Ageing in the Zebrafish Heart

David Burns

Submitted for the degree of Doctor of Philosophy

Institute of Genetic Medicine

Faculty of Medical Sciences

Newcastle University

June 2014

Abstract

With advancing age there is a progressive decline in the function of the heart. In humans reductions in stroke volume and cardiac output occurs often resulting in cardiac disease and subsequent death. The pathology found in the heart due to advancing age is attributed to a reduction in cardiomyocytes which causes cardiac dysfunction and heart disease, leading to heart failure. Zebrafish are a valuable tool in studying ageing and heart disease. As zebrafish age they gradually senesce. This is similar to humans and other mammals. However the response of the zebrafish heart to ageing has not been explored.

The zebrafish heart changes due to ageing, with increased fibrosis and ventricular wall thickness. I have established new assays to measure proliferation and apoptosis in zebrafish cardiomyocytes using multiplexing of thymidine analogues and cleaved caspase 3, respectively. Using these developed assays it was discovered that these changes may be caused by an observed increase in cardiomyocyte apoptosis. This was coupled with no change in cardiomyocyte proliferation. These changes may be mediated by an increase in natriuretic peptide expression. In response to exercise, cardiomyocyte proliferation increases signalled by increased *gata4*, *nkx2.5*, *tbx5*, and *mef2c* expression and a reduction of natriuretic peptide expression. In the long term these genetic and cellular changes in the heart in response to exercise may slow some of the pathological changes observed in the heart.

Acknowledgements

During the course of this thesis I experienced many ups and downs. I can safely say that I am a different person now to that which begun this PhD almost 4 years ago. The highs I enjoyed and the lows that I overcame not only made me a stronger, more confident person but also made me appreciate the importance of the people around me. Without this support network around me I never would have finished.

I would like to thank my supervisors Dr Bill Chaudhry, Professor Deborah Henderson and Mr Andrew Owens. Without their faith in my ability I would have never had the opportunity to do a PhD. Their guidance and experience were essential factors in helping me finish this thesis. Although I didn't always agree with them!

I am forever grateful to members of the current lab; Paul, Vipul, Simon, Nina, Tash, Lorraine and Lindsay, along with past members; Iain, Jun, Nicola, Tania, Mike, Hannah, Darren, Helen, Veronika and Nic and also Gavin, Amy-Leigh and many more people at the IGM, who were not in my lab group but still helped me out. Not only have these people taught me valuable lab techniques and organisational skills, but they also understood the difficulties of working in Science and helped me get through it. They were awesome for a good night out!

My family and friends deserve special praise for keeping me sane throughout the course of this PhD. My Mam and Dad, Christine and Dave Snr. and my sisters, Julie and Donna with their respective partners, Amy and Graham gave me lots of support during my studying. I am grateful to my niece and nephew, Cassie and Alex, who gave me an excuse to return to my childhood and act like a big kid.

My friends were a huge area of support. In particular; Scotty, Lewis, Paul, Ant, Gibby, Rob, Lee and Mark. They all always had time to go for a pint, watch the match or go on a night out in Krash. Also, my dog Scrappy and guinea pigs Led and Zep who always had time to listen to me moan!

Last, but by no means least, I would like to thank the zebrafish and the core staff at the IGM and the CBC who run the zebrafish facility. So long, and thanks for all the fish!

Contents

List of Figures

List of Abbreviations

Abbreviation	Meaning
ANP	atrial natriuretic peptide
APAF-1	apoptotic protease activating factor 1
ATG	autophagy related gene
ATP	adenosine tripohosphate
AVN	atrioventricular node
BAD	Bcl-2-associated death promoter
BCL-2	B-cell lymphoma 2
BL	body length
BMP	bone morphogenetic protein
BNP	brain natriuretic peptide
BrdU	5-bromodeoxyuridine
CaMKII	ca2+/calmodulin-dependant kinase II
cAMP	cyclic adenosine monophosphate
CDC	cell division cycle genes
CDKs	cyclin dependant kinases
cGMP	cyclic guanosine monophosphate
CldU	5-chlorodeoxyuridine
CNCs	cardiac neural crest cells
Ct	cycle threshold
DEPC	diethylprocarbonate
DMSO	dimethyl sulfoxide
dpa	days post amputation
dpf	days post fertilisation
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EMT	epithelial–mesenchymal transition
EPDCs	epicardial derived cells
FGF	fibroblast growth factor
FHF	first heart field
GMT	GATA4, MEF2C and TBX5
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
IdU	5-iododeoxyuridine
IL	interleukin
Isl	islet
LAMP-2	lysosome-associated membrane protein 2
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MAPKs	mitogen activated protein kinases
MEF	myocyte enhancer factor

MHC	myosin heavy chain
MIMS	multi-isotope imaging mass spectrometry
MMP	matrix metalloproteinase
NPR-C	natriuretic peptide clearance receptors
OFT	outflow tract
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEO	proepicardial organ
PFA	paraformaldehyde
PHH3	phosphorylated histone H3
PKA	protein kinase A
ROS	reactive oxygen species
RT-qPCR	reverse transcriptase-quantative polymerase chain reaction
SAN	sinoatrial node
SERCA	sarcoendoplasmic reticulum (SR) calcium transport ATPase
SHF	second heart field
SHH	sonic hedgehog
SR	sarcoendoplasmic reticulum
TdT	terminal deoxynucleotidly transferase
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumour necrosis factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor
ZIRC	Zebrafish International Resource Centre

Chapter 1 : Introduction

1.1. Anatomy of the heart

1.1.1. *Mammalian cardiac anatomy*

The heart is found in the midline of the thorax and is a cone shaped muscular pump consisting of different compartments which are separated by valves. In adult humans the heart weighs 300g and is approximately the size of a fist (Malliaras *et al.*, 2013). The base is located anteriorly to the descending aorta and oesophagus, facing posteriorly. The Inferior surface nestles on the diaphragm at the central tendon. Two thirds of the heart lies to the left of the midline and one third to the right. Enclosing the heart is a fibroseous sac, called the pericardium (Song *et al.*, 2012).

Entering the right atrium of the heart is the vena cava, which carries deoxygenated blood from the body (Whitaker, 2010) (Figure 1.1). The vena cava is split into two portions, the superior vena cava and inferior vena cava. The superior vena cava enters the superior portion of the right atrium and forms from the convergence of the right and left brachiocephalic veins. The inferior vena cava enters the right atrium via the diaphragm.

From the right atrium, blood then enters the right ventricle, via the tricuspid valve. The tricuspid valve has three cusps: posterior, anterior and septal. Fibrous chordate tendinae attaches the cusps to papillary muscles. The wall of the right ventricle is normally 3-5mm thick with interweaving strands of muscle called the trabeculae carneae. Trabeculae are found in both ventricles and improve the efficiency of the heart by preventing suction which may occur if the walls of ventricles were flat (Hollmann *et al.*, 2007). Blood travels superiorly leaving the right ventricle through the smooth infundibulum, also known as the conus arteriosus, and the pulmonary valve, heading to the lungs. Blood travelling from the lungs enters the left atrium via the pulmonary veins. There are four pulmonary veins, two for each lung. None of them contain valves.

From the left atrium, blood then passes through the mitral valve, into the left ventricle. The mitral valve is composed of two cusps; a large anterior cusp and a smaller posterior cusp. The wall of the left ventricle is approximately 10mm thick and is also roughened by trabeculae. Blood exits the left ventricle via the outflow tract, through the
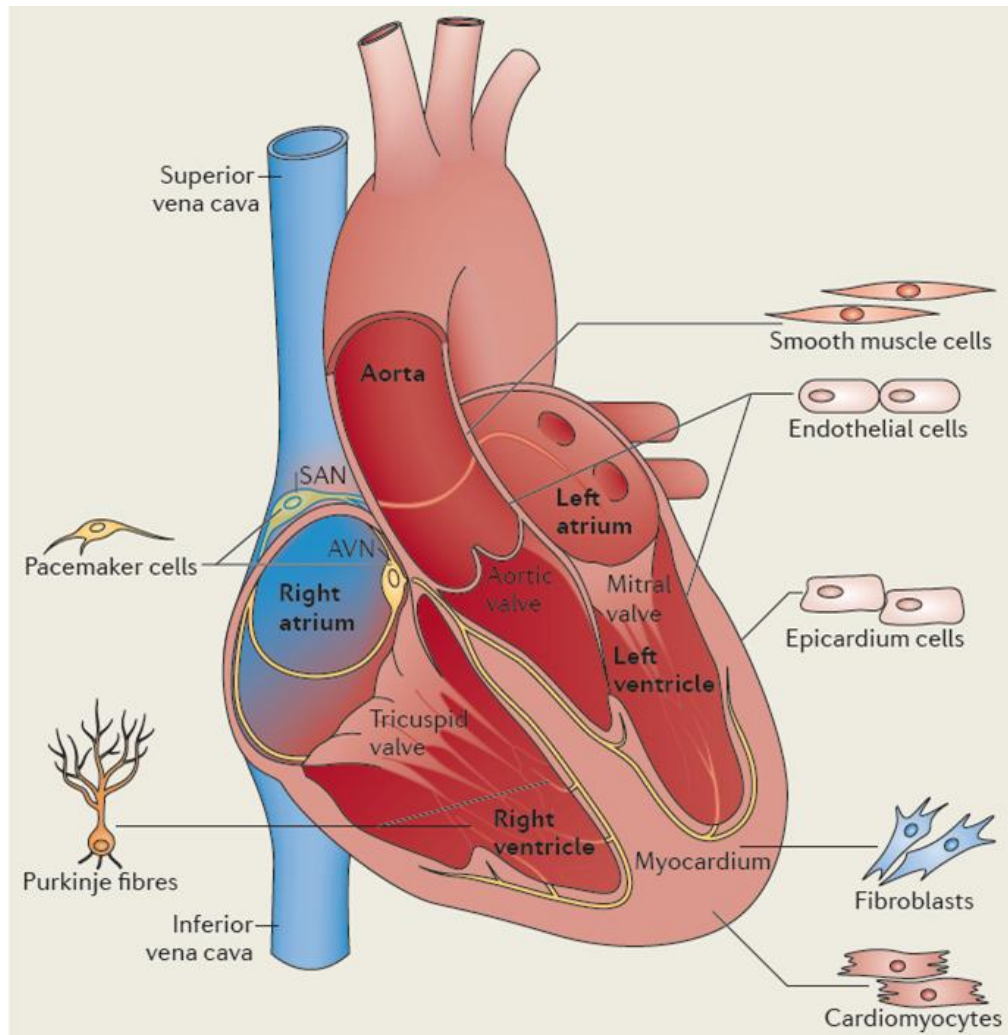
Figure 1.1:Anatomy of the mammalian heart
The mammalian heart consists of four chambers, two atria and two ventricles. The heart is primarily made up of cardiac fibroblasts and cardiomyocytes and also, to a smaller extent, smooth muscle cells, endothelium cells, epicardium cells and pacemaker cells. SAN= sino atrial node, AVN= atrio ventricular node

Adapted from Xin *et al.,* 2013

aortic valve. The aortic valve is composed of 3 cusps, one is anterior and two are posterior.

The right and left portions of the heart are sperated by a septum. Due to the thickness of the left ventricular wall, the septum bulges to the right. A fibrous skeletal framework which is composed of connective tissue, provides structural support to the heart. The fibrous skeleton is shaped like a figure of eight and is attached to the muscles of the four valves and four chambers. The skeleton physically and electrically separates the chambers and forms part of the septum.

The atrioventricular node is the source of electrical activation in the heart. The sinoatrial node is located in the wall of the right atrium, near the entrance of the superior vena cavae. The contractions spread along walls of the atria reaching the atrioventricular node, located in the right atrium in the left wall, and then the right and left atrioventricular bundles. The Purkinje fibres form a plexus, allowing a wave of excitation to spread to the walls of the ventricles. This allows the most inferior portion of the ventricles to contract first.

Blood is supplied to the heart by the coronary arteries. There are two coronary arteries; left and right, which are distributed on the surface of the heart. Blood is drained from the heart primarily by the great cardiac vein and to a lesser extent, the smaller cardiac veins. The heart is made up of many different cell types which forms a complete network of tissue (Figure 1.1).

1.1.1.1. Cardiomyocytes

In order to fulfil the primary function of pumping blood around the body to maintain the various tissues and organs with adequate blood perfusions, cardiomyocytes within the heart must contract and relax. In the adult heart cardiomyocytes form compact rod shaped cells arranged lengthways, from the apex to the base in the case of cardiomyocytes in the cardiac walls, so that upon contraction the cardiomyocytes shorten and all chambers of the heart contract in unison (Harvey and Leinwand, 2011). The functional unit of cardiomyocytes are the sarcomeres. Sarcomeres are composed of repeating units of actin (thin filaments) and myosin (thick filaments) which facilitate contraction. Gap junctions between cardiomyocytes allow the wave of contraction to spread throughout the heart by allowing signals to be passed between cardiomyocytes in an electrochemical fashion.

Contraction of cardiomyocytes is possible due to the accurate control of $Ca2+$ entry into and out of the sarcoplasmic reticulum and the cell by ion channels and $Ca2+$

exchangers. The sarcoplasmic reticulum is a specialised organelle found in muscle cells with the purpose of storing Ca2+ and releasing Ca2+. The electrical activity of the sarcolemma, the cell membrane of cardiomyocytes, regulates contractility via the release of Ca2+. The action potential of cardiomyocytes is initiated when the sarcolemma depolarizes and activation of voltage gated Ca2+ channels sustain the plateau phase. Ca2+ is released by the sarcoplasmic reticulum in response to Ca2+ entry to the cardiomyocyte via the sarcolemma, which is orchestrated by intracellular calcium recpetors, known as ryanodine receptors, which mediate Ca2+ induced Ca2+ release. The released Ca2+ interacts with troponin C, causing contraction of the myofilaments by enhancing the interactions between actin and myosin causing the cardiomyocyte to shorten (Williams *et al.*, 1992; Aronsen *et al.*, 2013). Ca2+ is removed from the cytosol by transportation through the sarcolemma via the Na+/Ca2+ exchanger (Hilgemann, 2004) and reuptake by the sarcoplasmic reticulum through the sarco-endoplasmic reticulum Ca2+ transporter (SERCA) (Bers *et al.*, 2003). The cycle of Ca2+ in the cardiomyocyte result in results in contraction which is orchestrated by electrical contact via gap junctions between cardiomyocytes and the cardiac conduction system.

When cardiac output needs to be increased, during exercise or stressful situations, for example, the heart rate and force of contraction can be increased by more efficient and quicker Ca2+ transport and cycling respectively. This can be regulated by the activation of cardiac sympathetic nerves which cause the release of norepinephrine which binds to β-adrenergic receptors. This leads to cAMP generation and downstream protein kinase A (PKA) activation. PKA, when activated, can phosphorylate many proteins important for Ca2+ handling in cardiomyocytes, such as ryanodine receptors (Reiken *et al.*, 2003), Ca2+ channels (Hulme et al., 2003) and phospholamban, which regulates SERCA (Brittsan *et al.*, 2000; Kranias and Hajjar, 2012).

1.1.1.2. Fibroblasts

Although cardiomyocytes are the main cellular components involved in contraction of the heart, cardiomyocytes in mouse and rat hearts make up only 56% and 30% of the total cells, respectively. In the human heart this figure is even less, at only 28% (Vliegen *et al.*, 1991; Banerjee *et al.*, 2007). In the mouse and rat heart fibroblasts make up 27% and 64% of the total cells respectively. In the human heart this is even more, 72% of cells are fibroblasts (Vliegen *et al.*, 1991; Banerjee *et al.*, 2007). This demonstrates the interspecies variation of the cellular components of the heart

9

Fibroblasts in the heart are arranged in strands and sheets running parallel to muscle fibres where they maintain structural integrity between the different cells in various layers of the myocardium (Kohl *et al.*, 2005). The structure of the heart is maintained by cardiac fibroblasts by the production of extracellular matrix, such as collagen I and III (Weber, 2004; Brown *et al.*, 2005; Camelliti *et al.*, 2005; Kwak, 2013). Whilst cardiac fibroblasts are fundamental in maintaining the structure of the heart, they also play a vital role in physiological functions via interactions with other cells and extracellular matrix (Sullivan and Black, 2013). In order to fill physiological roles within the heart cardiac fibroblasts can respond to electrical, mechanical and chemical stimuli (Kohl *et al.*, 2005; Banerjee *et al.*, 2006; Goldsmith *et al.*, 2014). Cardiac fibroblasts also play in integral role in pathological remodelling. Therefore cardiac fibroblasts have important roles in cardiac structure, function and pathophysiology.

During ageing and disease it has been reported that the number of cardiac fibroblasts increases (Camelliti *et al.*, 2006; Kelleher *et al.*, 2013). The number of cardiac fibroblasts can increase locally during cardiac repair and remodelling, for example after myocardial infarction there is an increase in fibroblasts in and around the infarct zone (Holmes *et al.*, 2005) and cardiac scar tissue has persistently high levels of fibroblasts (Sun and Weber, 2000).

Cardiac fibroblasts respond to specific growth factors, hormones and proinflammatory cytokines, levels of which are generally increased during cardiac remodelling. Cardiac fibroblasts respond to such stimulation by proliferating and becoming more invasive and remodelling the heart by secreting matrix metalloproteinases and producing extracelluar matrix (Weber, 2004; Brown *et al.*, 2005; Camelliti *et al.*, 2005). Cytokines, such as as TNFα and IL-1β, and growth factors are also secreted by cardiac fibroblasts, promoting further proliferation, further contributing to adverse cardiac remodelling via increasing extracellular matrix turnover and regulating activity of MMPs and TIMPs, often leading to excessive fibrosis (Mann and Spinale, 1998; Jugdutt, 2003).

1.1.1.3. Other cells in the heart

There are a few other cells that contribute to the population of cells forming the entire heart. Specialised cardiomyocytes that are able to conduct and generate action potentials make up the sinoatrial node and Purkinje fibres (Conn, 2011). Cardiac valves and the lumen side of blood vessels are formed by endothelial cells, making up the endocardium. Blood vessels, such as coronary arteries and the outflow vasculature, are

supplemented by smooth muscle cells. Cells from the epicardium can supply precursors to cardiac fibroblasts and vasculature.

1.1.2. Zebrafish

Zebrafish (Danio rerio) are emerging as a popular model for both ageing and cardiac diseases, however no one has reported changes in the hearts of zebrafish due to ageing. Using zebrafish as a model of diseases has various advantages over humans and other animal models which can help supplement the understanding of diseases in humans.

Zebrafish originate in rivers and streams in India and parts of Asia and are popular as pets kept in tropical aquariums. Female zebrafish weekly lay up to 300 eggs that are males fertilize externally, facilitating the studying of the developing embryo. The property of external development has helped establish zebrafish as a model vertebrate for developmental biology, used to supplement knowledge garnered from internally developing mammalian models. However zebrafish also possess other desirable properties making them an attractive model such as ease of transgenesis, having the whole genome mapped, relative simplicity compared to mammals and ethical reasons.

1.1.3. Anatomy of the adult zebrafish heart

There are various anatomical differences between the zebrafish and mammalian heart. Understanding the key differences can help interpret data obtained when analysing zebrafish hearts and help take advantage of specific caveats of zebrafish hearts in experimental design, which would otherwise be difficult or impossible in mammalian hearts.

