 (2011) 67-76.
[105] C. Pfefferli, A. Jaźwińska, The art of fin regeneration in zebrafish, Regeneration (Oxf) 2 (2015) 72-83.
[106] V. Kroehne, D. Freudenreich, S. Hans, J. Kaslin, M. Brand, Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors, Development 138 (2011) 4831-4841.
[107] K.D. Poss, L.G. Wilson, M.T. Keating, Heart regeneration in zebrafish, Science 298 (2002) 2188-2190.
[108] C. Jopling, E. Sleep, M. Raya, M. Martí, A. Raya, J.C. Izpisúa Belmonte, Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation, Nature 464 (2010) 606-609.
[109] K. Kikuchi, J.E. Holdway, A.A. Werdich, R.M. Anderson, Y. Fang, G.F. Egnaczyk, T. Evans, C.A. Macrae, D.Y. Stainier, K.D. Poss, Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes, Nature 464 (2010) 601-605.
[110] K. Ito, M. Morioka, S. Kimura, M. Tasaki, K. Inohaya, A. Kudo, Differential reparative phenotypes between zebrafish and medaka after cardiac injury, Dev Dyn 243 (2014) 1106-1115.
[111] F. Chablais, J. Veit, G. Rainer, A. Jaźwińska, The zebrafish heart regenerates after cryoinjury-induced myocardial infarction, BMC Dev Biol 11 (2011) 21.
[112] J.M. González-Rosa, V. Martín, M. Peralta, M. Torres, N. Mercader, Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish, Development 138 (2011) 1663-1674.
[113] K. Schnabel, C.C. Wu, T. Kurth, G. Weidinger, Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation, PLoS One 6 (2011) e18503.
[114] S. Curado, R.M. Anderson, B. Jungblut, J. Mumm, E. Schroeter, D.Y. Stainier, Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies, Dev Dyn 236 (2007) 1025-1035.
[115] S. Curado, D.Y. Stainier, R.M. Anderson, Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies, Nat Protoc 3 (2008) 948-954.
[116] J. Wang, D. Panáková, K. Kikuchi, J.E. Holdway, M. Gemberling, J.S. Burris, S.P. Singh, A.L. Dickson, Y.F. Lin, M.K. Sabeh, A.A. Werdich, D. Yelon, C.A. Macrae, K.D. Poss, The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion, Development 138 (2011) 3421-3430.
[117] E.R. Porrello, E.N. Olson, A neonatal blueprint for cardiac regeneration, Stem Cell Res 13 (2014) 556-570.
[118] J.D. Drenckhahn, Q.P. Schwarz, S. Gray, A. Laskowski, H. Kiriazis, Z. Ming, R.P. Harvey, X.J. Du, D.R. Thorburn, T.C. Cox, Compensatory growth of healthy cardiac cells in the presence of diseased cells restores tissue homeostasis during heart development, Dev Cell 15 (2008) 521-533.
[119] K.R. Chien, I.J. Domian, K.K. Parker, Cardiogenesis and the complex biology of regenerative cardiovascular medicine, Science 322 (2008) 1494-1497.

[120] E.R. Porrello, A.I. Mahmoud, E. Simpson, J.A. Hill, J.A. Richardson, E.N. Olson, H.A. Sadek, Transient regenerative potential of the neonatal mouse heart, Science 331 (2011) 1078-1080.
[121] B.J. Haubner, M. Adamowicz-Brice, S. Khadayate, V. Tiefenthaler, B. Metzler, T. Aitman, J.M. Penninger, Complete cardiac regeneration in a mouse model of myocardial infarction, Aging (Albany NY) 4 (2012) 966-977.
[122] E.R. Porrello, A.I. Mahmoud, E. Simpson, B.A. Johnson, D. Grinsfelder, D. Canseco, P.P. Mammen, B.A. Rothermel, E.N. Olson, H.A. Sadek, Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family, Proc Natl Acad Sci U S A 110 (2013) 187-192.
[123] E.J. van den Bos, B.M. Mees, M.C. de Waard, R. de Crom, D.J. Duncker, A novel model of cryoinjury-induced myocardial infarction in the mouse: a comparison with coronary artery ligation, Am J Physiol Heart Circ Physiol 289 (2005) H1291-1300.
[124] A. Darehzereshki, N. Rubin, L. Gamba, J. Kim, J. Fraser, Y. Huang, J. Billings, R. Mohammadzadeh, J. Wood, D. Warburton, V. Kaartinen, C.L. Lien, Differential regenerative capacity of neonatal mouse hearts after cryoinjury, Dev Biol 399 (2015) 91-99.