1.1.3.1. The circulatory system

The adult zebrafish heart consists of a single ventricle and atrium which is contained within a pericardial sac located between the pectoral bone and operculum, anteroventrally to the thoracic cavity (Figure 1.2) (Hu *et al.*, 2001). Blood returns to the heart via hepatic portal veins, anterior cardinal veins and posterior cardinal veins which enter the sinus venosus. Blood drains into the atrium which is located on the dorsal side of the ventricle. Blood is then pumped through the ventricle, along the bulboventricular
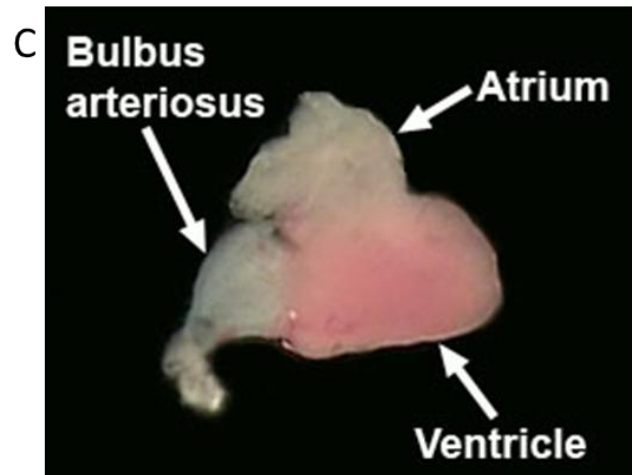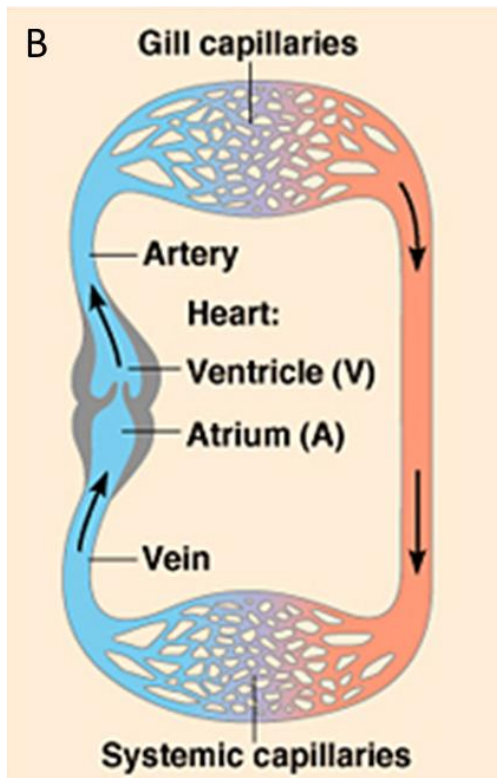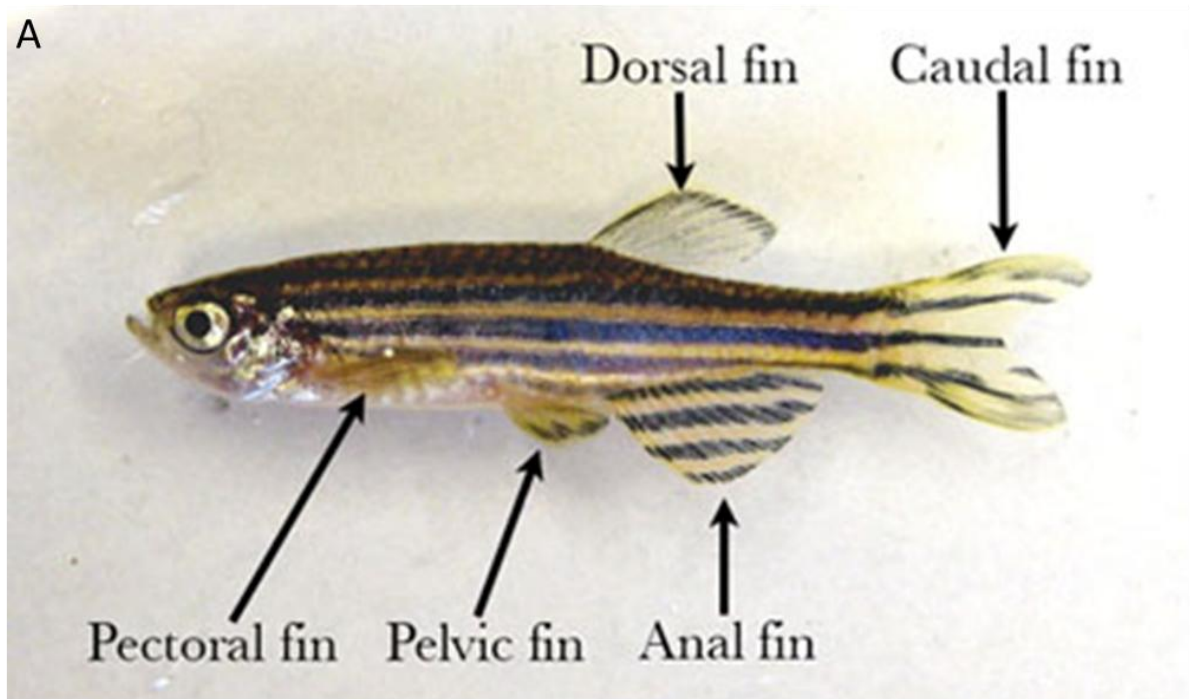
Figure 1.2:Anatomy of the zebrafish heart
An adult male zebrafish (A) has a closed loop circulatory system (B). This is common in all fish. Blue denotes deoxygenated blood and red oxygenated blood. Zebrafish hearts have two chambers; a ventricle and atrium (C). The bulbous arteriousus connects the ventricle to the aorta.

Adapted from Gupta and Mullins 2010.

orifice and into the bulbous arteriosus, a pear shaped appendage located between the ventricle and the ventral aorta. The ventral aorta is a cylindrical vessel connected to four pairs of afferent branchial arteries which branch off into the afferent branchial arterioles inside the gill filaments. The gill filaments are located under the operculum for protection, where oxygenated water flows over the filaments allowing oxygen to diffuse into the blood. Blood then flows into four pairs of efferent branchial arteries which converge to form the dorsal aorta.

1.1.3.2. The ventricle

The ventricle consists of an outer layer of epicardium, an inner layer of endocardium and muscular trabeculae in between. The muscular trabeculae forms a spongy network that spans the entire ventricle. When compared to higher vertebrates, the trabeculae in zebrafish hearts have fewer branching points, are longer and more radial and less organised. Trabeculae are vital in providing a large surface area to facilitate the exchange of nutrients and oxygen in the heart. Unlike mammals zebrafish do not have coronary vasculature so rely on the ventricles for gas and nutrient exchange. Trabecular folds reinforce the ventricle, particularly in the lumen where there is no trabeculation. At the anterior portion of the ventricle is the bulboventricular valve, which consists of two semilunar cusps; one on the right side of the ventricle and one on the left. The mass of the ventricle is directly proportional to the mass of the zebrafish, even throughout development (Barrionuevo and Burggren, 1999; Hu *et al.*, 2001), allowing for the development of higher blood pressure, increased cardiac stroke volume. The shape of the ventricle, pyramidal, allows pressure to develop, due to the small radius of curvature in the apex (Farrell and Jones, 1992). It is postulated that the shape of the ventricle is determined by the dynamic interactions between the lineage of the cells and the internal functional force acting upon them (Terracio *et al.*, 1988).

The majority of ventricular mass consists of the trabeculae, forming the spongy inner part of the heart. This is covered by a layer of compact myocardium. During development the compact myocardium forms first. Cardiomyocytes proliferate and then migrate towards the lumen to form the trabeculae (Bakkers *et al.,* 2008; Gupta and Poss 2012). The trabeculae maintain conformity of the ventricle during contraction and are essential for force generation. The ventricle has a relatively thin compact layer, when compared to higher vertebrates, with much more extensive and elongated trabeculae. There are many intertrabecular spaces (lacunae) which increases contractile efficiency

by increasing the pressure generated when compared to a heart with a smoothed wall (Taber *et al.*, 1993; Hu *et al.*, 2001). As coronary vasculature does not reach the trabeculae it is thought that the trabeculae receive oxygen and nutrition from luminal blood. Being long and slim gives the trabeculae a functional advantage of a shorter diffusion distance but may result in slower conduction velocity and a higher electrical resistance (Taber *et al.*, 1993; Hu *et al.*, 2001).

1.1.3.3.The atrium

The atrium is connected to the ventricle via fibrous cylindrical bridge, which encloses the atrioventricular orifice, on the dorsal side of the anterior portion of the ventricle (Hu *et al.*, 2001). The atrium has pectinate muscle radiating throughout the internal wall, originating near the atrioventricular orifice to the opposite wall. The atrioventricular valve consists of four leaflets located to the left, right, anterior and posterior to the atrioventricular orifice. The leaflets have no tension apparatus but are supported by papillary muscle and trabecular bands anchored to the atrioventricular junction. The pectinate muscle shortens during contraction, pulling the atrium towards the ventricle, thus forcing blood into the ventricle.

1.1.3.4.Zebrafish cardiomyocytes

Ventricular cardiomyocytes are larger than atrial cardiomyocytes. Despite the difference in size the percentage of mitochondria and proportion of myofibrils is similar. Myofibrils make up over half of the volume of cardiomyocytes and are centrally located, with mitochondria located to the periphery of sarcomeres. Trabeculae do not have a blood supply and therefore have abundant mitochondria in order to maximise energy production for cardiomyocyte contraction. Desmosomes connecting cells are frequently found throughout the ventricle and atrium. Cortical cardiomyocytes make up the spongy myocardium forming the trabeculae and primordial cardiomyocytes form compact myocardium at the walls (Gupta and Poss, 2012).

Cardiomyocyte size is thought to be inversely proportional to heart rate and maximal activity state and proportional to stroke volume (Poupa and Lindström, 1983), with the relatively fast heart rate of zebrafish and small mammals being attributed to small cardiomyocytes.

An experiment examining the origin of cardiomyocytes in zebrafish using multicolour clonal analysis demonstrated that all cortical cardiomyocytes in the adult heart originate from on average 8 original cardiomyoctes (Gupta and Poss, 2012).

These original cardiomyocytes have a clonally dominant phenotype reminiscent of stem cells.

1.1.3.5. Fibroblasts

Although the presence of fibroblasts have been reported in the regenerating zebrafish heart (Chablais *et al.*, 2011), it was only recently discovered that zebrafish hearts possess endogenous resident fibroblasts. Electron microscopy has identified a layer of cardiac fibroblasts and connective tissue between the compact myocardial layer and the spongy trabeculae (Lafontant *et al.*, 2013). This finding is in agreement with findings reported in hearts of tuna, atlantic salmon and rainbow trout which have similar heart physiology to zebrafish (Poupa *et al.*, 1974; Tota, 1978; Sanchez-Quintana *et al.*, 1996). In these fish between the spongy and compact myocardium there is a continuous layer of fibrous membrane. The fibroblasts and connective tissue may play a key role in maintain structural integrity by acting as an adhesive structure between the two distinct layers of the heart. This is in contrast to cardiac fibroblasts in mammalian hearts which are found throughout the heart and are the most abundant population of cells in the mammalian heart (Sullivan and Black, 2013). As the cardiac fibroblasts of zebrafish are confined to a discreet area of the heart, rather than interspersed throughout the heart like mammals, this would likely facilitate the studying of cells within the zebrafish heart. This compartmentalisation of cell populations in the heart allows cardiomyocytes to be identified with more confidence than in mammalian hearts, although a defined marker of cardiomyocytes is still required for correct identification.

1.1.3.6. Coronary Vessels

The coronary vessel provides the ventricle with oxygenated blood from the efferent branchial arch arteries via the coronary vessel located at the ventral surface of the bulbous arteriosus (Hu *et al.*, 2001). The coronary vessels form a network on the epicardium and penetrate and branch into the coronary capillaries reaching the compact myocardium but stopping before the subtrabecular layer. The structure of the coronary vessel is similar to that of higher vertebrates; the lumen is lined by a single layer of epithelial cells, spirally arranged pericytes make up the media and there is an outer layer of elastic lamina.

1.1.3.7.The Bulbous arteriosus

The bulbous arteriosus is a heavily ridged fibromuscular sructure which connects the ventricle to the aorta and consists of three distinct layers (Hu *et al.*, 2001). The outer layer (externa) is composed of layers of elastic lamina and smooth muscle. The middle layer (media) is made up of collagenous fibrils. The inner layer (intima) is composed of subendothelium, collagen, smooth muscle cells and elastic fibres. Despite containing muscle, the bulbous arteriosus is non-contractile but can stretch and does so at the same time as ventricular systole, returning to its original state upon diastole. This feature allows the bulbous arteriosus to act as an elastic reservoir, absorbing pressure during ventricular contraction and protecting the delicate vasculature of the gills and providing a constant flow of blood in the gills allowing optimum gas exchange (Johansen, 1965; Farrell and Jones, 1992). The bulbous arteriosus is analogous to the outflow tract of mammals and provides the same role (Malliaras *et al.*, 2013).

1.1.3.8.Cardiac function

The pressure developed by the atrium (systolic) is higher than that of the ventricle (diastole) which produces a pressure gradient (Hu *et al.*, 2001). Pressure in the atrium was lowest during the onset of atrial filling, coinciding with the initiation of ventricular ejection. Blood enters the ventricle early in atrial systole, for approximately two thirds of the cardiac cycle the ventricle is in diastole (397.7 milliseconds). The pressure in the ventricle rises rapidly due to closure of the atrioventricular valve, which in turn causes the opening of the bulboventricular valve, allowing blood to be pumped into the bulbous arteriosus. When ventricular pressure declines the bulboventricular valve closes.

1.2.Mammalian heart development and maturation

During the development of vertebrates the first organ to visibly develop is the heart. Cardiogenesis is a complex morphological process requiring migration of primordial heart cells, folding, looping, septation and subsequent maturation to produce a chambered heart. The initial stages of mammalian cardiac development are similar to those of zebrafish (discussed later). In the zebrafish heart septation does not occur and the heart has a single atrium and ventricle.

In the embryonic mouse, during the beginning of gastrulation, cardiac precursors, specified by the expression of cardiac specific transcription factors, migrate to the anterior primitive streak (Nakajima, 2010). Cardiac progenitors then undergo epithelial

to mesenchymal transition (EMT) ingressing through the anterior primitive streak forming the cardiac mesoderm. The cardiac precursors migrate laterally and anterior until they are located beneath the head folds forming a cardiac crescent at embryo day (E) 7.5 in mice (Figure 1.3) (Buckingham *et al.*, 2005; Aanhaanen *et al.*, 2011). The cardiac progenitors, which can be characterised by expression of *Nkx2.5*, *Gata4*, *Isl1* and *e/dHand*, move medially and ventrally until they fuse with the midline. This creates a linear heart tube by E8.0. The heart tube loops towards the right with the dorsal part becoming the inner curvature and ventral portion forming the outer curvature at E8.5. The morphology of the heart is almost completed by E10.5 with subsequent septation of the chambers and addition of the outflow track completed by E14.5 (Buckingham *et al.*, 2005; Aanhaanen *et al.*, 2011). During septation the systemic and pulmonary circulation separates through connection of the right ventricle to the pulmonary trunk and the aorta to the left ventricle respectively. Post septation the heart continues growing whilst many cells follow numerous, complex differentiating pathways to form the diverse, specialised cells of the mature heart.

The primitive linear heart tube is composed of endothelial cells which are separated from the external myocardial layer by a gel like matrix called cardiac jelly. Cardiac progenitors that produce the primitive heart tube are primarily found in the lateral most splanchnic mesoderm and are called the first heart field (FHF) progenitors. The second heart field (SHF) contributes to the primitive tube via addition of cardiac progenitors to the venous and arterial poles by migration from the pharyngeal and mediocaudal splanchnic mesoderm (Kelly *et al.*, 2001; Mjaatvedt *et al.*, 2001; Waldo *et al.*, 2001; Cai *et al.*, 2003). The FHF progenitors are the prominent source of the cardiomyocytes in the left ventricle, the SHF progenitors give rise to the whole right ventricle, a portion of the left ventricle, the proximal outflow tract and a large proportion of the atrial myocardium (Cai *et al.*, 2003; Buckingham *et al.*, 2005; Moretti *et al.*, 2006; Sun *et al.*, 2007).
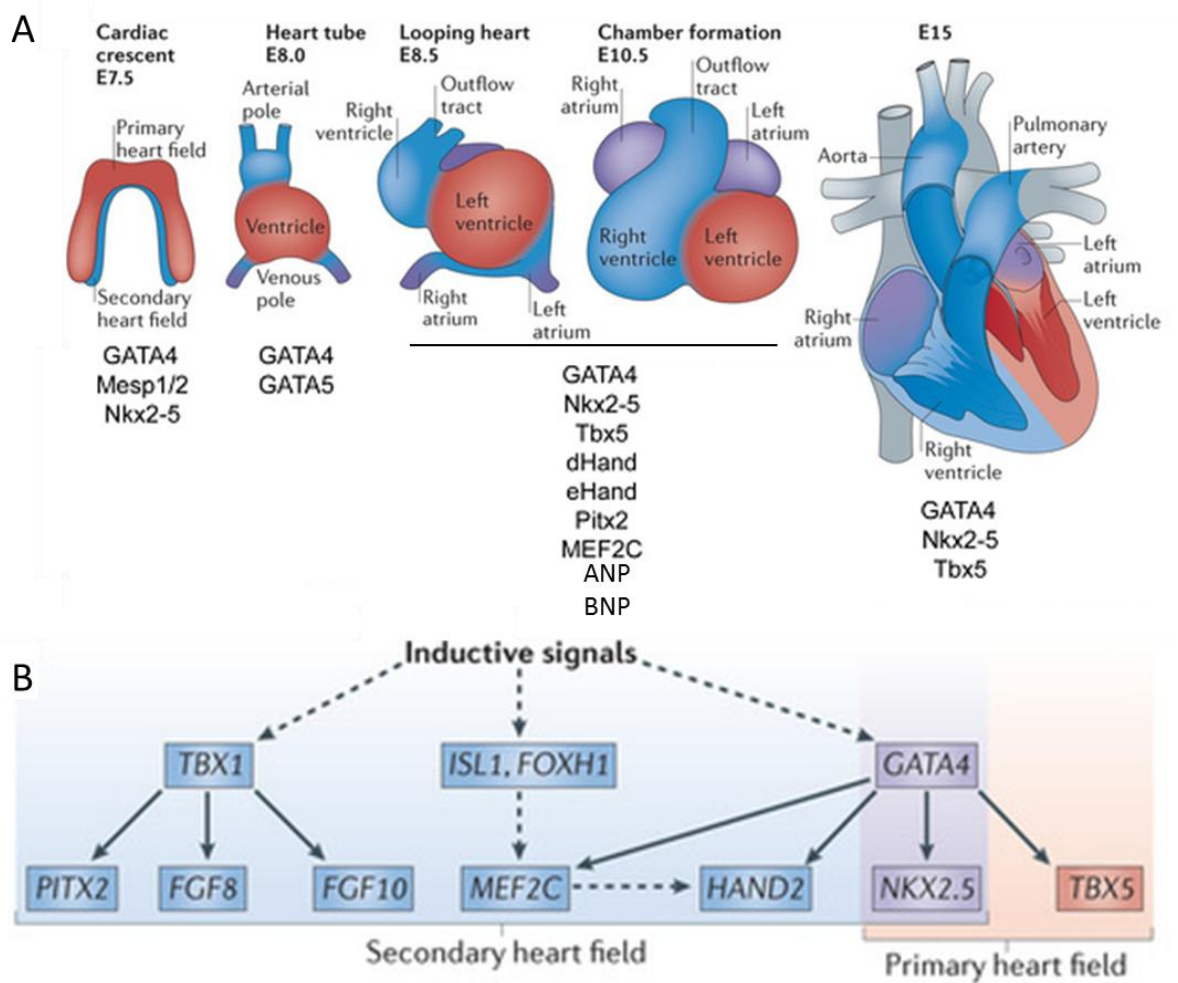
Figure 1.3:Mammalian heart development
A model of the key stages occurring in heart development from cardiac crescent through to maturation illustrating the predominant transcription factors expressed at each stage (A). The embryonic days are from mouse development. The signalling pathways involved in induction of cardiomyocytes (B).

Adapted from Xin *et al.,* 2013

At later stages addition of other cardiac cell populations occurs from various sources including the neural crest and epicardium. From the neural tube, cardiac neural crest cells (CNCs) migrate to the hearts arterial pole via the pharyngeal arches. CNCs are important in the remodelling of the outflow tract (OFT) and also add to the neurons and ganglia of the cardiac innervations and the smooth muscle layer of the aorticopulmonary septum (Snider *et al.*, 2007). Epicardial progenitors hail from the splanchnic mesoderm and form the proepicardial organ (PEO) at early stages. The PEO original lies adjacent to the sinus venosus and then migrates towards the myocardium, thus forming an outer layer termed the epicardium (Pérez-Pomares and de la Pompa,

2011). Few of the cells in the epicardium undergo EMT and form epicardial derived cells (EPDCs) which are vital in formation of interstitial fibroblasts, coronary vasculature and a minority may form cardiomyocytes (Cai *et al.*, 2008; Zhou *et al.*, 2008).

Trabeculation in the heart is initiated after looping. Trabeculation forms a network of projections into the lumen known as trabeculae (Chehrehasa *et al.*, 2009). Cardiomyocytes covered in a layer of endocardium make up the trabeculae. In the embryonic heart trabeculae play a vital role in increasing surface area required for diffusion of oxygen and nutrients until coronary vascularisation occurs (Ho *et al.*, 2002; Bustin *et al.*, 2009; Liu *et al.*, 2010). Remodelling of the trabeculae occurs with maturation of the cardiac wall coinciding with the proliferation of compact myocardium, conduction system maturation and formation of the coronary vasculature. Hypotrabeculation or hypertrabeculation in the ventricles is associated with impaired cardiac function in humans (Derveaux *et al.*, 2010; Taylor *et al.*, 2010).

Gene expression is tightly regulated throughout development of the mammalian heart. Gene expression controlling cardiogenesis is controlled temporally and spacially by a myriad of signalling networks (Kimmel and Meyer, 2010). Cardiac induction is initiated by many signalling pathways, most prominently Notch, WNT, bone morphogenetic protein (BMP) and sonic hedgehog (SHH). These signalling pathways control the expression of transcription factors that are essential for regulating downstream signalling cascades important in controlling the expression of genes essential for patterning and growth of the heart and also muscle specific genes. Transcription factors essential for heart development include GATA transcription factors, NKX2.5, MEF2c and TBX5 and the natriuretic peptides.

1.2.1. The fetal gene program

1.2.1.1. Natriuretic peptides

The hormones natriuetic peptides exert their effects on the endocrine and cardiovascular systems via actions on vasodilation, natriuresis, diuresis and rennin and aldosterone inhibition (Nishikimi *et al.*, 2006). The two main natriuretic peptides expressed in the heart are atrial natriuretic peptide (ANP), which is sometimes referred to as atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) which is also known as B-type natriuretic peptide. The genes of ANP and BNP are known as *NPPA* and *NPPB*, respectively Natriuretic peptides are first detected in the heart at E8.5 in the mouse, very early in cardiac development.

Cardiac expression of *NPPA* is restricted to the atria and *NPPB* is expressed in the ventricles and the atria. Upon secretion of ANP and BNP their N-terminals are cleaved activating the peptides which can bind to natriuretic pepide receptors. Receptors of natriuretic peptides are guanylyl cyclase receptors. When natriuretic peptides are bound to their receptors, guanylyl cyclase is activated which then converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (Gardner *et al.*, 2007). Termination of natriuretic peptide signalling is either by cleaving with endoperoxidases or removal from circulation by binding to natriuretic peptide clearance receptors (NPR-C) which subsequently internalises and then degrades the receptor-ligand complexes (Gardner, 2003; Richards, 2007). There is a negative feedback loop whereupon ANP transcriptionally represses the expression of its receptor via cGMP (Hum *et al.*, 2004). During embryonic development and early neonatally, ANP and BNP levels are high, whereas in healthy adult hearts ANP and BNP are absent (Gardner, 2003). ANP and BNP regulate cardiac growth during development by contributing to the regulation of cardiac transcription factors (Bruneau, 2011)

1.2.1.2. GATA transcription factors

GATA transcription factors consist of a DNA-binding domain, which is very highly conserved and is made up of a pair of zinc fingers which direct binding to the nucleotide motif (A/T)GATA(A/G), domains which mediate interactions with co-factors of transcription and a transcriptional activation domain (Molkentin, 2000). In vertebrates six GATA transcription factors have been discovered, due to expression patterns GATA transcription factors can be split into two subclasses; GATA-1, 2 and 3 are mainly expressed in cells with a haematpoietic lineage whereas GATA-4, 5 and 6 expression is in endoderm and mesoderm derived tissues like the liver, gut, lungs and heart (Molkentin, 2000). GATA4 is important in regulating development, differentiation, hypertrophic growth and survival in cardiomyocytes (Molkentin, 2000; Pikkarainen *et al.*, 2004). In the heart, GATA transcription factors have a similar expression profile.

Disruption of *Gata4* in mouse embryos causes lethality early in embryogenesis between days E7-9.5 because of problems in ventral and endoderm morphogenesis, however cardiac specific structural genes and tissue were still made in the embryos (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). When *Gata4* is specifically deleted in the murine heart embryonic lethality still occurs and the embryos posess hypoplastic ventricles (Pu *et al.*, 2004; Oka *et al.*, 2006). In humans cardiac septation abnormalities have been observed in people with a heterozygous mutation in *GATA4* (Garg *et al.*,

2003b). These observations underline the importance of GATA4 in cardiac development.

No cardiac defects have been obsereved in mice with null mutations in *Gata5*, however in zebrafish when *gata5* is knocked out a similar phenotype is observed to that of mice lacking Gata4 activity (Reiter *et al.*, 1999; Molkentin, 2000). The different response in zebrafish to GATA5 deletion compared to mice may be due to divergent evolutoion delegating different roles to the different GATA transcription factors. It may also be that the *Gata5* null mice do express a truncated form of the protein which may still function (Nemer *et al.*, 1999).

Gata6 null mice die at E5.5-7.5, before induction of the heart displaying defects in the extraembryonic endoderm (Morrisey *et al.*, 1998; Koutsourakis *et al.*, 1999). This has made studying the role of GATA6 in heart developmet difficult as GATA6 expression is a prerequisite in the earlier stages of development. However, *Gata6* null embryonic stem cells have been shown to be able to differentiate into myocardium when introduced into mouse embryos, indicating that GATA6 may not be essential for cardiac development (Koutsourakis *et al.*, 1999). In light of the experimental data it appears that GATA4 is the most important important GATA transcription factor for mammalian heart development with GATA5 being equally important in heat development in zebrafish.

1.2.1.3.MEF2 transcription factors

MEF2 was primarily identified as a myocyte specific enhancer binding factor in differentiated myotubes (Gossett *et al.*, 1989). MEF2 forms homo and heterodimers in order to gain DNA binding activity, the individual mefs are the products of four seperate genes; *MEF2A, B, C* and *D* (Molkentin and Olson, 1996; Black and Olson, 1998). The consensus sequence CTA(A/T)4TAG, which is located in the 5' transcriptional regulatory region of the majority of characterised cardiac and skeletal muscle structural genes, is the binding site of MEF2 dimers (Molkentin and Olson, 1996; Black and Olson, 1998). MEF2 signalling has prominent roles in heart development, muscle differentiation, bone development, neural crest development, T-cell development and many more (Potthoff and Olson, 2007).

The expression of many structural and contractile proteins is regulated by MEF2 in the heart. When a dominant negative Mef2c is overexpressed in cardiomyocblasts via the cardiac specific promoter Nkx2.5 (at approximately E7.5) in mice, differentiation of cardiomyocytes is inhibited (Karamboulas *et al.*, 2006).

Overexpression of a dominant negative *Mef2c* was shown to downregulate expression of Nkx2.5 and Gata proteins in cardiomyocytes, illustrating MEF2 plays a key role in regulating other cardiac transcription factors essential for cardiomyocyte differentiation. *Mef2c* is the first MEF2 expressed in chick and mouse, initially in the mesodermal precursors, in the secondary heart field, which go on to form the heart (Edmondson *et al.*, 1994). Not long after *Mef2c* expression, epression of other Mef2 transcripts occurs. Cardiac looping defects cause death at E9.5 in *Mef2c* null mice (Lin *et al.*, 1997), a spectrum of cardiac defects causes death perinatally in *Mef2a* null mice (Naya *et al.*, 2002). *Mef2d* null mice on the other hand, appear normal (Arnold *et al.*, 2007). Although embryonic leathality occurs due to defects in heart looping in *Mef2c* null mice, cardiomyocytes appear able to undergo differentiation pror to the occurance of looping defects (Lin *et al.*, 1997). When cardiac specific deletion of *Mef2c* occurs at E9.5, via the α myosin heavy chain promoter and cre recombinase, mice are viable (Vong *et al.*, 2005) suggesting that after the heart has looped MEF2C is dispensable, probably due to compensation of other MEF2s.

1.2.1.4.NKX2.5

NKX2.5 is a homeobox transcription factor that binds to the consensus sequences TNAAGTG and TTAATT. In heart development NKX2.5 plays a key role in regulating gene expression (Komuro and Izumo, 1993; Lints *et al.*, 1993). In early heart progenitor cells NKX2.5 expression is high during embryogenesis when cells are commiting to a cardiac lineage, in the adult heart *NKX2.5* continues to be expressed (Komuro and Izumo, 1993; Lints *et al.*, 1993; Kasahara *et al.*, 1998; Stanley *et al.*, 2002). Nkx2.5 deficient mouse models have been generated all of which die between E9-10 with an arrest of heart tube looping morphogenesis and a retardation of growth (Lyons *et al.*, 1995; Tanaka *et al.*, 1999; Biben *et al.*, 2000). Although *Nkx2.5* null embryos possess differentiated cardiomyocytes, expression levels of important cardiac transcriptional regulatory and structural genes is downregulated (Lyons *et al.*, 1995; Biben and Harvey, 1997; Zou *et al.*, 1997; Tanaka *et al.*, 1999; Bruneau *et al.*, 2000). NKX2.5 also appears important for conduction system development; heterozygotic mice or mice with cardiac specific deletions of *nkx2.5* have hypoplasia of the cardiac conduction system (Jay *et al.*, 2004; Pashmforoush *et al.*, 2004) such as anomalies in the atrioventricular node, ventricular septation and the conduction system (Schott *et al.*, 1998).

1.2.1.5.TBX5

TBX5 is part of the T-box transcription factor and is located on chromosome 12. Mutations in TBX5 can be responsible for Holt-Oram syndrome, a condition which afflicts bones in limbs in conjunction with cardiac defects (Basson *et al.*, 1997; Li *et al.*, 1997b). There have been many different mutations of TBX5 identifies in patients with Holt-Oram syndrome, all of which either alter gene dosage leading to haploinsufficiency or duplication, or produce normal levels of a defective protein. Haploinsufficiency of TBX5 results in ventricular and atrial septal defects, problems with trabeculation and cardiac isomerism (Basson *et al.*, 1994; Basson *et al.*, 1997; Basson *et al.*, 1999). When missense mutations occur in the T-box DNA binding domain, which is usually highly conserved, severe limb and cardiac malformations occur, mutations in the aminoterminal region cause the most severe cardiac defects and mutations in the carboxy terminal mainly cause defects in limbs (Basson *et al.*, 1994; Basson *et al.*, 1997; Basson *et al.*, 1999). The observed genotype-phenotype correlations may mean that TBX5 has different downstream targets in the heart when compared to the limb.

Various experiments have been conducted in order to elucidate the mechanism of action behind TBX5 insufficiency. When E15 chick hearts were injected with Tbx5 they developed smaller hearts with a reduction of trabeculae in the ventricle and thinner atrial walls (Hatcher *et al.*, 2001). Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) expression demonstrated that suppressed proliferation of cardiomyocytes may have been the reason for reduced heart size and reduction of trabeculae (Hatcher *et al.*, 2001). Further analysis in human embryos have demonstrated that in the heart, limbs and eyes tissue regions with high levels of TBX5 expression have low levels of proliferation and vice versa (Hatcher *et al.*, 2001). The inverse relationship between cellular proliferation and TBX5 expression suggests that TBX5 can function as a signal to arrest growth during organogenesis. Cessation of proliferation in the heart may certainly account for the increase trabeculae often observed in patients with Holt-Oram syndrome.

TBX5 has been demonstrated to interact with NKX2.5 so that can act in synergy to regulate the expression of target genes (Fan *et al.*, 2003; Garg *et al.*, 2003a). Many of the mutations in *TBX5* found in patients impair the ability of TBX5 to bind to NKX2.5 (Fan *et al.*, 2003). GATA4 has also been shown to interact with TBX5; a mutation in GATA4 found in some patients with cardiac septal defects prevented interaction with TBX5 (Garg *et al.*, 2003a). This illustrates that TBX5 has important

interactions with genes associated with the fetal gene program and may cause pathogenesis through its antiproliferative abilities.

1.2.1.6. GMT transcription factors

Interestingly, it appears that the transcription factors *GATA4*, *MEF2C* and *TBX5*, which are often collectively called GMT factors, can directly reprogram cardiac fibroblasts from mice into cells resembling cardiomyocytes, known collectively as induced cardiac like myocytes (Ieda *et al.*, 2010). This occurs with an efficiency of around 5-7% in vitro. When these induced cardiac like myocytes are grown in culture, contractions occur within 4-6 weeks. The inclusion of *HAND2* to the GMT factor cocktail appears to increase the efficiency to approximately 20% (Song *et al.*, 2012). After removal of the exogenously applied GMT factors, the induced cardiac like myocytes remain stable phenotypically and the expression of cardiac fibroblast specific genes is absent. This piece of data suggests that GMT factors are sufficient to promote cells, specifically cardiac fibroblasts, into cardiomyocytes and therefore 'synthesise' cardiomyocytes.

1.3. Mammalian cardiac maturation

Maturation of cardiomyocytes occurs in the weeks after birth. There is an increase in cardiomyocyte hypertrophy and binucleation (Li *et al.*, 1996; Soonpaa *et al.*, 1996). Sarcomeres also become more defined. There is a reported increase in type I to type IV collagen in adult hearts which is suggested to increase the compliance of the heart (Nakajima *et al.*, 2014). Indeed, in chick embryos it was demonstrated that in the embryonic heart, an initially large production of type IV collagen was followed by the increase in production of type I collagen after birth (Poddar *et al.*, 2013). A big increase in collagen gene expression has been reported in neonatal rat hearts following birth, accompanied by changes in the structure of collagen (Kumar *et al.*, 2014). This suggests that there is an increase the ratios and amount of collagen in the heart soon after birth, as the heart matures.

1.2.2. Hypertrophy

During the postnatal and adult period of life when cardiomyocyte proliferation almost ceases, cellular hypertrophy is the response cardiomyocytes make in order to fulfil increased workload and functional demands (Hill and Olson, 2008). Cardiomyocyte hypertrophy is also the adaptive response to loss of cardiomyocytes due to ageing. Many mechanisms have been implicated in cardiac hypertrophy which includes the

activation of neurohormonal factors, peroxisome proliferator activation in response to fatty acid oxidation and biomechanical stress due to increases in workload. Some neurohormonal factors which are thought to be invoved in cardiomyocyte hypertrophy are the catecholamines AngII, IGF-1 and ET-1 (Heineke and Molkentin, 2006). The cell detects biomechanical stress via multiple mechanisms which involve stretch sensitive ion channels, integrins and cytoskeletal proteins. The effects of some neurohormonal factors are mediated through tyrosine kinase receptors with the majority activating G-protein- coupled receptors which tend to converge in calcium signalling pathways. Alterations in calcium levels intracellularly can promote activation of MAPKs, protein kinases or phosphatises. Downstream these can then activate the ERK1/2, the calcineurin/NFAT pathway and the CaMKII/histone deacetylase pathways (Heineke and Molkentin, 2006; Muslin, 2008). In response to calcium or hypertrophy, transcription factors such as Mef2, Gata4 and NFAT are upregulated (Wei *et al.*, 1998; Diedrichs *et al.*, 2004; Wilkins *et al.*, 2004). These transcription factors regulate the expression of many cardiac genes such as SERCA2, ANP, troponins, αMH,cacineurin and desmin. Levels of calcium handling proteins such as SERCA2 and phospholamban are also upregulated by activation of the calcineurin/NFAT pathway (Prasad and Inesi, 2011). It is thought that hypertrophy in young and old animals occurs via the same mechanism, however in senescent cardiomyocytes this ability is lost (Chimenti *et al.*, 2003).

1.2.3. Binucleation

Two distinct temporally cycling phases have been observed in mammalian cardiomycoytes (Li *et al.*, 1996; Soonpaa *et al.*, 1996; Jonker *et al.*, 2007). The primary phase happens in the fetal heart and consists of cardiomyocyte proliferation. Most cardiomyocytes enter quiescence after birth with the rate of cardiomyocyte division reducing throughout gestation. In neonatal hearts the second phase of cycling is predominant which results in cardiomyocytes becoming binucleated due to the failure of cytokinesis (Li *et al.*, 1996). In conjunction with binucleation, cardiomyocytes tend to withdraw from the cell cycle but still possess the ability to re-enter the cell cycle, often due to cellular stresses. The change from hyperplasia to hypertrophy in cardiomyocytes is still mechanistically unclear but appears correlated with binucleation of cardiomyocytes. Cardiomyocytes that are binucleating appear to properly go through all of the stages of mitosis, except during telophase when abscission fails to follow furrow ingression (Li *et al.*, 1997a; Engel *et al.*, 2006b). Binucleation can be observed

25

in all mammals, one difference between species is the timing of binucleation, for example in mice and rats binucleation of cardiomyocytes occurs soon after birth (Li *et al.*, 1996; Soonpaa *et al.*, 1996; Jonker *et al.*, 2007) and in the final third of gestation in humans and sheep (Kim *et al.*, 1992; Jonker *et al.*, 2007).

The proportion of binucleated cardiomyocytes varies greatly between species; humans have 23-57% of cardiomyocytes binucleated (Schmid and Pfitzer, 1985; Olivetti *et al.*, 1996; Kajstura *et al.*, 2012a), sheep have around 70% (Burrell *et al.*, 2003; Thornburg *et al.*, 2011) and rodents have about 90% (Li *et al.*, 1996; Soonpaa *et al.*, 1996). In contrast to this, the hearts of zebrafish and newts are almost exclusively mononucleated (Matz *et al.*, 1998; Kikuchi and Poss, 2012). The reason for this difference between species in percentage of cardiomyocytes that are binucleated is currently unknown but may be due to species specific haemodynamic demand (Jonker *et al.*, 2007; Jonker *et al.*, 2010).

There remains a possibility that some cardiomyocytes may be more capable of proliferation than others within the adult heart in order to maintain homeostasis rather than all cardiomyocytes possessing an equal ability of proliferation. As binucleation is associated with loss of proliferation competence it may be that cardiomyocytes that are diploid and mononucleated have a higher proliferation potential. It may be that the few mononucleated cardiomyocytes found in mammalian hearts act as a potential reserve and upon stress may proliferate or binucleate in order to compensate contractile force within the heart. However it is not clear whether cardiomyocytes which proliferate need to dedifferentiate into a more fetal or stem like state or whether they already exist in a fetal or stem like state.

Sarcomere disassembly has also been associated with cardiomyocyte dedifferentiation and subsequent proliferation (Jopling *et al.*, 2011). There are cases of cardiomyocyte division in fetal (Ahuja *et al.*, 2004), neonatal (Engel *et al.*, 2005; Engel *et al.*, 2006a), and adult cardiomyocytes (Engel *et al.*, 2005) whilst still possessing a functioning contractile sarcomere. Just like how in cell division in other cells the cytoskeleton undergoes disassembly when entering mitosis (Heng and Koh, 2010), the disassembly of the sarcomere appears to be an unavoidable event. Reversible disassembly of cardiomyocytes can be achieved via treatment with actin and tubulin destabilizing compounds (Rothen-Rutishauser *et al.*, 1998), illustrating that the sarcomere can be readily disassembled and reassembled and is dependent on cytoskeletal orgainisation. Failure of the sarcomere to fully disassemble may be what causes binucleation, as the sarcomere could act as a mechanical barrier during mitosis,

preventing furrow ingression and mitosis (Li *et al.*, 1997b), however this is yet to be proven (Engel *et al.*, 2006a) and the theory of sarcomere disassembly in order for mitosis in cardiomyocytes may be premature in mammals, although this phenomenom has clearly been demonstrated in zebrafish cardiomyocytes (Jopling *et al.*, 2010).

1.2.4. Zebrafish heart development and maturation

Similar to mammals, migration of the cardiac precursors occurs after gastrulation forming a single heart tube in the embryonic zebrafish (Figure 1.4) (Stainier, 2001). The heart tube loops towards the left and differentiates to form a defined ventricle and atrium with an atrioventricular valve from endocardial cushions. The ventricle and atrium are composed of myocardium and endocardium and have myosin heavy chain isoforms that are specific to each chamber (Schoenebeck and Yelon, 2007). Although the genetic determinants leading to the formation of the heart tube are similar between mammals and zebrafish (Stainier, 2001), septation does not occur, in contrast to mammals and amphibians. Genes involved in mammalian heart development also play prominent roles in zebrafish heart development such as nkx2.5, gata4 and tbx5 (Bakkers, 2011).

Mesodermal angioblasts translocate forming a primitive vascular loop at the midline in cardiac development, later becoming the cardinal vein and aorta. Angiogenic sprouting occurs from the axial aorta and cardinal vein forming vascular throughout the zebrafish embryo (Lawson and Weinstein, 2002). The aortic arches remain symmetrical and attach to the gills, and lateral aortae which eventually merge forming one dorsal aorta. Similar observations have been made in embryonic mammals prior to aortic arch extension, regression and remodelling with the same key genes involved in vasculogenesis in both zebrafish and mammals, such as sonic hedgehog, notch and vascular endothelial growth factor (vegf) (Lawson *et al.*, 2002). At about 20 hours post fertilisation cardiac contraction is first observed, with circulation of the blood occurring at around 24 hours post fertilisation. The conduction system in hearts of adult zebrafish appears to be specialised with two trabeculae bands conducting atrioventricular contraction (Sedmera *et al.*, 2003). Separate atrial and ventricular depolarisation waves have been reported in embryos from 5 days post fertilization (Forouhar *et al.*, 2004). The mechanism and onset of trabeculation in zebrafish hearts is thought to be very comparible to that of mammals (Schwartz *et al.*, 1991). As chamber development occurs, compaction and thickening of the myocardial walls occurs along with the

formation and thickening of trabeculae (Schwartz *et al.*, 1991; Liu *et al.*, 2010; Jeppesen *et al.*, 2012).
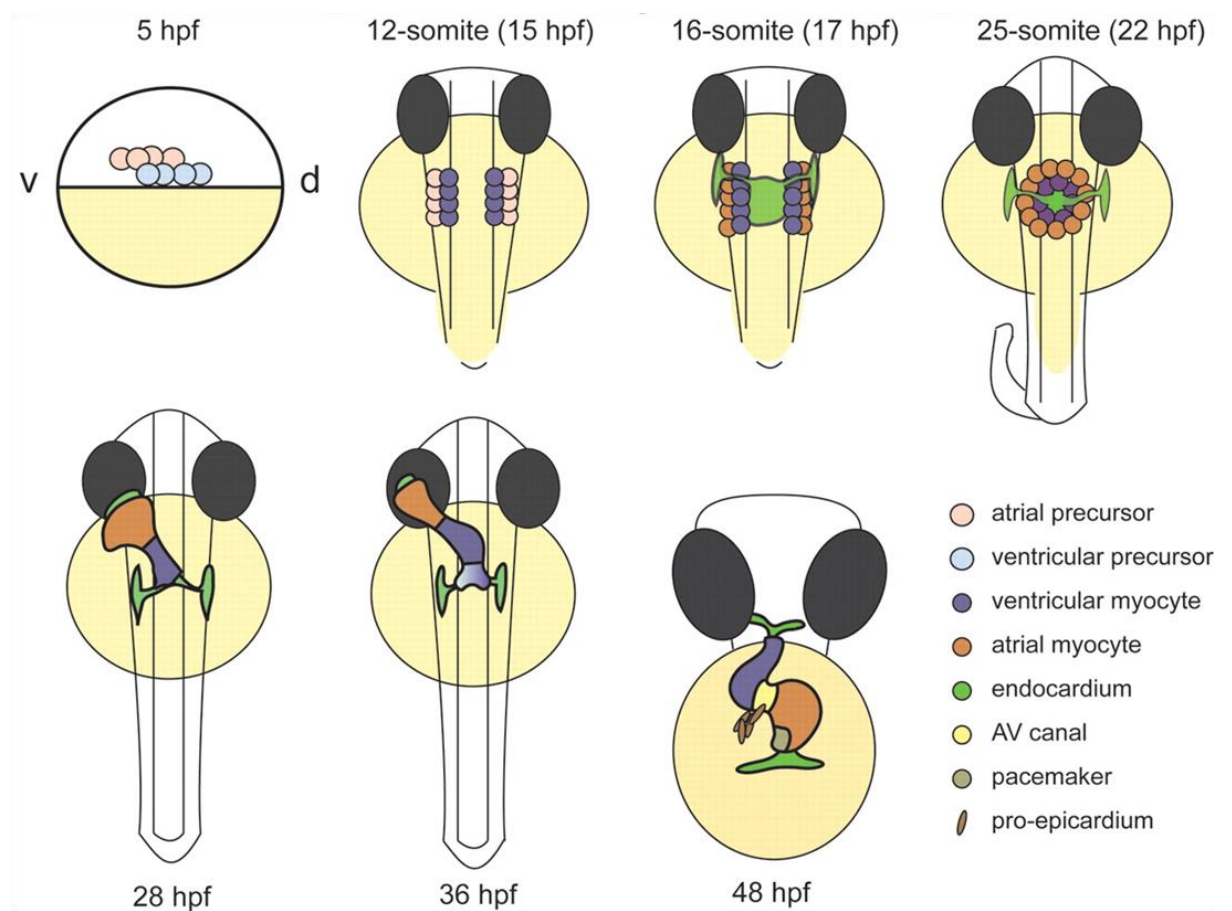
Figure 1.4 : Heart development in zebrafish
Atrial and ventricular precursor cells arrange assymetrically in at the midline in the embryo at 5 hours post fertilisation (hpf). The precursor cells express cardiac myosins and differentiate at 15hpf, continued expansion of cardiac tissue occurs by differentiation. At 17dpf endocardial cells migrate into the heart with the heart becoming a cardiac disc 22hpf. Morphogenesis of the heart continues with the heart becoming a linear tube at 28hpf. Expansion of the heart continues by differentiation of cardiomyocytes at the atrial pole of the heart tube with the heart tube looping to form an 'S-shape' at 48hpf.