[125] S.E. Senyo, M.L. Steinhauser, C.L. Pizzimenti, V.K. Yang, L. Cai, M. Wang, T.D. Wu, J.L. Guerquin-Kern, C.P. Lechene, R.T. Lee, Mammalian heart renewal by pre-existing cardiomyocytes, Nature 493 (2013) 433-436.
[126] O. Bergmann, S. Jovinge, Cardiac regeneration in vivo: mending the heart from within?, Stem Cell Res 13 (2014) 523-531.
[127] H.E. Macmahon, Hyperplasia and Regeneration of the Myocardium in Infants and in Children, Am J Pathol 13 (1937) 845-854.845.
[128] I.I. Kots, G.G. Abakumov, L.G. Vdovenko, I.K. Cheremushnikov, [Myocardial lesions in diphtheria], Sov Med (1991) 52-53.
[129] C.P. Adler, H. Friedburg, G.W. Herget, M. Neuburger, H. Schwalb, Variability of cardiomyocyte DNA content, ploidy level and nuclear number in mammalian hearts, Virchows Arch 429 (1996) 159-164.
[130] O. Bergmann, S. Zdunek, K. Alkass, H. Druid, S. Bernard, J. Frisén, Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover, Exp Cell Res 317 (2011) 188-194.
[131] G.W. Herget, M. Neuburger, R. Plagwitz, C.P. Adler, DNA content, ploidy level and number of nuclei in the human heart after myocardial infarction, Cardiovasc Res 36 (1997) 45-51.
[132] M.H. Soonpaa, K.K. Kim, L. Pajak, M. Franklin, L.J. Field, Cardiomyocyte DNA synthesis and binucleation during murine development, Am J Physiol 271 (1996) H2183-2189.
[133] M. Mollova, K. Bersell, S. Walsh, J. Savla, L.T. Das, S.Y. Park, L.E. Silberstein, C.G. Dos Remedios, D. Graham, S. Colan, B. Kühn, Cardiomyocyte proliferation contributes to heart growth in young humans, Proc Natl Acad Sci U S A 110 (2013) 1446-1451.
[134] J. Kajstura, K. Urbanek, S. Perl, T. Hosoda, H. Zheng, B. Ogórek, J. Ferreira-Martins, P. Goichberg, C. Rondon-Clavo, F. Sanada, D. D'Amario, M. Rota, F. Del Monte, D. Orlic, J. Tisdale, A. Leri, P. Anversa, Cardiomyogenesis in the adult human heart, Circ Res 107 (2010) 305-315.

[135] A.P. Beltrami, K. Urbanek, J. Kajstura, S.M. Yan, N. Finato, R. Bussani, B. Nadal-Ginard, F. Silvestri, A. Leri, C.A. Beltrami, P. Anversa, Evidence that human cardiac myocytes divide after myocardial infarction, N Engl J Med 344 (2001) 1750-1757.
[136] O. Bergmann, R.D. Bhardwaj, S. Bernard, S. Zdunek, F. Barnabé-Heider, S. Walsh, J. Zupicich, K. Alkass, B.A. Buchholz, H. Druid, S. Jovinge, J. Frisén, Evidence for cardiomyocyte renewal in humans, Science 324 (2009) 98-102.
[137] J. Kajstura, M. Rota, D. Cappetta, B. Ogórek, C. Arranto, Y. Bai, J. Ferreira-Martins, S. Signore, F. Sanada, A. Matsuda, J. Kostyla, M.V. Caballero, C. Fiorini, D.A. D'Alessandro, R.E. Michler, F. del Monte, T. Hosoda, M.A. Perrella, A. Leri, B.A. Buchholz, J. Loscalzo, P. Anversa, Cardiomyogenesis in the aging and failing human heart, Circulation 126 (2012) 1869-1881.
[138] O. Bergmann, S. Zdunek, A. Felker, M. Salehpour, K. Alkass, S. Bernard, S.L. Sjostrom, M. Szewczykowska, T. Jackowska, C. Dos Remedios, T. Malm, M. Andrä, R. Jashari, J.R. Nyengaard, G. Possnert, S. Jovinge, H. Druid, J. Frisén, Dynamics of Cell Generation and Turnover in the Human Heart, Cell 161 (2015) 1566-1575.
[139] E. Graham, O. Bergmann, Dating the Heart: Exploring Cardiomyocyte Renewal in Humans, Physiology (Bethesda) 32 (2017) 33-41.
[140] W.F. Libby, E.C. Anderson, J.R. Arnold, Age Determination by Radiocarbon Content: World-Wide Assay of Natural Radiocarbon, Science 109 (1949) 227-228.
[141] B. Kromer, S.W. Manning, P.I. Kuniholm, M.W. Newton, M. Spurk, I. Levin, Regional 14CO2 offsets in the troposphere: magnitude, mechanisms, and consequences, Science 294 (2001) 2529-2532.