Adapted from Bakkers *et al.,* 2011

After initial cardiac development, cardiac growth continues linearly with the length of zebrafish (Bertaso *et al.*, 2013). Cardac growth in zebrafish is a consequence of cardiomyocyte hyperplasia via the proliferation of exising cardiomyocytes. This is in contrast to mammals, where ardiac growh postnatlly is achieved through hypertrophy and binuleation (Soonpaa et al., 1996, Li et al., 1996).

In response to increased space or nutrients zebrafish can rapidly increase growth (Wills *et al.*, 2008). This rapid increase in growth coincides with cardiac growth via cardiomyocyte proliferation. In this scenario cardiomyocyte proliferation has been shown to occur via increased *aldah2* (the zebrafish orthologue of *RALDH2*) expression in the epicardium (Wills *et al.*, 2008). RALDH2 is the rate limiting enzyme for the production of retinoic acid, which is essential for growth and patterning of organs. In mouse heart the ablation of retinoic acid signalling in the epicardium prevents cardiac development (Chen *et al.*, 2002)

1.3. Ageing

The definition of 'ageing' can be relative to the area of interest. In this thesis I am using ageing to mean the time induced gradual accumulation of detrimental changes over many levels resulting in reduction of whole organism functionality. (Weinert and Timiras, 2003). Ageing has long been thought of as a progressive disorder with organ specific and cumulative functional consequences. Various diseases have been associated with ageing including Alzheimer's and Parkinson's disease, diabetes, dementia and various cancers. Moreover, cardiovascular-related morbidity and mortality are strongly associated with ageing (Lakatta, 1993; Najjar *et al.*, 2005).

1.3.2. *Ageing in the mammalian heart*

Functional and anatomical changes occur in the heart in response to ageing (Lakatta, 1993; Fleg *et al.*, 1995; Lakatta, 1999; Chang *et al.*, 2000; Schwartz, 2007). Often the left ventricle becomes hypertrophic, thickening of the heart wall is sometimes experienced, the diameter of the left ventricular chamber may differ compared to younger hearts and the heart may change shape becoming more globular than ellipsoid (Hees *et al.*, 2002).

There is often a reduction in cardiomyocytes as a percentage of total cells in the heart and cardiomyocytes are generally enlarged (Anversa *et al.*, 1986; Olivetti *et al.*, 1991). At end diastole the volume of blood in the heart can increase and pressure can be decreased in the left ventricle at the end of systole in elderly hearts compared to

younger hearts. Cardiac valves become stiffer and thicker in response to ageing with accompanied accumulation of extracellular matrix proteins. Pacemaker cells are often lost in the sinoatrial node with the conduction system accumulating fatty deposits and becoming fibrotic. A slower heart rate without increased stroke volume generally accompanies these maladaptive responses with a reduction in overall cardiac output. Arrhythmias occur more often in the elderly (Schwartz, 2007) mainly affecting diastole due to slower diastolic relaxation leading to diastolic heart failure (Hees *et al.*, 2002). In spite of the many age related changes in the heart, as long as the heart is disease free it is able to distribute an adequate blood supply in the body. On the other hand the aged heart is less capable of responding appropriately to increased work load, often due to stress, with reduced maximum oxygen consumption (VO2max) causing shortness of breath (Schwartz, 2007).

In the context of ageing reduced heart function, stress tolerance and manifestation of many diseases can be attributes to the reduction and altered function of cardiomyocytes. Cardiomyocytes are reduced due to apoptosis and necrosis (Anversa *et al.*, 1990a; Kajstura *et al.*, 1996) and hypertrophy, changes in activation, contraction and relaxation and the inability to sufficiently replace lost cardiomyocytes occurs during ageing (Figure 1.5) (Swynghedauw, 1999). At a molecular level a lot of these changes due to ageing are attributed to activation of genes that were active during development. This has lead to the 'fetal type gene programme hypothesis' (Figure 1.6).
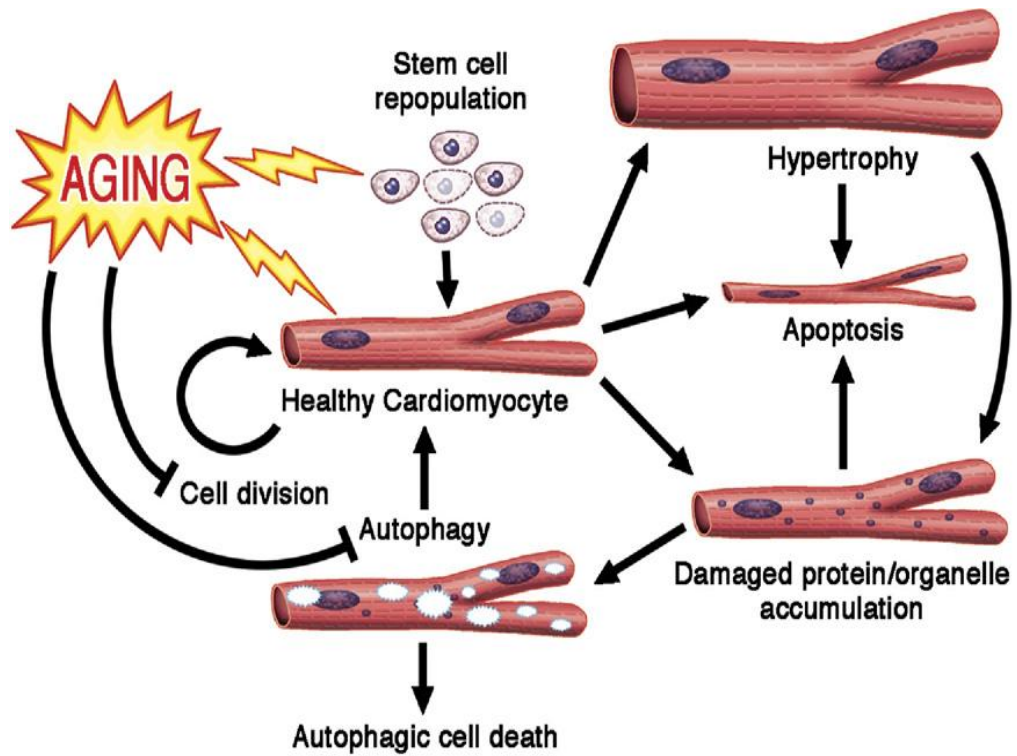
Figure 1.5: The affects of ageing on cardiomyoytes
Loss of cardiomyocytes is the main characteristic of ageing at a cellular level in the heart, due to an increase in apoptosis and a possible reduction in proliferation. The increase in apoptosis is partly due to an increase in accumulation of damaged organelles which are too much to remove by autophagy. In order to keep the heart functioning cardiomyocytes undergo pathological compensatory hypertrophy which puts the cardiomyocyte under increasing metabolic demand, causing more oxidative damage to organelles.

Adapted from Shah *et al.,* 2010
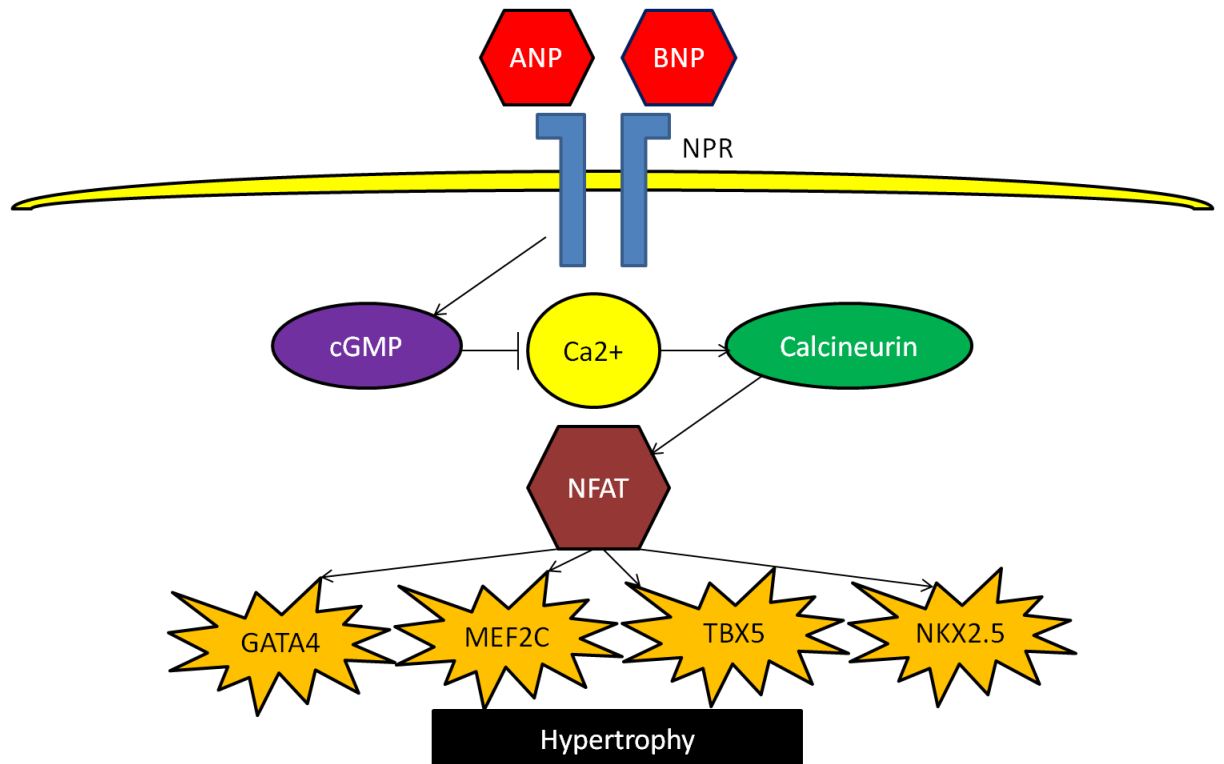
Figure 1.6: The fetal gene program
The natriuretic peptides ANP and BNP bind to natriuretic peptide receptors (NPRs) on the surface of cells causing an increase in cGMP. This inhibits calcium signalling leading to a decrease in expression of cardiac transcription factors. The overexpression of ANP and BNP during pathological hypertrophy is thought to be a failed attempt to prevent patholoical hypertrophy.

1.2.2. *Re-activation of the fetal gene program in response to ageing*

During pathological cardiac remodelling the fetal gene program is often reactivated (Figure 1.6). Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), contractile protein isoforms expressed in the fetal heart, such as β-myosin heavy chain and α-actin, and smooth muscle genes, for example smooth muscle actin, are genes that are expressed in the fetal heart that are also expressed in pathologic hearts but not expressed in healthy hearts after birth (Oka *et al.*, 2007a; Barry *et al.*, 2008). The expression of the fetal gene program is thought to occur in response to pathological stress and cause molecular changes which leads to pathological changes in the heart.

A microarray conducted on isolated cardiomyocytes from young (4 months old) and old (20 months old) mice identified 43 transcripts that significantly change with age (Bodyak *et al.*, 2002). Natriuretic peptides and transcription factors *Nkx2.5* and *Gata4* mRNA levels were reduced in aged hearts.

1.2.2.1.*Natriuretic peptides*

During embryonic development and early neonatally, ANP and BNP levels are high, whereas in healthy adult hearts ANP and BNP are absent (Gardner, 2003). When hypertrophic stimuli is present *NPPA* and *NPPB* expression is upregulated, via the transcription factor *GATA4*, with the role of reducing the response to the hypertrophic stimuli (Gardner, 2003; Richards, 2007). When *Nppa* is deleted in mice hypertrophy and hypertension occur under normal resting conditions and the response to pressure and volume overload is exaggerated when compared to wild-type control mice undergoing the same treatment (Wang *et al.*, 2003; Mori *et al.*, 2004). When hypertrophy does occur in *Nppa* deficient hearts the expression of extracellular matrix proteins is increased, illustrating that ANP could decrease the extent of pathological remodelling in response to hypertrophy via reducing the amount of extracellular matrix deposited (Wang *et al.*, 2003). Overexpression of natriuretic peptides in transgenic mice resulted in a decrease in blood pressure without any noticible cardiac phenotypes (Ogawa *et al.*, 1994). The anti-hypertrophic effects of ANP are thought to occur independent of changes in blood pressure; Anp deficient mice that consumed a low salt diet did not develop high blood pressure but still progressed to hypetrophy (Feng *et al.*, 2003). *Nppb* deletion in mice has been demonstrated to cause interstitial ventricular fibrosis, however unlike *Nppa* deletion, the deletion of *Nppb* did not cause hypertension or hypertrophy (Tamura *et al.*, 2000b).

Deletion of *Npr-a*, the receptor for ANP and BNP prevents their functionality system (Lopez *et al.*, 1997). Hypertension, hypertrophy, fibrosis and heart failure occurs in *Npr-a* null mice (Oliver *et al.*, 1997; Kishimoto *et al.*, 2001; Knowles *et al.*, 2001). The effects of Npr-a absence is also independent of elevations in blood pressure; *Npr-a* null mice treated with drugs to control hypertension progress to develop cardiac hypertrophy due to impaired natriuretic peptide signalling (Knowles *et al.*, 2001). Certain polymorphisms in *NPR-A* have been associated with an increase in left ventricular mass in humans with essential hypertension (Rubattu *et al.*, 2006). It may be that the polymorphism in *NPR-A* may somehow impair natriuretic peptide signalling.

The downstream signalling pathways that inhibit hypertrophy in natriuretic peptide signalling have not been full elucidated. Mitogen activated protein kinases (MAPKs) are thought to play a prominent role in exerting the effects of natriuretic peptides; p38Mapk and Erk activity is inhibited by Anp in smooth muscle cells and hearts of mice deficient in Npr-a possess higher basal levels of phosphorylated (activated) Akt, p38Mapk and Erk (Sharma *et al.*, 2002; Kilic *et al.*, 2005). cGMP appears to be a ubiquitous mediator of natriuetic peptide signalling. Anti-hypertrophic effects, similar to those exerted by natriuretic peptides, are observed when adult cardiomyocytes are stimulated with a cGMP analogue (Calderone *et al.*, 1998; Rosenkranz *et al.*, 2003). Anti-hypertrophic effects are also observed in cultured rat ventricular cardiomyocytes which are stimulated by nitric oxide via cGMP (Calderone *et al.*, 1998). The serine/threonine kinase, cGMP dependant protein kinase-1 (PGK-1), is thought to be the mediator of cGMP in the context of hypertrophy. In the heart PGK-1 exerts its affects by inhibiting Ca^{2+} channels, subsequently preventing calcineurin activating NFAT, which is a pivotal transcription factor for induction of hypertrophy (Sepulveda *et al.*, 1998). Indeed, mice deficient in Npr-a have higher intracellular Ca^{2+} in cardiomyocytes which causes an increase in calcineurin and Ca^{2+}/calmodulin-dependant kinase II (CaMKII) signalling (Kilic *et al.*, 2005).

If ANP and BNP can cause reductions in blood pressure, hypertrophy and fibrosis then why does hypertrophy occur in patients with high levels of ANP and BNP? It is hypothesized that natriuretic peptides do slow down the progress of hypertrophy however they are insufficient in preventing progress to hypertrophy in the context of prolonged exposure to overloading of pressure or volume. During some cases of congestive heart failure it has been reported that NPR-C, a receptor that metabolizes natriuretic peptides, can be upregulated reducing the affectiveness of ANP and BNP (Kuhn *et al.*, 2004).

High upregulation of BNP is so prevalent in cases of hypertrophy that it can be used as a biomarker in diagnosing and managing heart disease (Savarese *et al.*, 2013). BNP levels in the plasma of patients are useful in predicting hospitalisation and mortality due to heart failure and plasma BNP levels can guide clinical decision making leading to a better outcome for patients (Kim *et al.*, 1992; Koglin *et al.*, 2001; De Denus *et al.*, 2004; Moe, 2006).

1.2.2.2. Cardiac transcription factors

1.2.2.2.1. GATA transcription factors

In the adult heart *GATA4* expression acts as an important transcriptional regulator of many genes in the heart, such as *ANP*, *BNP*, α *MHC* and *β MHC* amongst others (Molkentin, 2000; Pikkarainen *et al.*, 2004). In cardiomyocyte cultures the expression of antisense GATA4 mRNA inhibited the expression of some cardiac specific genes, illustrating a direct role in maintaining regulation of transcription in adult hearts (Charron *et al.*, 1999). In response to hypertrophic stimuli GATA4 can mediate gene expression in the heart (Hasegawa *et al.*, 1997; Herzig *et al.*, 1997; Morimoto *et al.*, 2000; Liang *et al.*, 2001). In cardiomyocyte cultures the overexpression of *GATA4* was sufficient enough to cause hypertrophy (Liang *et al.*, 2001). Also in culture, expression of antisense mRNA or dominant negative GATA4 blocks transcription of genes regulated by GATA4 and prevents hypertrophy which would otherwise be induced by endothelin-1 and phenylephrine (Charron *et al.*, 1999; Liang *et al.*, 2001). In transgenic mice, a mild cardiac specific overexpression of *Gata4* caused hypertrophy (Liang *et al.*, 2001).

Transgenic mice with a cardiac specific reduction in expression of *Gata4* by 70% or 95% survived to adulthood but had an increased rate of cardiomyocyte apoptosis and developed heart failure and cardiac dilation (Oka *et al.*, 2006). In response to doxorubicin treatment, heterozygous *Gata4* null mice respond with a greater increase in cardiac apoptosis (Hautala *et al.*, 2001).

GATA4 is thought to be important in physiological growth, for example in response to exercise, rather than postnatal growth; mice with a cardiac specific deletion of *Gata4* were compromised in their ability to develop cardiac hypertrophy in response to pressure overload, whereas *Gata4* loss did not affect the growth of hearts postnatally (Oka *et al.*, 2006).

Stimuli which induce cardiac hypertrophy, such as pressure overload, agiotensin II, phorbol esters, phenylephrine and isooproterenol, phosphorylate GATA4, enhancing

its transcriptional activity (Hasegawa *et al.*, 1997; Morimoto *et al.*, 2000; Hautala *et al.*, 2001; Kitta *et al.*, 2001; Liang *et al.*, 2001; Kerkelä *et al.*, 2002). Serine 105 phosphorylation enhances the transcriptional potency and ability to bind to DNA in GATA4, whereas a mutation of serine 105 to alanine prevents transcriptional activity (Liang *et al.*, 2001). The phosphorylation of serine 105 in GATA4 can occur via p38MAPK and ERK1/2 signalling (Charron *et al.*, 1999; Liang *et al.*, 2001). As p38MAPK and ERK1/2 receive signals from many upstream pathways, the phosphorylation of serine 105 is believed to be an important point of convergance in regulating myocardial hypertrophy. Glycogen synthase kinase 3β (GSK3β) can negatively regulate GATA4 via phosphorylation suppressing its transcriptional activity (Jonker *et al.*, 2010). As many diverse signalling pathways can stimulate or inhibit the transcriptional activity of GATA4, this underlines the importance of GATA4 in converging signals induced by cardiac stress into an effective hypertrophic response.

1.2.2.2.2. *Myocyte enhancer factor 2 (MEF2)*

Some evidence has suggested that MEF2 signalling may be important in regulating hypertrophic growth of the heart in adults. When hypertrophy occurs due to pressure and volume overload DNA binding activity of MEF2 is increased (Molkentin and Markham, 1993; Nadruz Jr *et al.*, 2003). In cultured cardiomyocytes stretching has been shown to enhance DNA binding activity of MEF2 (Nadruz Jr *et al.*, 2005; Shyu *et al.*, 2005). In vivo when *Mef2a* or *Mef2c* was mildly overexpressed under the control of the αmhc promoter transgenic mouse hearts developed contractile dysfunction and dilation of the ventricular chamber with the predisposition of developing exasperated cardiac hypertrophy after pressure overloading (Xu *et al.*, 2006). Overexpression of Mef2 did not appear to cause 'classic' hypertrophy as isolated cardiomyocytes displayed an increase in lenth rather than cross sectional area, which is suggestive of cardiac dilation with the serial addition of sarcomeres (Xu *et al.*, 2006). In cultured cardiomyocytes a dilative phenotype was also observed in response to overexpression of *MEF2A* or *MEF2C* due to adenoviral transduction; neonatal cardiomyocytes degenerated sarcomeres and elongated (Xu *et al.*, 2006). Overexpression of *MEF2* indicates that rather than directly affecting cardiac hypertrophy *MEF2* regulates the serial addition of sarcomeres in cardiomyocytes, which can lead to cardiac dilation.

1.2.2.2.3. NKX2.5

NKX2.5 is thought to help regulate cardiac hypertrophy as *NKX2.5* expression is upregulated by stress stimulation and pressure overload. In a model of right ventricular pressure overload in felines or isoproterenol or phenylephrine mediated hypertrophy in mice *Nkx2.5* expression was upregulated (Thompson *et al.*, 1998; Saadane *et al.*, 1999). However, in transgenic mice over expressing *Nkx2.5* under the control of the ubiquitously expressed *β-actin* promoter, hearts were not hypertrophied despite the increased expression genes associated with cardiac hypertrophy, such as natriuretic peptides (Takimoto *et al.*, 2000). This suggests that although NKX2.5 can regulate the expression of some hypertrophic markers within the heart, NKX2.5 overexpression is not sufficient alone to cause cardiac hypertrophy. It may be that NKX2.5 can modulate hypertrophy in the adult heart as NKX2.5 has been shown to interact with other cardiac transcription factors including GATA4 (Durocher *et al.*, 1997; Sepulveda *et al.*, 1998; Shiojima *et al.*, 1999). NKX2.5 has also been shown to interact with the co factor calmodulin binding transactivator (CAMTA), which can regulate natriuretic peptide expression and mediate cardiomyocyte hypertrophy (Song *et al.*, 2006). Taken together, this data suggests that NKX2.5 is a vital regulator of gene expression in the heart but may only function as a modulator in the response to hypertrophy.

There may also be a role for NKX2.5 as a cardiac survival factor. When a dominant negative *Nkx2.5* is expressed in the murine heart cardiac dysfunction and degeneration occurs (Toko *et al.*, 2002). In response to doxorubicin injection increased apoptosis and a more poronounced cardiac dysfunction occurs in the *Nkx2.5* dominant negative hearts when compared to control hearts injected with doxorubicin (Toko *et al.*, 2002). The cardioprotective effects of NKX2.5 has been queried as overexpression in the heart leads to organ failure in murine hearts by 4 months of age, with hearts displaying abnormalities in conduction (Kasahara *et al.*, 2003). It appears that there is an optimum level of NKX2.5 expression, with too much or too little being detrimental, particularly for the conduction system.

1.2.2.3. Summary of re-expression of the fetal gene program

It appears that there are many different genes with various roles that are expressed in the embryonic heart during development and subsequently redeployed suring pathological ageing. Some of the genes are involved in the structure of the heart (*MHCs*), calcium handling (*SERCA*), hormonal (natriuretic peptides) and transcription factors (*GATA4*, *MEF2*, *NKX2.5*). Changes in expression of these genes has fundamental and far

reaching effects on downstream signalling pathways, which affect the behaviour of the cells the heart is comprised of.

1.2.3. Fibrosis

Fibrosis, the excessive formation of connective tissue, is a common feature associated with cardiac ageing (Biernacka and Frangogiannis, 2011). Fibrosis is not just limited to the heart and occurs in other organs due to ageing such as the liver (Hinton and Williams, 1968; Gagliano *et al.*, 2007), lungs, the pancreas and kidneys (Abrass *et al.*, 1995; Gagliano *et al.*, 2000).

Both animal models (Mukherjee and Sen, 1993; Kwak, 2013) and human studies (Song *et al.*, 1999; Burkauskiene *et al.*, 2006; Chung *et al.*, 2013) have demonstrated increased collagen deposition and fibrosis in the context of ageing in the heart. Collagen is a protein that is abundant in fibrous tissue. Myocardial stiffness is caused by an increase in fibrosis and in conjunction with inefficient cardiac relaxation can lead to diastolic dysfunction (Burlew, 2004; Olzinski *et al.*, 2013).

Studies of animal models have consistently demonstrated the age related increase of myocardial collagen. In rats left ventricular collagen content has been shown to increase from 5.5% total protein in young animals to 12% in older animals. Collagen content has also been shown to significantly increase with age in hearts of mice (Lin *et al.*, 2008) and rabbits (Orlandi *et al.*, 2004). It has also been shown that the proportion of different types of collagen changes; in rats there was an increase in the proportion of type III collagen to type I collagen in the myocardium (Mays *et al.*, 1988).

In human hearts similar observations have been made with collagen content increasing in the heart in the context of normal ageing (Gazoti Debessa *et al.*, 2001). Collagen content has been shown to increase by almost 50% between the ages of 30 and 70 years of age, as shown by autopsy studies of non-diseased hearts (Gazoti Debessa *et al.*, 2001).

The amount of collagen in the heart is regulated by the fine balance between matrix preservation, controlled by tissue inhibitors of metalloproteinases (TIMPs), and matrix degradation, controlled by metalloproteinases (MMPs). Differences in regulating MMPs due to ageing have been described in rat hearts with aged rats having reduced cardiac MMP-1 and MMP-2 expression and activity (Robert *et al.*, 1997; Kwak *et al.*, 2011). This has also been described in the livers of rats (Gagliano *et al.*, 2002) where although there was a net increase in collagen, the transcription of type III collagen was reduced and transcription of type I remained the same throughout the

animals life span. This suggests that fibrosis due to ageing occurs due to dysregulation of MMPs and matrix degrading pathways leading to subsequent 'mishandling' of collagen rather than an increase in collagen expression.

Fibrosis is an important contributor to the pathological reduction in cardiac function due to ageing. Initially fibrosis appears to compensate for the loss of cardiomyocytes but causes ventricular stiffening leading to diastolic dysfunctions. Understanding the changes in gene expression involved in induction of fibrosis is fundamental in understanding why alterations in extracellular matrix occurs in the ageing heart.

1.2.4. Transforming growth factor-β (TGF-β)

The cytokine TGF-β is important in proliferation, apoptosis, cell migration, differentiation and extracellular matrix production (Annes *et al.*, 2003; Baudino *et al.*, 2006; Sales *et al.*, 2006; Hinck, 2012). TGF-β can also stimulate synthesis of collagen and is an anti-inflammatory cytokine (Siwik *et al.*, 2000). There are three functionally and structurally related isoforms of TGF-β; TGF-β1, TGF-β2 and TGF-β3 (Annes *et al.*, 2003). In response to injury leukocytes and platelets release TGF-β which stimulates extracellular matrix component synthesis, such as collagens, integrins, fibronectin, and proteoglycans aiding tissue repair (Annes *et al.*, 2003). TGF-β mediates synthesis of collagen by increasing collagen expression and reducing the breakdown of collagen by decreasing matrix metalloproteinases (MMPs) activity levels or increasing levels of tissue inhibitors of matrix metalloproteinases (TIMPs), favouring a net increase in extracellular matrix deposition, particularly collagen (Siwik *et al.*, 2000). In transgenic mouse hearts, overexpressing *Tgf-β1* was reported to increase collagen type I and III, increase protein levels of Timp-1, 2 and 3 and also decrease mRNA and protein levels of collagenase (Seeland *et al.*, 2002).

Synthesis of TGF-β may occur as a physiological response to mechanical loading or exercise in skeletal muscle (Gavin and Wagner, 2001), smooth muscle (Gutierrez and Perr, 1999), circulating blood (Heinemeier *et al.*, 2003) and the heart (Calderone *et al.*, 2001). For instance, it has been reported that in the rat left ventricle *Tgf-β1* expression was increased after voluntary exercise in conjunction with physiological cardiac hypertrophy (Calderone *et al.*, 2001). Chronic, excessive over expression of TGF-β has been associated with fibrostic diseases, such as lung fibrosis, cardiac fibrosis and also scarring (Annes *et al.*, 2003).

In cardiovascular disease, TGF-β signalling may stimulate the accumulation of extracellular matrix proteins. In transgenic mice, overexpression of *Tgf-β* lead to cardiac fibrosis and hypertrophy (Rosenkranz *et al.*, 2002). Similarly, mice heterozygous for a *Tgfβ-1* missense mutation had a reduction in age induced myocardial fibrosis compared to WT littermates (Brooks and Conrad, 2000). As a result, mice deficient in *Tgf-β* had a shorter duration of cardiac contraction and less myocardial stiffness, which may be why significantly more Tgf-β deficient mice survived to the time of examination, 24 months, compared to WT mice (Brooks and Conrad, 2000). This body of data suggests that TGF-β signalling is an important mediator of extracellular matrix deposition and subsequently disease in the heart, particularly in response to loss of cardiomyocytes.

1.2.5. *Ageing in zebrafish*

The average lifespan of a zebrafish is approximately 3 years (Gerhard *et al.*, 2002) As zebrafish have regenerative abilities and some degree of indetermined growth, the use of zebrafish as a model of ageing is often questioned. However, with age, zebrafish gradually senesce, similarly to humans and other mammals. As zebrafish age they sometimes display spinal curvature which is due to muscular atrophy, rather than bone demineralisation, as demonstrated by electron microscopy, with older zebrafish muscle tissue myofibrillar disorganisation, sparse abnormally shaped mitochondria and absence of lipid storage deposits (Gerhard *et al.*, 2002). Zebrafish vertebrae showed no hallmarks of deformations due to compression, demineralisation of bone or osteophyte formation. Oxidised protein accumulation has also been identified in muscle of aged zebrafish along with increased β-galactosidase activity in the skin which is associated with senescence (Kishi *et al.*, 2003; Tsai *et al.*, 2007; Kishi *et al.*, 2008). In livers of aged zebrafish there is increased accumulation of lipofuscin, also known as ageing pigment, (Kishi *et al.*, 2008) which is similar to what has been reported in humans and mice (Jung *et al.*, 2007). Lipofuscin can also be detected in the retinal pigment epithelium of aged zebrafish along with lesions which are reported to be similar in age-related macular degeneration in humans. Zebrafish can develop cataracts and retinal atrophy due to ageing (Kishi *et al.*, 2008). Age related declines are also reported in reproductive and regenerative abilities (Kishi *et al.*, 2003; Tsai *et al.*, 2007; Kishi *et al.*, 2008).

1.3. The cell cycle

In most cases, in order to grow or replace lost cells in organisms, cells must proliferate and re-enter the cell cycle. Eukaryotic cells alternate from stages of genome doubling (synthesis, S-phase) and splitting of genomes (mitosis, M-phase) to produce daughter cells containing the same chromosomal content. The cell cycle is a series of signal controlled events from G1>S>G2>M, with quiescent cells in G0 (Figure 1.7A).

When cells are stimulated to proliferate by mitogens, signalling cascades are activated allowing the cell to proliferate (Alberts, 2007). Genes involved in control of the cell cycle are called CDC-genes (cell division cycle genes). The first step in the initiation, progress from G1 into S-phase is regulated by CDK1 (cyclin dependant kinase 1). CDK1 is one of many cyclin dependant kinases (CDKs). Cyclins are proteins that are formed and degraded at specific points throughout the cell cycle in which levels of cyclins will vary periodically. Cyclins bind to CDKs regulating there activity via phosphorylation. Controlled periodic cyclin synthesis and degradation is an important control mechanism of the cell cycle as levels of CDKs remain relatively constant throughout all stages of the cell cycle.

Intermediate filaments vimentin, desmin and lamins disassemble during mitosis under the duress of CDC2 kinase via phosphorylation of serine residues at the n-terminal. Dissassembly occurs at the end of prophase occurring at the same time as dissolution of the nuclear envelope. When cell division is completed they are dephosphorylated prior to the nucleus forming around the chromosomes of daughter cells.

Checkpoints stringently regulate the process of the cell cycle. The G1/S and G2/M checkpoints are vital in preserving fidelity of the genome. Deregulation of the cell cycle results in many malignancies including various types of cancer.
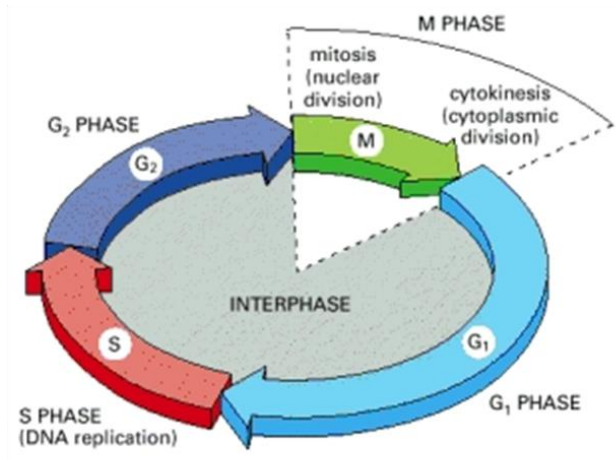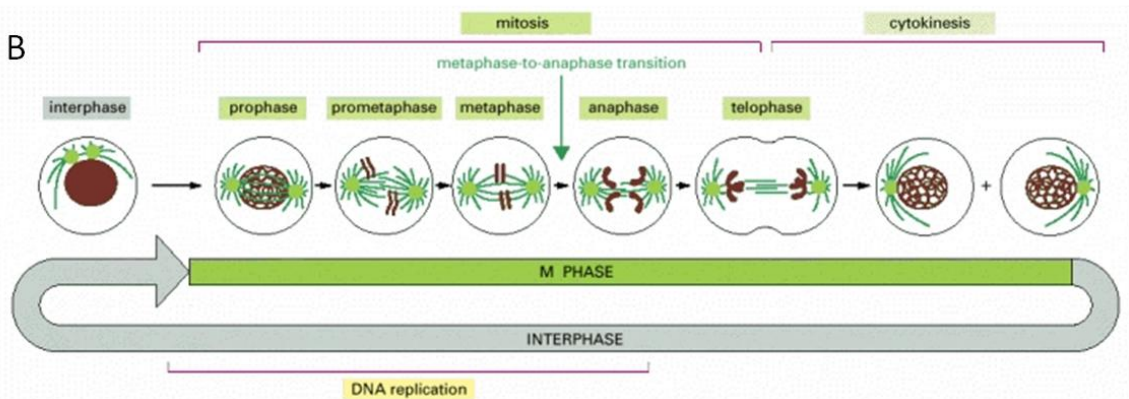
Figure 1.7: The cell cycle
In the cell cycle (A) M-phase is the stage when the cell undergoes mitosis. In interphase the cell continuosly grows. Interphase can be split into three distinct phases; S-phase, where DNA replication occurs and G1 and G2-phase, the gaps between M and S-phase and S and M-phase, respectively. M-Phase (B) consists of division of the nucleus (mitosis) and division of the cell (cytokinesis). The key stages of mitosis are depicted, showing the transition from prophase to anaphase, and also the key stages of cytokinesis.

Adapted from Alberts, 2002

1.3.2. Mitosis

Mitosis is the process whereby the cell divides splitting its nucleus and giving each of its progeny, daughter cells, a complete set of chromosomes (Figure 1.7B)(Alberts, 2007). In order for this to occur chromosomal DNA must be replicated prior to the splitting of cells, cytokinesis. Molecules that stimulate cells to proliferate are termed mitogens and are mainly proteins that activate signal transduction pathways which utilize mitogen activated protein kinases (MAPKs). Mitogens can be growth factors, cytokines, cellular stress proteins, neurotransmitters, cell adhesion ligands and hormones.

Prophase is the first stage in the cell cycle that resting (interphase) cells undergo. The nuclear chromatin becomes organized and condenses into thick strands eventually becoming chromosomes during prophase. Microtubules forming the cytoskeleton disassemble while the mitotic spindle forms around the nucleus at opposite poles of the cell.

The nuclear envelope breaks down in prometaphase into small membranous vesicles. Chromosomes proceed to condense and the nucleolus disappears. The mitotic spindle, which consists of microtubules, enters the nuclear region and kinetochores start to form on the centromeres.

In metaphase chromosomes align along the metaphase plate via the kinetochores. The chromosomes separate durning anaphase which proceeds as soon as chromosomes are aligned on the metaphase plate.

Chromosomes are pulled apart during anaphase by the spindle apparatus, migrating to opposite poles. Polar microtubules elongate further whilst kinetochores begin to disappear.

Telophase commences when the daughter chromosomes reach the spindle poles and are redistributed into chromatin. Cytokinesis, the division and subsequent cleavage of cytoplasm, occurs late in anaphase and throughout telophase. The nuclear membrane reassembles around each set of chromosomes when chromosomes are separated and relocated to the spindle poles.

1.3.3. Cardiomyocyte proliferation

1.3.3.1. Cardiomyocyte proliferation in humans

It has previously been believed that the heart is a post mitotic organ, where growth is achieved by cell hypertrophy rather than proliferation. However recent evidence has argued against this viewpoint. During the Cold War due to nuclear bomb testing

between 1955 and 1963, the levels of the radioactive isotope of carbon-12, carbon-14, was increased for a short period until the restriction of nuclear bomb testing. This created a spike of carbon-14 in the atmosphere which was incorporated into proliferating cells (Liebl, 2011). One experiment aiming to measure the rate of cardiomyocyte proliferation utilised this carbon-14 spike in a similar way to a thymidine analogue pulse chase experiment (Figure 1.8A) (Bergmann *et al.*, 2009). It was reported that annual cardiomyocyte turnover decreased throughout life from 1% at the age of 25 to 0.45% for 75 year olds, with around 50% of cardiomyocytes remaining throughout life. Although one criticism of this experiment may be that the assumption that carbon-14 is instantly and efficiently incorporated into human tissues may not be true (Broecker *et al.*, 1959) which may mean the cardiomyocyte proliferation rate is underestimated. Another criticism of this study is that the marker used to identify cardiomyocytes, cardiac troponin, may only be expressed in senescent cardiomyocytes (Kajstura *et al.*, 2010a; Kajstura *et al.*, 2010b). Another problem arises in the analysis of the data, data from young people (19 to 42 years old) was analysed differently to data from older people (50-73 years old) because of the changes in atmospheric carbon-14 after the cessation of nuclear bomb testing. This arguably creates two data sets. Also, 6 of the 12 samples examined showed signs of cardiac pathology, with one of the samples actually coming from someone who died of myocardial infarction. This can raise the question of whether this data is actually representative of the general population. A re-evaluation of the same data by a different group concluded that the average rate of cardiomyocyte proliferation was actually 18% per year (Kajstura *et al.*, 2010b). Also, 6 of the 12 samples examined showed signs of cardiac pathology, with one of the samples actually coming from someone who died of myocardial infarction. This can raise the question of whether this data is actually representative of the general population.

The halogenated thymidine analogue iododeoxyuridine (IdU) is incorporated into dividing cells during mitosis and can then be subsequently detected via immunohistochemistry (Tuttle *et al.*, 2010). IdU is also used as a radiosensitiser in patients with cancer (Kinsella, 1992). One group used this fortunate coincidence to assess cardiomyocyte proliferation in 8 hearts that were collected post mortem from patients with cancer who were receiving IdU for therapeutic reasons (Kajstura *et al.*, 2010b). It was discovered that IdU positive cardiomyocytes in individuals ranged from 2.5% to 46%, with an average yearly cardiomyocyte proliferation rate of 22%. Such a range may be due to the different ages of hearts used (20-74 years old), different doses and durations of IdU 'pulsing' and differences in time of death after IdU treatment.
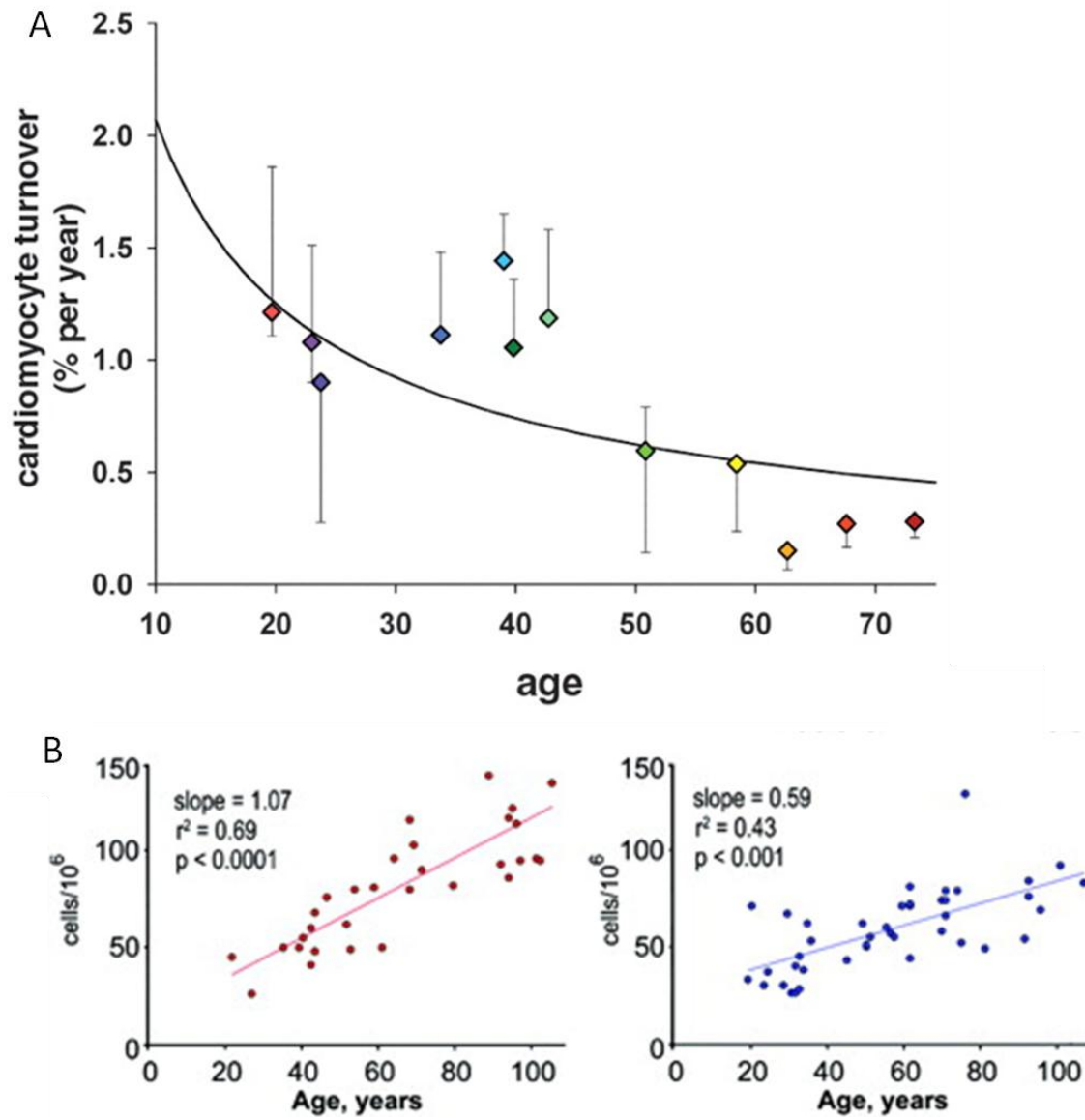
Figure 1.8: Cardiomyocyte proliferation in response to ageing
Bergmann's data using carbon-14 integration in order to assess cardiomyocyte proliferation at different ages illustrates a decline in proliferation due to ageing (A) (Bergmann et al., 2009). On the other hand, Kajstura's data using markers of mitosis and cytokinesis to assess cardiomycotye proliferation at different ages, demonstrates an increase in cardiomyocyte proliferation due to ageing in both men and women (B) (Kajstura et al., 2010a).