[142] I. Levin, S. Hammer, B. Kromer, F. Meinhardt, Radiocarbon observations in atmospheric CO2: determining fossil fuel CO2 over Europe using Jungfraujoch observations as background, Sci Total Environ 391 (2008) 211-216.
[143] H.Y. Mok, E.R. Druffel, W.M. Rampone, Chronology of cholelithiasis. Dating gallstones from atmospheric radiocarbon produced by nuclear bomb explosions, N Engl J Med 314 (1986) 1075-1077.
[144] M. Salehpour, N. Forsgard, G. Possnert, Accelerator mass spectrometry of small biological samples, Rapid Commun Mass Spectrom 22 (2008) 3928-3934.
[145] D.D. Harkness, Further investigations of the transfer of bomb 14 C to man, Nature 240 (1972) 302-303.
[146] W.F. LIBBY, R. BERGER, J.F. MEAD, G.V. ALEXANDER, J.F. ROSS, REPLACEMENT RATES FOR HUMAN TISSUE FROM ATMOSPHERIC RADIOCARBON, Science 146 (1964) 1170-1172.
[147] K.L. Spalding, R.D. Bhardwaj, B.A. Buchholz, H. Druid, J. Frisén, Retrospective birth dating of cells in humans, Cell 122 (2005) 133-143.
[148] K.L. Spalding, E. Arner, P.O. Westermark, S. Bernard, B.A. Buchholz, O. Bergmann, L. Blomqvist, J. Hoffstedt, E. Näslund, T. Britton, H. Concha, M. Hassan, M. Rydén, J. Frisén, P. Arner, Dynamics of fat cell turnover in humans, Nature 453 (2008) 783-787.
[149] K.L. Spalding, O. Bergmann, K. Alkass, S. Bernard, M. Salehpour, H.B. Huttner, E. Boström, I. Westerlund, C. Vial, B.A. Buchholz, G. Possnert, D.C.

Mash, H. Druid, J. Frisén, Dynamics of hippocampal neurogenesis in adult humans, Cell 153 (2013) 1219-1227.
[150] O. Bergmann, S. Jovinge, Isolation of cardiomyocyte nuclei from post-mortem tissue, J Vis Exp (2012).
[151] A. Raulf, H. Horder, L. Tarnawski, C. Geisen, A. Ottersbach, W. Röll, S. Jovinge, B.K. Fleischmann, M. Hesse, Transgenic systems for unequivocal identification of cardiac myocyte nuclei and analysis of cardiomyocyte cell cycle status, Basic Res Cardiol 110 (2015) 33.
[152] V. Srsen, X. Fant, R. Heald, C. Rabouille, A. Merdes, Centrosome proteins form an insoluble perinuclear matrix during muscle cell differentiation, BMC Cell Biol 10 (2009) 28.
[153] K. Wei, V. Serpooshan, C. Hurtado, M. Diez-Cuñado, M. Zhao, S. Maruyama, W. Zhu, G. Fajardo, M. Noseda, K. Nakamura, X. Tian, Q. Liu, A. Wang, Y. Matsuura, P. Bushway, W. Cai, A. Savchenko, M. Mahmoudi, M.D. Schneider, M.J. van den Hoff, M.J. Butte, P.C. Yang, K. Walsh, B. Zhou, D. Bernstein, M. Mercola, P. Ruiz-Lozano, Epicardial FSTL1 reconstitution regenerates the adult mammalian heart, Nature 525 (2015) 479-485.
[154] D.C. Zebrowski, S. Vergarajauregui, C.C. Wu, T. Piatkowski, R. Becker, M. Leone, S. Hirth, F. Ricciardi, N. Falk, A. Giessl, S. Just, T. Braun, G. Weidinger, F.B. Engel, Developmental alterations in centrosome integrity contribute to the post-mitotic state of mammalian cardiomyocytes, Elife 4 (2015).
[155] T.M. Mayhew, A. Pharaoh, A. Austin, D.G. Fagan, Stereological estimates of nuclear number in human ventricular cardiomyocytes before and after birth obtained using physical disectors, J Anat 191 (Pt 1) (1997) 107-115.
[156] A.F. Quigley, R.M. Kapsa, D. Esmore, G. Hale, E. Byrne, Mitochondrial respiratory chain activity in idiopathic dilated cardiomyopathy, J Card Fail 6 (2000) 47-55.
[157] B. Guertl, C. Noehammer, G. Hoefler, Metabolic cardiomyopathies, Int J Exp Pathol 81 (2000) 349-372.
[158] A.R. Moslemi, N. Selimovic, C.H. Bergh, A. Oldfors, Fatal dilated cardiomyopathy associated with a mitochondrial DNA deletion, Cardiology 94 (2000) 68-71.
[159] S.N. Schreiber, R. Emter, M.B. Hock, D. Knutti, J. Cardenas, M. Podvinec, E.J. Oakeley, A. Kralli, The estrogen-related receptor alpha (ERRalpha) functions in PPARgamma coactivator 1alpha (PGC-1alpha)-induced mitochondrial biogenesis, Proc Natl Acad Sci U S A 101 (2004) 6472-6477.
[160] W.A. Alaynick, R.P. Kondo, W. Xie, W. He, C.R. Dufour, M. Downes, J.W. Jonker, W. Giles, R.K. Naviaux, V. Giguère, R.M. Evans, ERRgamma directs and maintains the transition to oxidative metabolism in the postnatal heart, Cell Metab 6 (2007) 13-24.
[161] J. Luo, R. Sladek, J.A. Bader, A. Matthyssen, J. Rossant, V. Giguère, Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta, Nature 388 (1997) 778-782.
[162] J.D. Moreno, C.E. Clancy, Pathophysiology of the cardiac late Na current and its potential as a drug target, J Mol Cell Cardiol 52 (2012) 608-619.
[163] S.V. Thomas, P.P. Kathpalia, M. Rajagopal, C. Charlton, J. Zhang, D.C. Eaton, M.N. Helms, A.C. Pao, Epithelial sodium channel regulation by cell surface-

associated serum- and glucocorticoid-regulated kinase 1, J Biol Chem 286 (2011) 32074-32085.
[164] L. Yang, G. Frindt, F. Lang, D. Kuhl, V. Vallon, L.G. Palmer, SGK1-dependent ENaC processing and trafficking in mice with high dietary K intake and elevated aldosterone, Am J Physiol Renal Physiol 312 (2017) F65-F76.
[165] D. Pearce, The role of SGK1 in hormone-regulated sodium transport, Trends Endocrinol Metab 12 (2001) 341-347.
[166] Q. Wang, J. Shen, I. Splawski, D. Atkinson, Z. Li, J.L. Robinson, A.J. Moss, J.A. Towbin, M.T. Keating, SCN5A mutations associated with an inherited cardiac arrhythmia, long QT syndrome, Cell 80 (1995) 805-811.
[167] E. Zaklyazminskaya, S. Dzemeshkevich, The role of mutations in the SCN5A gene in cardiomyopathies, Biochim Biophys Acta 1863 (2016) 1799-1805.
[168] H. Abriel, Cardiac sodium channel Na(v)1.5 and interacting proteins: Physiology and pathophysiology, J Mol Cell Cardiol 48 (2010) 2-11.
[169] C.A. Ahern, J.F. Zhang, M.J. Wookalis, R. Horn, Modulation of the cardiac sodium channel NaV1.5 by Fyn, a Src family tyrosine kinase, Circ Res 96 (2005) 991-998.
[170] N.M. Ashpole, A.W. Herren, K.S. Ginsburg, J.D. Brogan, D.E. Johnson, T.R. Cummins, D.M. Bers, A. Hudmon, Ca2+/calmodulin-dependent protein kinase II (CaMKII) regulates cardiac sodium channel NaV1.5 gating by multiple phosphorylation sites, J Biol Chem 287 (2012) 19856-19869.
[171] D.R. Brickley, C.A. Mikosz, C.R. Hagan, S.D. Conzen, Ubiquitin modification of serum and glucocorticoid-induced protein kinase-1 (SGK-1), J Biol Chem 277 (2002) 43064-43070.
[172] Y. Ruan, N. Liu, R. Bloise, C. Napolitano, S.G. Priori, Gating properties of SCN5A mutations and the response to mexiletine in long-QT syndrome type 3 patients, Circulation 116 (2007) 1137-1144.
[173] K.D. Poss, Getting to the heart of regeneration in zebrafish, Semin Cell Dev Biol 18 (2007) 36-45.
[174] M.S. Dickover, R. Zhang, P. Han, N.C. Chi, Zebrafish cardiac injury and regeneration models: a noninvasive and invasive in vivo model of cardiac regeneration, Methods Mol Biol 1037 (2013) 463-473.
[175] V.J. Bezzerides, C. Platt, C. Lerchenmüller, K. Paruchuri, N.L. Oh, C. Xiao, Y. Cao, N. Mann, B.M. Spiegelman, A. Rosenzweig, CITED4 induces physiologic hypertrophy and promotes functional recovery after ischemic injury, JCI Insight 1 (2016).
[176] O. Bergmann, S. Zdunek, J. Frisén, S. Bernard, H. Druid, S. Jovinge, Cardiomyocyte renewal in humans, Circ Res 110 (2012) e17-18; author reply e19-21.