Due to the nature of this experiment it is not really possible to control for these factors and the only way to overcome this may be to assess a larger number of people, which again is difficult. It was also reported that the lifespan of cardiomyocytes was 4.5 years on average (Kajstura *et al.*, 2010b). Interestingly, the annual turnover of cardiac fibroblasts and endothelial cells was deemed to be 20% and 13% with an average cellular lifespan of 5 and 8 years respectively, suggesting these cell populations are less proliferative and longer lived than cardiomyocytes in healthy hearts.

In a separate study, Kajstura et al., measured the proliferation and apoptosis of cardiomyoctes in 42 male and 32 female hearts from people aged 19 to 104 years old, whose mortality was not related to cardiac disease (Figure 1.8B) (Kajstura *et al.*, 2010a). Proliferation was detected using the markers of S-phase Ki67 and phosphorylated histone H3 and the marker of M-phase, aurora B kinase. Although each of these markers possess certain drawbacks on their own, using a panel of markers over the course of cell division improves the reliability. It appeared that cardiomyocyte proliferation increased with age in both males and females. In males annual cardiomyocyte proliferation at 20 years was 7%, 60 was 12% and 100 was 32%. In females annual cardiomyocyte proliferation was 10% at 20 years, 14% at 60 years and 40% at 100 years. It was estimated that cardiomyocytes are replace 11 times in men and 15 times in women from during the period of 20-100 years of age (Kajstura *et al.*, 2010a). This is in contrast to what Bergmann *et al.,* 2009 reported, they found cardiomyocyte proliferation decreased with age and the magnitude of proliferation was much lower.

In a follow up experiment, Bergmann *et al.,* 2009 tested the reliability of cardiac troponin to identify all cardiomyocytes, not just a scenescent subpopulation as implied by Kajstura et al., giving reliability to their previous experiment using carbon-14 and cardiac troponin to measure cardiomyocyte proliferation (Bergmann *et al.*, 2009). It was demonstrated that when examining cardiac cell nuclei from the heart using flow cytometry and antibodies against pericentriolar material and cardiac troponin-T and cardiac troponin I there was no significant difference between pericentriolar material and cardiac troponin-T or cardiac troponin I (Bergmann *et al.*, 2011). This implies that using cardiac troponins to identify all cardiomyocytes is a reliable method and cardiac troponins do not only label scenescent cardiomyocytes, given credibility to the carbon-14 experiment (Bergmann *et al.*, 2009). Bergmann et al., also analyse the IdU data generated by Kajstura et al., (Kajstura *et al.*, 2010b). They report that the annual cardiomyocyte proliferation rate was too high to fit into any of their models and that

radiotherapy that the patients were receiving may have lead to DNA repair, so some of the IdU positive cardiomyocytes may be due to DNA repair rather than cardiomyocyte proliferation.

In order to address the discrepancies reported in terms of the amount of cardiomyocyte proliferation occurring and age associated changes in cardiomyocyte proliferation, an independent group re-examined data from Bergmann's carbon-14 experiment and Kajstura's experiment using markers of proliferation, using multiple mathematical models (Elser and Margulies, 2012). Using a hybrid mathematical model it was concluded from both data sets that cardiomyocyte proliferation does increase with age form 4-6% to 15-22% as long as each successive generation of cardiomyocytes has a higher rate of apoptosis than the previous generation (Elser and Margulies, 2012) (Figure 1.9). It was concluded that problems with the original studies were that they rely too heavily on assumptions within their respective mathematical modelling whereas the re-analysis of both datasets using a hybrid model explored different assumptions and evaluated plausible outcomes to reconcile discrepancies with estimated proliferation rates.

The debate over the amount of cardiomyocyte proliferation that occurs and the impact ageing has on turnover will undoubtedly continue. Regardless of the magnitude of cardiomyocyte proliferation a key paradigm has been established; cardiomyocyte proliferation does occur under normal physiological conditions in the adult human heart, however this is evidently not enough to ameliorate the loss of cardiomyocytes due to ageing, even if cardiomyocyte proliferation does increase with age.

1.3.3.2.Cardiomyocye proliferation in mice

Proliferation of cardiomyocytes also occurs in adult murine hearts. The proliferation of murine cardiomyocytes was first described in 1965. It was reported that the annual proliferation rate of 32 day old mice was 2.2% (Petersen and Baserga, 1965). Using titrated thymidine to measure cell turnover and a cardiac specific nuclear localised beta-galactosidase reporter to label cardiomyocytes, the yearly cardiomyocyte proliferation rate was determined to be 1.09% (Soonpaa and Field, 1997). Using BrdU pulsing to measure proliferation and α-actinin to identify cardiomyocytes, it was determined that 0.05% of cardiomyocytes were BrdU positive after three days of labelling (Boström *et al.*, 2010). This would give a daily cardiomyocyte proliferation rate of 0.16% and yearly turnover of approximately 6%. Another group used BrdU
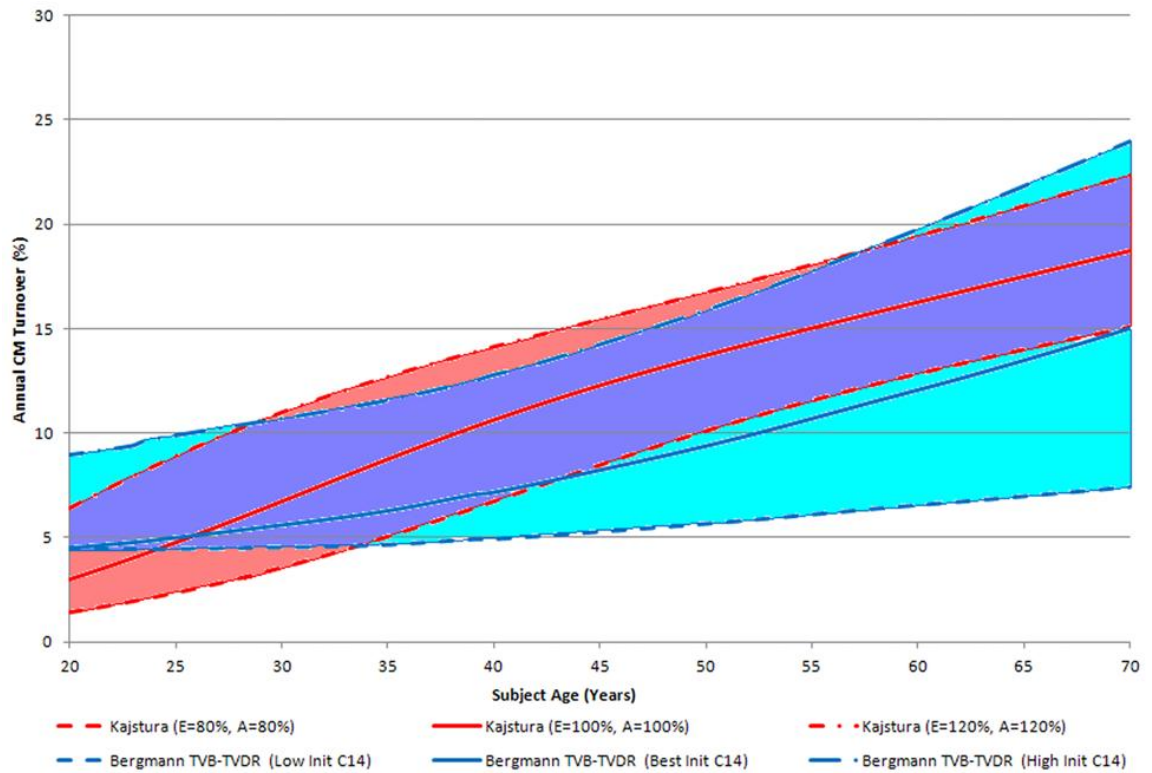
Figure 1.9: Hybrid mathematical model of cardiomyocyte turnover in the human heart

The hybrid model by Elser and Margulies, 2012 merged the data collected from C14 dating of cardiomyocytes by Bergmann et al., 2009 and Ki67 expression collected by Kajstura et al., 2010. The data from Kajstura et al., 2010 is plotted with three different variables regarding the assumption of the frequency of apoptosis ('A') and the expansion exponent ('E'). Data from the Bergmann et al., 2009 study is also plotted using three different assumptions based on the levels of atmospheric C14.

Adapted from Elser and Margulies, 2012

labelling and tropomysoin to identify cardiomyocyte proliferation (Bersell *et al.*, 2009). They discovered 0.15% of cardiomyocytes to be BrdU positive after 4 days BrdU labelling. This would give a daily cardiomyocyte proliferation rate of 0.375% and yearly rate of 13.6%. In these two studies the proliferation rate may have been over estimated, as the markers used to label cardiomyocytes, α-actinin and tropomyosin are not exclusive to cardiomyocytes, so other cell types may give false positive counts. Another study used genetic fate mapping to label cardiomyocytes and BrdU pulsing to determine that annual cardiomyocyte proliferation in 3-4 week old mice was 1.3% (Malliaras *et al.*, 2013). Overall this data implies that adult murine hearts are not post mitotic as cardiomyocyte proliferation does occur. However the mice used in these studies were all relatively young.

Pulse chasing experiments using multi-isotope imaging mass spectrometry (MIMS) have been conducted in order to try and elucidate the cardiomyocyte proliferation rate in different aged mice. MIMS is method that allows the user to image and measure stable isotope labels in areas of cells smaller than $1\mu m^3$ (Steinhauser *et al.*, 2012). To put this into perspective, the length of an average murine cardiomyocyte is 90μm (Hinrichs *et al.*, 2011). This means that labelling is extremely accurate due to the ability to be able to resolve cardiomyocyte cell borders. Pulse chasing was carried out by administering ^{14}N to mice for 8 weeks. It was determined that daily cardiomyocyte proliferation in neonatal mice (4 days old) was 1%, in young adults (10 weeks old) was 0.015% and in old adults (22 months) was 0.005% (Senyo *et al.*, 2013). Extrapolating from these values would give yearly cardiomyocyte proliferation rates of 365%, 5.5% and 1.8% in neonatal, young adult and old adult mice, respectively. These newly generated cardiomyocytes were determined to originate from the proliferation of pre-existing cardiomyocytes. This data is in agreement with that of Bergmann *et al.,* in that it is also concluded that cardiomyocyte proliferation declines in later life. It is not surprising that the proliferation rate of neonatal murine cardiomyocytes is remarkably higher than that of adult mice, as neonatal mice (under one week old) can muster a sufficient regenerative response to replace cardiac tissue lost after 20% ventricular resection (Porrello *et al.*, 2011).

However, as humans and mice have such different lifespans it is not really fair to compare cardiomyocyte proliferation in terms of yearly turnover. A much more meaningful way which would allow more direct comparisons would be to compare cardiomyocyte proliferation in terms of a percentage of life expectancy. I will use 100 years as the expected disease free life expectancy for humans, not accounting for

differences in sex. The average life expectancy of mice in laboratory conditions is 2.15 years, or 25.8 months, or 785 days (Green, 1966). Converting the data from Senyo et al., 2013 into a percentage of elapsed life expectancy rather than daily or yearly cardiomyocyte proliferation means that the neonatal mice (4 days old), young adult mice (10 week old or 70 days) and old adult mice (22 months) were 0.5%, 8.9% and 85.27% of the way through their expected lifespan, respectively. None of these ages falls onto the age ranges plotted by Elser and Margulies, 2012. However a cardiomyocyte proliferation rate of 1.8% in mice at 85.27% of their elapsed life span appears considerably lower than that of aged humans. In humans the range of cardiomyocyte proliferation at 70% of elapsed potential lifespan is between 7-24% (Figure 1.9).

Overall, this body of data suggest that cardiomyocyte proliferation does occur in mice. It would also be very interesting to see the spread of cardiomyocyte proliferation in mice. Currently there is only data on cardiomyocyte turnover in very young or very old mice. From this data it appears that proliferation does decrease with age. However, at a very young age proliferation would be expected to be high, as cardiomyocyte proliferation has often been reported as the main mechanism for cardiac growth in very early life. It may be that proliferation decreases in mice as they mature and then actually increases with advancing age, as in the Kajstura et al., 2010 model and the hybrid model (Elser and Margulies, 2012). Similar problems appear to arise in quantifying the amount of proliferation occurring as occurs in quantifying cardiomyocyte proliferation in humans. This again will be due to the differences in labelling methods used in both labelling proliferation and cardiomyocytes. The proliferation of cardiomyocytes appears to be low in humans and especially in mice when compared to proliferation of cells in other tissues (Michalopoulos, 2011; Andersson *et al.*, 2012). One reason for this may be the preference of cardiomyocytes to become hypertrophied and binuclear in order to grow in response to stress (Li *et al.*, 1996; Soonpaa *et al.*, 1996). However, in zebrafish cardiomyocytes are exclusively mononuclear (Poss *et al.*, 2002; Poss, 2007) and have a tendancy to proliferate in response to growth promoting stimuli (Jean *et al.*, 2012). This relative simplicity of zebrafish hearts in comparison to mammalian hearts makes them an ideal model for studying cardiomyocyte turnover as there would be an absence of noise from the occurance of hypertrophy and binucleaton which would occur in mammalian hearts.

1.3.3.3. Sources of new cardiomyocytes

It has been shown that cardiomyocytes can be stimulated to proliferate by exogenous factors in culture (Bersell *et al.*, 2009; Ieda *et al.*, 2010). These two areas of cardiomyocyte proliferation suggest that certain factors may be able to stimulate cardiomyocyte proliferation in whole organisms. Identifying these factors that can promote cardiomyocyte proliferation would be valuable in overcoming the limited regenerative response in human hearts.

Sources of new cardiomyocytes could consist of proliferation of mature pre-existing cardiomyocytes (Kajstura *et al.*, 1998), division of dedifferentiated cardiomyocytes (Jopling *et al.*, 2010; Kikuchi *et al.*, 2010) or from a pool of cardiac progenitor cells (Hsieh *et al.*, 2007). There is evidence to support all three as sources of new cardiomyocytes in different models and it may be that certain sources or multiple sources may be more beneficial or practical for regenerating damaged myocardium.

Division of mature cardiomyocytes is a rare event probably due to cardiomyocytes being tightly packed together, their highly organised sarcomeric protein and constantly undergoing contraction and relaxation making proliferation difficult. When proliferation has been reported after myocardial injury molecular and cytoskeletal dedifferentatation is often observed prior to cell division (Oberpriller and Oberpriller, 1974; Jopling *et al.*, 2010; Kikuchi *et al.*, 2010; Porrello *et al.*, 2011) which helps facilitate proliferation.

Increases in cardiac mass during development are due to the initial differentiation of cardiomyocytes followed by cardiomyocyte proliferation. The proliferative ability of cardiomyocytes is greatly reduced perinatally and instead hypertrophy is largely responsible for increases in cardiac mass. Up until recently the human heart was thought of as a post mitotic organ, this has since been disproved (Bergmann *et al.*, 2009; Kajstura *et al.*, 2010a; Kajstura *et al.*, 2010b). The source of new cardiomyocytes is currently still up to debate but as of yet no bona fide stem cells have been identified in the human heart which suggests proliferation occurs due to replication of already differentiated cardiomyocytes as in post-natal mice (Porrello *et al.*, 2011) and zebrafish (Jopling *et al.*, 2010). Promoting this proliferative response in humans could help treat and augment many cardiac diseases.

1.3.4. Potential problems measuring cardiomyocyte proliferation

The paradigm that the heart is a post mitotic organ has now been disproven. The main topic of debate is now not whether cardiomyocytes proliferate but the magnitude of

proliferation. Variations in rates of cardiomyocyte turnover comes from a variety of factors, the key factors being the marker used for measuring proliferation and the marker used for identifying cardiomyocytes (Soonpaa *et al.*, 2013).

The choice of cell cycle marker is key, as different markers of proliferation are expressed for different periods of the cell cycle. For instance, the period of S-phase lasts considerably longer than M-phase. This means that assays relying on DNA synthesis to identify proliferation, for example thymidine analogue incorporation, would give higher proliferation rates than assays that rely on the presence of mitotic figures, such as aurora b kinase. Markers which are expressed throughout the cell cycle, such as Ki67 or PCNA, would suggest even higher renewal rates.

Another source contributing to discrepancies in reported cardiomyocyte turnover is the identification of cardiomyocytes. There are many markers of cardiomyocytes available. Some, such as MEF2C are nuclear localised, whereas others can be cytoplasmic, such as Troponin-T. Differences in location of expression may influence the identification of cardiomyocytes. One of the gold standard ways of identifying proliferating cardiomyocytes was believed to be using immunofluorescence of Troponin T and a cell membrane marker, such as wheat germ agglutinin. When used in conjunction with confocal microscopy this method can be used to find nuclei of cardiomyocytes in sections of tissue (Leri *et al.*, 2005). However, in reality if the nuclei of a non-cardiomyocyte is close (less than 0.5µm) to the cytoplasm of a cardiomyocyte the resolution of confocal microscopy is insufficient to distinguish the two (Centonze and White; Pawley, 2010). This may give false positive identification of cardiomyocytes in some instances.

It appears that there are many caveats to selecting the most appropriate marker of both proliferation and cardiomyocytes. Selecting the most robust method for measuring turnover in zebrafish is vital.

1.3.5. *Markers of proliferation*

There are many markers of proliferation available to date, choosing the most suitable marker to robustly measure proliferation in zebrafish cardiomyocytes is vital.

1.3.5.1. Ki67

Ki67 is a protein that is associated with cellular proliferation and ribosomal RNA transcription (Prevedello *et al.*, 2005). Ki67 can be detected in all stages of the cell cycle except from G0 and is therefore present in all dividing cells (Forones *et al.*, 2005).

Ki67 is expressed in the nucleus and is commonly used in pathology and tumour prognosis (Schlüter *et al.*, 1993).

Studies have shown that ki67 expression correlates well with other markers of proliferation such as BrdU and proliferating cell nuclear antigen (PCNA) (Sakai *et al.*, 2002). Some studies have suggested that Ki67 is a superior marker of proliferation than PCNA (Pierce *et al.*, 1998). However, it has been reported that not all cells expressing Ki67 are actively replicating, especially in cells which are overexpressing p53 or p21 (Van Oijen *et al.*, 1998).

Although immunofluorescence using anti-Ki67 antibodies to detect proliferating zebrafish cells (Kimmel and Meyer, 2010; Goldshmit *et al.*, 2012) and human cardiomyocytes (Kajstura *et al.*, 2010a), there are no reports of Ki67 being utilised in the detection of proliferating zebrafish cardiomyocytes.

1.3.5.2. Proliferating cell nuclear antigen (PCNA)

PCNA is an important part of the DNA replication machinery and is involved in elongation, recombination, methylation and repair of DNA (Kisielewska *et al.*, 2005). PCNA is nuclear localised and expressed during s-phase of the cell cycle (Motiwale *et al.*, 2005). The detection of PCNA using immunofluorescence has been used extensively in zebrafish cardiomyocytes (Jopling *et al.*, 2010; Kikuchi *et al.*, 2011b; Schnabel *et al.*, 2011; Wang *et al.*, 2011)

1.3.5.3. PHH3

The compaction of chromosomes in the nucleus limits the number of proteins that can interact with DNA affecting mitosis. Chromatin fibre is composed of nucleosomes, repetitive units made up of octamers of core histones, two of each: H2A, H2B, H3 and H4 (Hans and Dimitrov, 2001). The compaction of chromosomes is maintained by histone-histone and histone-DNA interactions. Chromosome condensation during mitosis is achieved by phosphorylation of serine 10 on histone H3 which relaxes DNA compaction and allows DNA synthesis to occur (Hans and Dimitrov, 2001).

Highly specific antibodies have been developed which bind exclusively to phosphorylated histone H3 (PHH3) (Hendzel *et al.*, 1997; Wei *et al.*, 1998). Histone H3 is phosphorylated in mitosis while the chromosome is condensed; from prophase to early telophase (Hans and Dimitrov, 2001). Anti-PHH3 antibodies have been successfully used to detect cardiomyocyte proliferation in zebrafish cardiomyocytes (Jopling *et al.*, 2012).

1.3.5.4.Thymidine analogues

Thymidine analogues, such as radioactive thymidine and halogen based nucleoside analogues are incorporated into the DNA of dividing cells during S-phase at the expense of the nucleotide thymidine (Taupin, 2007). Thymidine analogues have to be exogenously administered, in laboratory animals this may be done via drinking water or intra peritoneal injections (Muskhelishvili *et al.*, 2003; Tuttle *et al.*, 2010). This can be disadvantageous compared to other markers of proliferation as administration of substances is not a prerequisite which may have problems such as determining the dose, method and timing of administration of thymidine analogues. On the other hand this can be a huge advantage as it means that careful planning of timing the delivery of two different analogues can label populations of proliferating cells at different timepoints (Tuttle *et al.*, 2010) rather than merely at the time the sample was fixed.

Thymidine analogue incorporation is irreversible; once a cell has been labelled with a thymidine analogue it will remain incorporated in the DNA and if proliferation occurs again this will be passed on to subsequent daughter cells. Importantly, thymidine analogue treatment does not appear to increase apoptosis or necrosis and does not affect cell viability (Diermeier *et al.*, 2004).

The first experiments for labelling proliferating cells involved tagging thymidine with a radioactive probe, such as tritium, and then detecting the radioactive probe using autoradiography or scintillation (Taylor *et al.*, 1957; Messier, 1958). The use of the halogen based thymidine analogue 5-bromodeoxyuridine (BrdU) superseded tritium labelled thymidine with the development of antibodies specific for BrdU allowing for the immunological detection of BrdU (Gratzner *et al.*, 1975; Gratzner, 1982; Taupin, 2007). Using BrdU proved to be advantageous over tritium labelled thymidine in for many reasons such as convenience and the ability to use BrdU with cell specific markers, allowing cell fate and lineage analysis to be possible (Gratzner, 1982; Taupin, 2007; Li *et al.*, 2008).

5-chlorodeoxyuridine (CldU) and 5-iododeoxyuridine (IdU) are also halogenated thymidine analogues similar to BrdU which can be detected by specific antibodies allowing multiplexing to occur in order to identify proliferating cells at different points in time (Yokochi and Gilbert, 2007; Tuttle *et al.*, 2010). Labelling and the efficacy to DNA is considered to be the same with all halogenated thymidine analogues.

During S-phase 5-ethynyl-2'-deoxyuridine (EdU) is incorporated into DNA, upon application and can be subsequently detected via a 'click' chemical reaction in which a fluorescent dye is covalently bound rather than antibody detection (Salic and Mitchison, 2008). The detection of halogenated antibodies via antibodies requires cell and nuclear membranes to be permeabilised and DNA denaturation in order for the antibodies to gain access, whereas EdU does not; the fluorescent probe is small and permeable enough to penetrate membranes and can be detected in double stranded DNA (Hua and Kearsey, 2011). This means that whole mount tissue and thicker sections can be used with less prerequisite knowledge required. However EdU is a relatively new technology and other 'EdU analogues' have not yet been developed allowing for multiplex labelling.

BrdU labelling has been used in zebrafish in many different studies to look at proliferation in various organs in adults and embryos such as the heart (Poss *et al.*, 2002), brain (Rowlerson *et al.*, 1997), liver (Her *et al.*, 2011) and retina (Zou *et al.*, 2013) amongst others. There are two main methods of administering BrdU in zebrafish; intraperitoneal injections (Poss *et al.*, 2002; Raya *et al.*, 2003) or immersion (Rowlerson *et al.*, 1997; Grandel *et al.*, 2006; Kaslin and Brand, 2013). Each method has its own particular advantages and disadvantages. Injection of BrdU uses a relatively small volume of BrdU but is much more invasive and the pulsing time can be variable. Immersion requires a much greater volume of BrdU but is much less invasive and the pulse time can be user determined.

1.3.5.5.Summary of markers of proliferation

Overall it seems like there are many different methods for labelling and measuring proliferation. This is most likely due to the different models and cell lines tested in each scenario. The major use for proliferation markers appears to be in tumour pathogenesis where they can be a valuable indicator of disease progress. In these cases proliferation is relatively high compared to cardiomyocyte proliferation.

The most important aspect of deciding which marker to use will be during which stages of the cell cycle the marker is expressed for and whether labelling proliferating at different timepoints would be valuable. Using thymidine analogues to label proliferating cardiomyocytes appears to be the best option in this instance for various reasons. Firstly, the length of thymidine analogue pulsing is user determined. As cardiomyocytes are not rapidly ycling cells this means that I can increase the pulsing time accordingly to increase the amount of proliferating cells counted. This is

important as many proliferating cells should be counted in order to accurately reflect the overall level of cardiomyocyte proliferation and to spot subtle changes in proliferation. If a different marker was used, for instance PCNA which is only expressed in S-phase, it would be expected that a very small minority of cardiomyocytes would be PCNA positive, meaning possibly hundreds of thousands of cardiomyocytes may need to be counted in order to count a suitable number of proliferating cardiomyocytes which would be extremely labour intensive. Failure to count sufficient numbers of cardiomyocytes could greatly skew the results, for example if only 1 proliferating cardiomyocytes were counted out of a total of 1,000 cardiomyocytes in one zebrafish and 2 in 1000 in another, proliferation would appear to have doubled, even though it may not be the case. Whereas with thymidine analogues the amount of cardiomyocytes proliferating in a 24 hour labelling period would be expected to be greater so more cardiomyocytes can be counted giving greater accuracy, for example if there was 100 proliferating cardiomyocytes in 1000 cardiomyocytes counted in one zebrafish and 200 in 1000 in another the risk of error would be reduced and it would be likely that cardiomyocyte proliferation had doubled. The other advantage of using thymidine analogues is that two different analogues can be used and identified separately allowing proliferation to be measured at different time points. This is a huge benefit as it would allow me to measure cardiomyocyte proliferation before and after particular intereventions aiming to increase cardiomyocyte proliferation.

1.3.6. Cardiomyocyte loss

There are three mechanisms by which cells die, apoptosis, necrosis and autophagic cell death. Understanding the mechanisms behind the loss of cells will be fundamental in interpreting data concerning loss of cardiomyocytes.

1.3.6.1. Apoptosis

In multicellular organisms each cell is a part of a highly organised system. The number of cells in this system is highly regulated by not only a tight control on cell proliferation but also which cells are programmed to die and therefore undergo apoptosis (Figure 1.10). If cells are no longer needed in the organism they will initiate intracellular death programs and commit suicide. In healthy adult tissues cell death and division should be in equilibrium; if this paradigm is unfulfilled then the tissue will grow which may be pathogenic (hyperplasia) or shrink (hypoplasia).

Apoptosis differs to necrosis, the process where cells are damaged by an acute injury and burst causing an inflammatory response, often damaging neighbouring cells. In contrast apoptosis is relatively clean: the cytoskeleton collapses causing the cell to shrink and condense and DNA is broken down. The shrunken cell displays altered receptors on its surface allowing neighbouring cells or macrophages to recognise it and phagocytose the apoptotic cell. This process not only prevents any unnecessary damage occurring to neighbouring cells, but also allows the components of the apoptosed cell to be recycled by the phagocytosing cell.
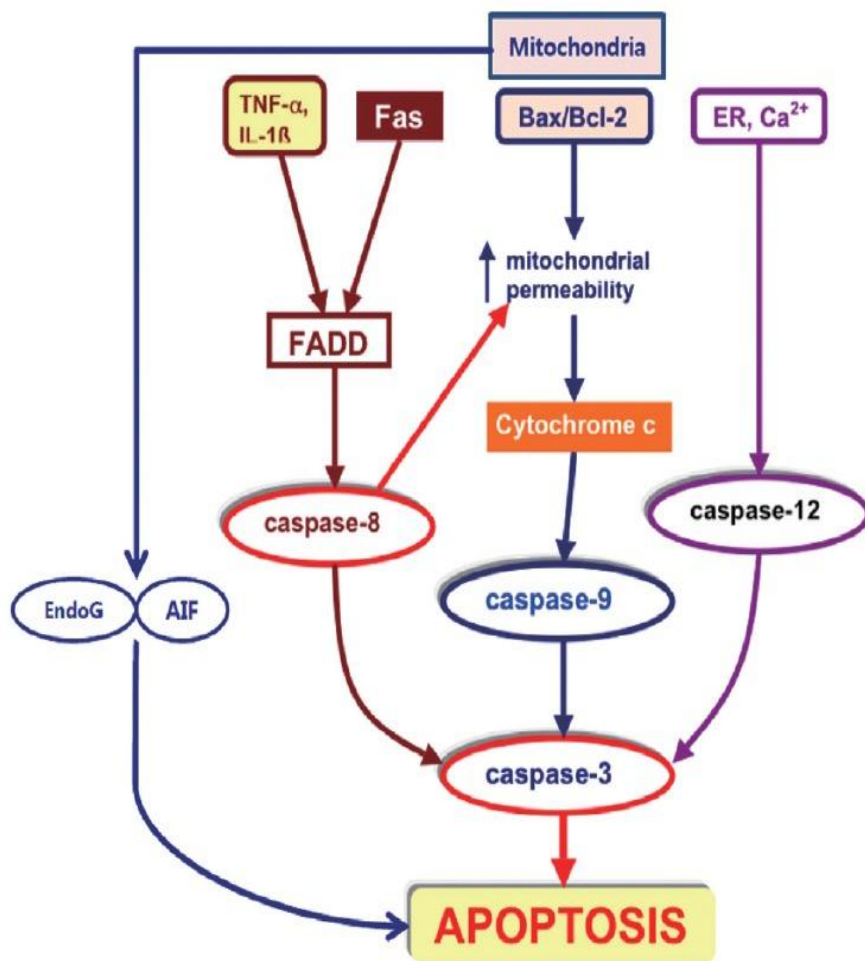
Figure 1.10: Apoptosis
An overview of the signalling pathways involved in apoptosis including the extrinsic (FAS mediated) pathway and the extrinsic pathway.

Adapted from Kwak, 2013.

Caspases are responsible for the bulk of intracellular signalling involved in apoptosis. Caspases are proteases that contain a cysteine residue at their active site (Alberts, 2007). Caspases are present in cells in an inactive form, procaspases, which when cleaved at aspartic acid sites by other caspases become active caspases. When a caspase has been cleaved they can cleave other procaspases which results in an amplifying signal cascade. Some caspases can cleave other proteins in the cell such as DNases which activate them and causes the DNase to brak down nuclear DNA and some may cleave important structural proteins such as nuclear laminins causing the nucleus to collapse. This cascade therefore facilitates the rapid breakdown of cellular components, readying the cell in a tidy was for phagocytosis by another cell (Alberts, 2007).

The activity of caspases is tightly regulated inside the cell. The initial signal is often triggered by adaptor proteins which cause initiator procaspases to form a complex which causes the initiator procaspases to cleave each other and therefore become active. Other downstream procaspases can then be rapidly cleaved causing them to become active and allowing them to cleave other procaspases or proteins amplifying the signal within the cell.

Procaspases can be activated externally (extrinsic pathway) or internally (intrinsic pathway). Extrinsically apoptosis is triggered by activation of a death receptor on the cell surface. For example killer lymphocytes posses a ligand called FAS which binds to a death receptor found on target cells (Alberts, 2007). Upon activation FAS recruits adaptor proteins that cause procaspase-8 to form a complex and mutually activate one another and subsequently activate downstream procaspases. Some apoptotic cells may produce both the Fas ligand and receptor causing an intracellular apoptotic cascade.

Intrinsically, damaged or stressed cells can apoptose by causing mitochondria to release the electron carrier cytochrome c where it can bind to and activate the adaptor protein APAF-1 in the cytosol (Alberts, 2007). This mechanism is active in most forms of apoptosis and operates in conjunction with extrinsic apoptosis to further amplify and accelerate the caspase cascade. The intrinsic pathway often requires p53 which causes the transcription of the BCL-2 family of proteins which promote the release of cytochrome c.

Some of the BCL-2 proteins such as BCL-2 and BCL-XL prevent the release of cytochrome c from the mitochondria and therefore prevent apoptosis whereas BAD, BAX and BAK promote the release of cytochrome c (Alberts, 2007). If *BAX* and

BAK are not being expressed this is usually sufficient for cells to resist apoptosis even when other pro-apoptotic stimuli is present. Understanding the mechanisms of apoptosis is essential in order to understand the changes in frequency of apoptosis in response to ageing and in the heart.

1.3.6.2. Ageing and Apoptosis

The propensity of many different tissue types to increase the incidence of apoptosis is a common feature of ageing, especially in organs where the frequency of cell proliferation is relatively low (Hasty and Christy, 2013; Moskalev *et al.*, 2014). For example, in the brain there is an increase in DNA fragmentation and pro apoptotic Bax protein due to ageing (Higami and Shimokawa, 2000). In the heart this paradigm is especially true, apoptosis due to ageing may cause a 30% reduction of cardiomyocytes when analysed independently of hearts with genuine cardiac diseases (Olivetti *et al.*, 1991; Liu *et al.*, 1998; Higami and Shimokawa, 2000). In rats cardiomyocyte apoptosis and necrosis was deduced to be the cause of ventricular dysfunction and subsequent failure (Kajstura *et al.*, 1996) and it has also been observed in rats that *Bcl-2* and *Bax* expression is increased in older hearts (Liu *et al.*, 1998). An increase in DNA fragmentation, cleaved caspase 3 and Bax is also observed in rat hearts (Kwak *et al.*, 2006) and also skeletal muscle (Song *et al.*, 2006) due to ageing. However, increased cardiomyocyte apoptosis in rats has also been observed due to ageing without changes in levels of Bax and Bcl-2 protein (Nitahara *et al.*, 1998). These results suggest that although the frequency of cardiomyocyte apoptosis increases with age this may not always be dependent on BAX and BCL-2 signalling.

The effects of increasing apoptosis due to ageing is exaggerated in tissues with relatively low rates of proliferation such as the brain, skeletal muscle and the heart as lost cells are not replaced. Various markers of apoptosis have been observed to be increased in hearts of aged rats (Phaneuf and Leeuwenburgh, 2002). Protein levels of cytochrome c in the cytosol was increased in rats aged over 16 months when compared to 6 months old rats, where the levels of cytocrome c in the mitochondria remained unchanged, suggesting the mitochondria may become more permeable due to ageing. In the same experiment it was also demonstrated that anti apoptotic Bcl-2 was decreased whereas Bax levels remained the same in the context of ageing, further muddying the waters regarding the role of Bcl-2/Bax signalling in ageing associated apoptosis.

On the other hand, tissues with high rates of proliferation, such as the colon mucosa and liver appear to have reduced apoptosis with advancing age. In colon

mucosa the frequency of apoptosis was reduced due to ageing accompanied by a reduction in the levels of pro-apoptotic proteins caspase 3, caspase 9 and BAK with a reduction in anti-apoptotic BCL-XL (Xiao *et al.*, 2001). In the liver DNA fragmentation and levels of cytochrome c were higher in young rats than older rats (Suh *et al.*, 2002; Zhang *et al.*, 2002). These findings suggesting a decrease in apoptosis in highly proliferative tissue may contribute to the higher cancer risk in these tissues (Suh *et al.*, 2002).

1.3.6.3.Apoptosis in the heart

Apoptosis in the heart can increase under many different circumstances, such as during heart failure, ischemia-reperfusion, doxorubicin treatment and ageing (Olivetti *et al.*, 1997; Higami and Shimokawa, 2000; Childs *et al.*, 2002; Powers *et al.*, 2002; Kajstura *et al.*, 2010a; Tacar *et al.*, 2013). Studies in humans and animals demonstrate that there is a decrease in total cardiomyocytes in the heart with advancing age which results in reactive hypertrophy of the surviving cardiomyocytes and an increase in fibrosis (Figure 1.11) (Olivetti *et al.*, 1991; Phaneuf and Leeuwenburgh, 2002; Kwak *et al.*, 2006; Kajstura *et al.*, 2010a; Kwak *et al.*, 2011).

Damage to mitochondrial DNA causing defects in the electron transport chain can cause upregulation of ROS leding to an increase in cardiomyocyte apoptosis has been reported to be a cause of some cardiomyopathies (Mignotte and Vayssiere, 1998; von Harsdorf *et al.*, 1999; Anderson *et al.*, 2011; Li *et al.*, 2012; Mughal *et al.*, 2012). In cardiomyocytes mitochondria can make up as much as 25% of the volume of the cell, as in tissues that are highly aerobic, such as the heart, energy is in high demand, requiring the constant supply of ATP by mitochondrial respiration (Lemasters *et al.*, 1999; Primeau *et al.*, 2002; Li *et al.*, 2012). Tumour necrosis factor-α (TNF-α) has also been implicated in cardiomyocyte apoptosis by inducing nitric oxide (NO) expression via nitric oxide synthase (iNOS) (Ing *et al.*, 1999; Song *et al.*, 2000).

In the heart ischemia reperfusion results in apoptosis which occurs during myocardial infarction and heart disease (Powers *et al.*, 2002; Quindry *et al.*, 2012). Preconditioning the heart by inducing brief periods of ischemia can provide protection from the pathogenesis of a longer duration of ischemia and reperfusion (Murry *et al.*, 1986; Taylor and Starnes, 2003). NF-κB may mediate the observed resistance to apoptosis gained from preconditioning andupregulate Bcl-2 (Maulik *et al.*, 1998; Das *et al.*, 1999) as the DNA binding activity of NF-κB is increased and levels of Bcl-2 increased in response to preconditioning (Maulik *et al.*, 2000). This idea is also

reinforced by the observation that overexpression of *Bcl-2* in transgenic mice reduced the extent of injury and improved cardiac function after reperfusion injury (Brocheriou *et al.*, 2000).

In the heart the balance of BAX/BCL-2 is thought to be an important tipping point in increasing apoptosis in cardiomyocytes (Condorelli *et al.*, 1999; Pollack *et al.*, 2002). It was observed in a model of chronic pressure overload in rats that upregulation of *Bax* causing an increase in Bax compared to Bcl-2, lead to apoptosis of cardiomycotyes and left ventricular hypertrophy and dysfunction (Condorelli *et al.*, 1999). Bcl-2 has been reported to inhibit apoptosis after reoxygenation in cardiomyocytes by preventing mitochondria releasing cytochrome c (Kang *et al.*, 2000).

Apoptosis of cardiomyocytes has been demonstated to contribute to the severity of congestive heart failure. Patients with cardiac failure have been observed to have an increased frequency of cardiomyocyte apoptosis with an increase in BCL-2 expressed (Olivetti *et al.*, 1997; Saraste *et al.*, 1999). It appears that the ratio of BAX to BCL-2 is important in apoptosis of cardiomyocytes, with BAX inducing apoptosis and BCL-2 inhibiting apoptosis. It does not seem as simple a scenario as BAX being increased and BCL-2 decreased causing apoptosis in cardiomyocytes, in some circumstances it may be that the opposite is true during apoptosis as a compensatory mechanism that has ultimately failed. Regardless of the underlying mechanisms at play, the literature unanimously suggests cardiomyocyte apoptosis is increased in ageing and diseased hearts and that measuring BAX or BCL-2 expression is not a reliable way of measuring proliferation.
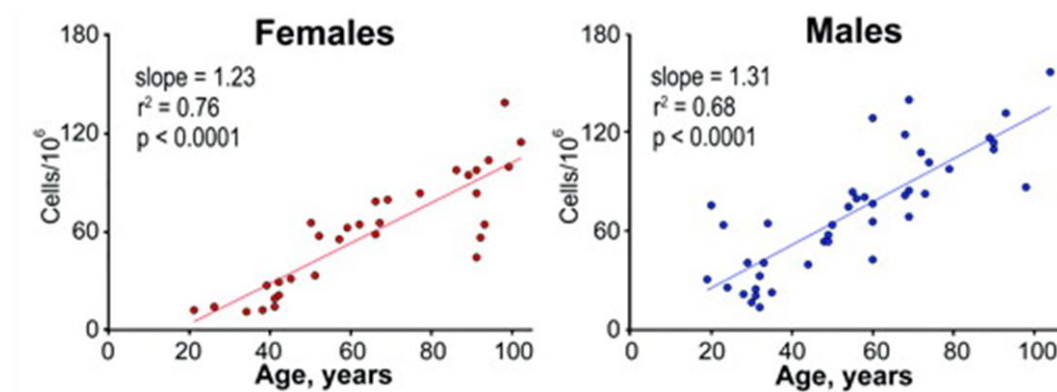
Figure 1.11: Apoptosis in human cardiomycoytes due to ageing
Apoptosis increases in cardiomyocytes with age in both females and males. **dUTP nick end labeling (TUNEL)** as a marker of apoptosis

Adpted from Kajstura *et al.,* 2010

Suprisingly, even when apoptosis in the heart is increased due to ageing, there is no significant change in the ratio of heart weight when compared to body weight between young and old rats (Kwak *et al.*, 2006). This may be because the heart responds to loss of cardiomyocytes by reactive hypertrophy in the remaining cardiomyocytes in both humans and animals (Olivetti *et al.*, 1991; Phaneuf and Leeuwenburgh, 2002) and also increasing connective tissue, which increases wall thickness (Anversa *et al.*, 1990b; Lakatta, 2002; Kwak *et al.*, 2011) therefore there is not necessarily a loss in cardiac mass.

1.3.6.4.Markers of apoptosis
There appears to be many different markers of apoptosis available, choosing the most appropriate marker for measuring cardiomyocyte apoptosis in zebrafish is essential.

1.3.6.4.1. *Annexin V*
Annexin V is used to mark cells in early stages of apoptosis. Soon after initiation of apoptosis cells translocate phosphatidylserine to the surface of the cell. Annexin V has a high affinity for phosphatidylserine and binds to it when it is on the surface of cells (Zhang *et al.*, 1997).

1.3.6.4.2. *Caspase-3*
Caspase -3 is a caspase that is involved in apoptosis and becomes activated by cleavage acting downstream of caspase-8 and caspase-9 (Alberts, 2007). Caspase-3 cleaves many different proteins including other caspases, kinases, cytoskeletal proteins and members of the Bcl-2 family (Feng *et al.*, 2005).

1.3.6.4.3. *Terminal transferase dUTP nick end labelling (TUNEL) assays*
TUNEL assays can be used to label cells in the later stages of apoptosis as it detects DNA degradation (Gavrieli *et al.*, 1992). When DNA is degraded it leaves exposed 3'-hydroxyl termini at the ends. These breaks can be labelled with fluorescent tagged deoxyuridine triphosphate nucleotides (F-dUTP). Terminal deoxynucleotidly transferase (TdT) can then be used to catalyze addition of deoxyribonucleoside triphosphates to the exposed 3' hydroxyl ends of degraded DNA. Adding the deoxythymidine analogue 5-bromo-2'deoxyuridine 5'-triphosphate (BrdUTP) to the TdT reaction labels the broken sites of the DNA which can then be detected via standard immunohistochemical techniques using an anti-BrdU antibody. TUNEL

assays can often be variable depending on different length of different treatment times and techniques used in the assay and can also detect necrotic cells alongside apoptotic cells (Charriaut-Marlangue and Ben-Ari, 1995).

1.3.6.4.4. Electron microscopy

Electron microscopy can be used to detect apoptotic cells due to changes in cells such as DNA fragmentation and structural changes in the mitochondria (Arbustini *et al.*, 2008). However, electron microscopy is more expensive and technically demanding than immunofluorescence. Tissue processing for electron microscopy would also limit the amount of other applications that the tissue could subsequently afterwards.

1.3.6.4.5. Summary of markers of apoptosis

Generally markers of apoptosis vary in terms of where expression is found in the cell such as nuclear and cytoplasmic localised (caspase-3 and TUNEL), or on the cell membrane (annexin-V). Also the time in apoptosis when they are present; early (annexin-V), mid (caspase-3) or late (TUNEL). Careful consideration must be taken when selecting markers of apoptosis taking into account the methods being used and the purpose of the labelling, in this instance for measuring apoptosis in zebrafish cardiomyocytes.

1.3.6.5.Necrosis

Necrosis is distinguishable from apoptosis due to distinct changes in morphology; the cell membrane is damaged, the cell and organelles swell and there is a loss of ATP. Inflammation is often caused after necrosis due cellular contents being released, which can cause further pathological damage (Whelan *et al.*, 2010). Chemical or physical trauma to cells often causes necrosis. This has lead to necrosis often being considered a passive form of cell death caused by accident (Vanlangenakker *et al.*, 2008). However there is some evidence that necrosis may be a controlled, programmed form of cell death, similar to apoptosis (Vande Velde *et al.*, 2000; Krysko *et al.*, 2008; Cho *et al.*, 2010).

One of the key early events of necrosis distinguishable from apoptosis is the changes in permeability of the inner mitochondrial membrane. Changes in permeability of the mitochondria is also a feature of apoptosis, however in apoptosis it is the outer mitochondrial membrane which becomes more permeable, not the inner mitochondrial membrane, as is the case in necrosis (Krysko *et al.*, 2008; Kung *et al.*, 2011). It has

been hypothesized that if mitochondrial pore opening occurs when ATP levels are normal within the cell apoptosis is activated. Whereas necrosis occurs instead if ATP levels are below a critical threshold or suddenly decline, as occurs in necrosis induced by the loss of an electrochemical gradient on the inner mitochondrial membrane as observed in myocardial infarction and acute ischemia (Lemasters *et al.*, 1999). Disruption of an electrochemical gradient across the mitochondrial membrane causes swelling of the inner mitochondrial membrane. This swelling leads to rupture of the outer mitochondrial membrane which ultimately releases cytotoxic mitochondrial proteases within the cytoplasm of the cell. The rupture of the outer mitochondrial membrane also causes the cell membrane rupture, releasing its contents (Krysko *et al.*, 2008). An overt inflammatory response occurs at the site of the necrotic injury by neighbouring cells, caused by proteins and enzymes released due to the initial cell necrosis, such as troponin-T and lactate dehydrogenase. This is a vital feature markedly different from apoptosis which does not cause an inflammatory response (Krysko *et al.*, 2008).

Necrosis has been reported alongside apoptosis as a mechanism of cellular death, leading to a reduction of cardiomyocytes in patients with heart failure (Guerra *et al.*, 1999). In this study heart failure caused necrosis to increase 27 and 13 fold in cardiomyocytes of men and women, respectively (Guerra *et al.*, 1999). Although, in the same study, apoptosis was found to increase 85 and 35 fold in cardiomyocyte of men and women, respectively, due to heart failure. This body of data suggests that necrosis may contribute in reducing cardiomyocyte numbers, leading to heart failure and that mitochondrial permeability transition pores and aberrant Ca2+ handling might play a critical role in whether cardiomyocytes undergo apoptosis or necrosis.

1.4. Autophagy

Autophagy is the process where cells break down, or 'eats' (phagy) some of itself (auto). In autophagy, contents of the cytoplasm, which can be organelles or aggregates of proteins that are ubiquitinated, become sequestered in vacuoles called autophagosomes and then digested in lysosomes (Mizushima *et al.*, 2008; Levine and Kroemer, 2009; Nakatogawa *et al.*, 2009). Autophagy serves to rid the cell of dysfunctional proteins and organelles and can also provide nutrients to the cell during periods of starvation. Autophagy can also play a role in the immune response, specifically in sequestering invading microorganisms and in antigen presentation.

Many factors can initiate an autophagic response, such as withdrawal of growth factors and nutrient deprivation (Cao *et al.*, 2009). Autophagic stimuli activates PI3K which binds to Beclin 1. This causes other autophagy proteins to assemble phagophores in a process called nucleation. Autophagy related gene (ATG) proteins cause the phagophore to expand, engulfing cytoplasmic contents to be autophaged. This process creates a structure with two membranes, the autophagosome. Fusion of the autophagosome with a lysosome then occurs, forming an autolysosome. In autolysosomes the sequestered cytoplasmic contents are digested into fundamental nutrients, such as fatty acids and amino acids, which can be re-used by the cell.

Autophagy is a double edged sword, although autophagy can be beneficial under certain cellular stresses, excessive autophagy may lead to cell death. The differences between cell death induced by autophagy and cell death in a cell with features of autophagy is not entirely clear cut (Kroemer and Levine, 2008), the main reason being that autophagy is a cellular response to stress, many forms of stress can be pro apoptotic (Levine and Kroemer, 2009). Autophagy has a beneficial effect on reducing disease burden in many conditions, such as in Huntingtin's disease, where autophagy removes pathogenic protein fragments in neurons (Sarkar *et al.*, 2008). In hepatocytes autophagy can increase the removal of mutant α-1-antitrypsin, accumulation of which can promote carcinogenesis (Perlmutter, 2008). There is also some evidence that autophagy may be an anti-ageing process in Caenorhabditis elegans (Hars *et al.*, 2007), yeast (Alvers *et al.*, 2009a; Alvers *et al.*, 2009b) and human cell lines (Gamerdinger *et al.*, 2009). The duality of autophagy is particularly true in the heart.

1.4.2. Autophagy in the heart

Cardiomyocytes are relatively long lived and as a result accumulate many damaged organelles and defective proteins. Autophagy is therefore an important method of maintaining viability and function by renewing damaged organelles. Disruption of autophagy in the heart can therefore cause many problems. An example of this is Danon disease, which is caused by a deficiency of lysosome-associated membrane protein 2 (LAMP-2) which is essential in delivering sequestered cargo to lysosomes. Patients with Danon disease have skeletal myopathy and cardiomyopathy (Maron *et al.*, 2009; Ruivo *et al.*, 2009). Autophagy is also essential in desmin related cardiomyopathies, myopathies that are the result of mutations in desmin and related proteins (Wang *et al.*, 2001). Features of desmin related cardiomyopathies include aggregation of proteins, disorganised myofibrils and contractile dysfunction often

resulting in sudden cardiac death. In a mouse model of desmin related cardiomyopathy a robust autophagy response has been reported, when autophagy was blunted by a Becn1 haploinsufficiency in this model, the progress to heart failure was accelerated (Tannous *et al.*, 2008).

Autophagy is also a key part of ageing in the heart as the accumulation of damaged organelles, particularly mitochondria, is often reported in aged cardiomyocytes (Terman *et al.*, 2003; Sheydina *et al.*, 2011). Mitochondria occupy up to 40% of the volume of cardiomyocytes and in conjunction with their role in generating energy, also generate and neutralise reactive oxygen species and are involved in apoptosis and calcium handling. Due to ageing the production of reactive oxygen species increases which causes more damage to the mitochondria (Juhaszova *et al.*, 2005; Green *et al.*, 2011). In aged cells mitochondria often have structural damage and are enlarged (Terman *et al.*, 2003; Bratic and Larsson, 2013). Removing damaged mitochondria via autophagy is critical in ensuring proper function of cardiomyocytes. It may be that due to cumulative damage caused by ageing, autophagy cannot keep up with the progressive accumulation of damaged organelles leading to declines in function and increasing disease susceptibility. Mice with a mutation in mitochondrial DNA polymerase, causing an increased accumulation of damaged mitochondria, exhibited a premature ageing phenotype consisting of alopecia, weight loss, enlarged hearts and a reduction in life span (Trifunovic *et al.*, 2004).

Some studies have managed to link autophagy directly to ageing. When *Atg8* is overexpressed in the nervous system of drosophila lifespan is extended by 50% (Simonsen *et al.*, 2008). In *C.elegens* the life extending affects of calorie restriction was shown to be mediated by autophagy as preventing autophagy in animals with reduced calorie intake did not significantly increase life span (Hansen *et al.*, 2008; Tóth *et al.*, 2008). In rats, a calorie restricted diet increased the incidence of autophagic vesicles and an upregulation of autophagy associated proteins, such as Beclin1, in the heart (Wohlgemuth *et al.*, 2007). This body of evidence suggests that autophagy can be reduced due to ageing and increasing autophagy can reduce ageing phenotypes.

Increase autophagy in the heart has been reported in response to many forms of stress, including starvation, pressure overload and ischemia. The role of autophagy in response to these stressors has not been completely resolved in terms of whether autophagy is contributing pathogenesis by causing cell death or reducing disease by improving cell survival. Research has been conflicting and suggests cardiac autophagy can be of benefit and burden depending on the context and level of activation

(Rothermel and Hill, 2008). For example, in mice, autophagy induced by pressure overload appears to be detrimental as it contributes to death of cardiomyocytes and leads to cardiac dysfunction (Zhu *et al.*, 2007). On the other hand, in pigs, autophagy promoted recovery after mild ischaemia (Yan *et al.*, 2005), probably by removing damaged organelles and preventing protein aggregates from accumulating. It appears that in the heart autophagy can be good and bad depending on the circumstances and differentiating beneficial autophagy from maladaptive autophagy is not possible.

1.5. Scenescence

Cellular senescence is the loss of the cells ability to proliferate and has been strongly associated with the shortening of telomeres (Fossel, 2000; Campisi, 2003; Itahana *et al.*, 2004; Rodier *et al.*, 2005). Characteristics of cardiomyocyte ageing include telomere shortening, loss of proliferative potential and expression of age associated proteins like p16INK4a (Chimenti *et al.*, 2003). Cardiomyocyte populations can be very heterogenous, with senescent and non-senescent populations being present in young (3 months old) murine hearts (Rota *et al.*, 2007). Human cardiomyocytes appear to undergo senescence after 20-25 passages in vitro, independent of telomere shortening (Ball and Levine, 2005).

The transition from proliferating to mainly quiescent cardiomyocytes is poorly understood at a molecular level. Murine cardiomyocytes are initially highly proliferative then withdraw from the cell cycle, undergoing bi-nucleation and exhibiting properties associated with contact inhibition (Biehl *et al.*, 2009). Proliferation was restored in early post-proliferative cardiomyocytes by knockdown of retinoblastoma protein (pRb) and deletion of pRb in adult mice caused an increase in cardiomyocyte DNA synthesis (MacLellan *et al.*, 2005). Similarly, cyclin D overexpression and cyclin dependant kinase 2 (CDK2) overexpression resulted in approximately 100 fold and 200 fold increases in DNA synthesis, respectively (Soonpaa *et al.*, 1996; Pasumarthi *et al.*, 2005). These results demonstrate that cardiomyocyte proliferation is tightly regulated by the G1 restriction checkpoint.

1.6.1. Telomeres

Telomeres are short repetitive sequences of DNA (5′-TTAGGGn-3′) located at ends of chromosomes (Blackburn, 1991). Shortening of telomeres occurs with cell division as approximately 60 base pairs are lost when the cell divides because of the end-replication problem; the failure of DNA polymerase replicating the chromosome end (Harley *et al.*,

1990; Bodnar *et al.*, 1998). Because of this phenomenon telomeres are like a mitotic clock recording the number of times a cell divides becoming a gold standard as a marker of ageing (López-Otín *et al.*, 2013).

The main role of telomeres are to protect the end of chromosomes from DNA damage by maintaining a sufficient length (De Lange, 2009); telomeres shortened past a critical point the ends of chromosomes are analogous to DNA double strand breaks (De Lange, 2005; Wright and Shay, 2005; De Lange, 2009). As aged cells have shorter telomeres due to cumulative proliferation, the telomeres can be regarded as double stranded breaks resulting in the cell entering senescence (Smogorzewska *et al.*, 2000; Wright and Shay, 2001; Blasco, 2007; De Lange, 2010; Cesare *et al.*, 2013). Accumulation of cells with a senescent phenotype is a major contributor to dysfunction in tissues due to ageing, although senescence can occur through mechanisms independent to telomere shortening, shortened telomeres is a primary cause (Tümpel and Rudolph, 2012; Tchkonia *et al.*, 2013).

1.6.2. Telomerase

Telomerase is a holoenzyme made up of a few proteins and a RNA moiety forming a ribonucleoprotein complex which functions as a reverse transcriptase (Greider and Blackburn, 1987). The main components which enable telomerase to possess enzymatic activity are TERT, dyskerin and TERC (Cohen *et al.*, 2007). TERT is believed to be the rate-limiting component of telomerase; TERT is only expressed in cells with telomerase activity, whereas *TERC* and *DKC1* expression has been observed in cells without telomerase activity (Feng *et al.*, 1995; Heiss *et al.*, 1998). Ectopic expression of TERT has also been demonstrated to be sufficient to induce telomerase activity in cells which otherwise have no telomerase activity (Counter *et al.*, 1998).

Telomerase facilitates the extension of chromosome ends by binding at the guanosine rich 3' end of the telomere and synthesising TTAGGG repetitions (Nicholls *et al.*, 2011). The whole reaction cycle of telomerase consists of three steps; recognising and binding to the substrate; extension by adding a single TTAGGG sequence and then repeating the process by translocating or ending the process by dissociation away from the telomere (Nicholls *et al.*, 2011).

In many models increased telomerase expression has been associated with a reduction in ageing or an increase in life span. Overexpression of *Tert* in transgenic mice resulted in a 10% extension in life span compared to wild-type control littermates (Gonzalez-Suarez *et al.*, 2005; Tomás-Loba *et al.*, 2008).

In mice, the loss of telomerase activity is associated with a shortened life span and genomic instability compromising the capacity to react to stressful stimuli, such as wound healing (Rudolph *et al.*, 1999; Jaskelioff *et al.*, 2011). These studies illustrate that in mouse models increasing telomerase activity has anti-ageing effects and lack of telomerase activity leads to an accelerated ageing phenotype.

1.6.3. Telomerase in zebrafish

In zebrafish, telomeres are a similar length to those of humans (Anchelin *et al.*, 2013) and expression of telomerase and length of telomeres have been demonstrated to decline due to ageing (Anchelin *et al.*, 2011). Studies have shown that knocking out telomerase reduces lifespan and causes ageing associated phenotypes such as gastrointestinal atrophy, premature infertility, spinal curvature and liver and retina degeneration (Anchelin *et al.*, 2013; Henriques *et al.*, 2013). The phenotypes observed were likely due to a decrease in proliferation of cells, increased DNA damage and senescent cells and an increase in premature apoptosis mediated by p53 (Anchelin *et al.*, 2013; Henriques *et al.*, 2013). Restoring the activity of telomerase increased telomere length and restore a normal lifespan (Anchelin *et al.*, 2013). This body of literature suggests that changes in telomeres and telomerase in zebrafish are similar to other experimental models.

1.7. p38 Mitogen activated protein kinase (MAPK)

In response to fluctuations in the properties of the external environment, cells can adapt many functions for survival such as metabolism and proliferative potential. Changes in the external environment that a cell may need to adapt to include varying concentrations of growth factors, cytokines, cell damaging agents, nutrients and physical stimulation, such as osmolarity. In order to respond to these environmental cues cells can initiate four separate subfamilies of mitogen-activated protein kinases (MAPKs): p38, JNK, ERK1/2 and ERK5. MAPK receive their signals from MAPK kinases (MAPKKSs) which in turn receive their signals from MAPK kinase kinases (MAPKKKs). MAPKKKs are activated by Ras/Rho GTP-binding proteins which respond to extracellular stimulation (Chang and Karin, 2001). Upon activation MAPKs are translocated to the nucleus whereupon they exert their effects via phosphorylating their specific target molecules.

In mammals there are four isoforms of p38MAPK: α, β, γ and δ (Cuenda and Rousseau, 2007). p38MAPKα is the most studied isoform as it is expressed in most

types of cells. The isoforms are often subdivided as α is most homologous to β and γ most homologous to δ. Also the α and β isoforms are both inhibited by SB203580 or SB202190 whereas γ and δ are not (Cuenda *et al.*, 1997; Goedert *et al.*, 1997; Kuma *et al.*, 2005). It has been shown that SB203580 binds to p38MAPKα by blocking an ATP binding domain (Eyers *et al.*, 1998; Gum *et al.*, 1998). Mice have been produced that are deficient in p38MAPKα, however these mice die at midgestation (Adams *et al.*, 2000; Mudgett *et al.*, 2000; Tamura *et al.*, 2000a), demonstrating the importance of p38MAPK in development.

1.7.1. p38MAPK in the heart

In the heart p38MAPKα is the most prevalent; p38MAPKβ has not been detected and γ and δ has only been detected at low levels (Lemke *et al.*, 2001). When human hearts with advanced heart failure or compensated hypertrophy were studied, only hearts with heart failure had any detectable levels of p38MAPK activity (Cook *et al.*, 1999). This suggests that p38MAPK is only active in the diseased heart.

In the stressed myocardium various studies have suggested that p38MAPK has a damaging role and inhibiting p38MAPK may offer protection. In mice p38MAPK activity has been shown to contributed to cell death (Kaiser *et al.*, 2005). p38MAPK signalling has been shown to lead to myocardium stiffness and reduced contractility and promotes certain ventricular remodelling processes in heart failure (Liao *et al.*, 2002). Using transgenic mice which expressed a dominant negative form of p38MAPK made it possible to discover that its signalling pathway has an anti-hypertrophic and may act via NFAT transcription factors to inhibit calcineurin-mediated hypertrophy (Braz *et al.*, 2003).

Inhibiting p38MAPK in perfused hearts during ischemia has been shown to reduce apoptosis and necrosis by hypoxia (Kaiser *et al*., 2005). Studies of ischaemic preconditioning have shown that p38MAPK has a role in cardioprotection (Sanada and Kitakaze, 2004). It has been demonstrated that activation and phosphorylation of p38MAPK correlated with the level of protection from ischaemic preconditioning (Weinbrenner *et al.*, 1997). SB203580 has been shown to have cardioprotective effects via inhibiting p38MAPK as SB203580 can block contractility during cardiac reperfusion which prevented necrosis of cardiomyocytes (Sumida *et al.*, 2005).

It has been demonstrated that p38MAPK inhibition in conjunction with FGF1 administration acted synergistically and increased mitosis of cardiomyocytes in rats with acute myocardial injury (Engel *et al.*, 2006a). FGF1 and SB203580 administration

resulted in improved cardiac function with reduced scarring and wall thinning 3 months post injury. Sb203580 administration alone appears to increase cardiomyocyte mitosis however administering FGF1, also, appears to increase angiogenesis which may aid the survival of new cardiomyocytes by providing a source of nutrients and oxygen.

Tissue specific deletion of p38MAPKα implicate an involvement of p38MAPKα in proliferation and survival of cardiomyocytes (Nishida *et al.*, 2004; Engel *et al.*, 2005). Knock out mice have been produced for the other three isoforms and double p38MAPKγ/δ knock out which are viable and fertile (Beardmore *et al.*, 2005; Sabio *et al.*, 2005).

p38MAPK inhibition appears to play a prominent role in heart disease and can have cardioprotective properties, improving cardiomyocyte survival and increasing cardiomyocyte proliferation. It appears feasible that inhibiting p38MAPK may lead to therapeutic advances in heart disease, and studying other compounds which would act in synergy or make the effects of p38MAPK inhibition heart specific would be beneficial.

1.7.2. p38MAPK in Zebrafish

In Zebrafish there are two orthologs of p38MAPKα: p38mapka and p38mapkb (also known as MAPK14a and MAPK14b, respectively), which are located on chromosomes 8 and 11 and share 86% and 84% homology with the human p38MAPK-α (Krens *et al.*, 2006). There are no orthologues of the β, δ, or γ isoforms in zebrafish. RT-PCR and in situ hybridisation has shown that p38mapka is expressed ubiquitously throughout development and in adult zebrafish, whereas p38mapkb is only transiently expresses between the 16 cell stage and 30% epiboly and not at all in adult zebrafish (Krens *et al.,* 2006).

Recently conditionally active and dominant negative p38MAPK transgenic zebrafish have been produced (Jopling *et al.*, 2012). Activating p38MAPK in regenerating hearts responding to ventricular resection was shown to inhibit regeneration and cause scar formation. However, inhibition of p38MAPK was shown to have no affect on the rate of regeneration compared to wild type zebrafish. This suggests that indeed zebrafish do need another stimulus to increase proliferation leading to regeneration in resected hearts, much like mice. This opens the possibility of improving regeration by inhibiting p38MAPK and in conjunction with another factor or cocktail of factors.

1.8. Exercise and cardiovascular health

Exercise training has many health benefits (Fiechter *et al.*, 2013), particularly to the cardiovascular system (Soonpaa *et al.*, 2013). A clear link has been demonstrated between physical activity and mortality (Hinits *et al.*, 2012; Kajstura *et al.*, 2012b). In terms of reducing morbidity and mortality of cardiovascular disease exercise is one of the most effective and cheapest interventions. Brisk walking for as little as 45-75 minutes each week appears to be eough to have an effect on reducing the risk of cardiac disease (Manson *et al.*, 2002; Nelson *et al.*, 2007). Many studies have illustrated a dose responsive paradigm between cardiovascular benefit and physical activity. However optimal dosage, frequency, duration, intensity and type of exercise used for maximal cardiovascular benefit remains debatable (Lee and Skerrett, 2001; Manson *et al.*, 2002; Tanasescu *et al.*, 2002).

Exercise provides multifactoral benefits to health. Many studies show that exercise is effective at reducing body mass index by reducing adipocyte mass and positively affects glucose uptake by skeletal muscle, cholesterol profiles and sensitivity to insulin (Thompson *et al.*, 2012). Many beneficial changes have been observed in vasculature in response to exercise, particularly aerobic exercise, such as improvements in arterial compliance, enhanced vasodilation and reductions in both systolic and diastolic blood pressure (Hambrecht *et al.*, 2000; Pescatello *et al.*, 2004; Nualnim *et al.*, 2012). Whilst all of these changes due to exercise have a downstream positive effect on cardiovascular health there are other, more direct benefits to cardiovascular health offered by partaking in physical activity.

In response to increased pressure and workload the heart becomes bigger by growing. There are two mechanisms to achieving growth, increasing cell size (hypertrophy) or increasing the number of cells (hyperplasia). In the heart there are two distinct types of hypertrophy: pathological, which is associated with disease and physiological, which is associated with post natal growth, pregnancy induced growth and exercise (Bernardo *et al.*, 2010). In contrast to pathological hypertrophy, which has been discussed previously, during physiological hypertrophy the structure of the heart is maintained and all parts grow in proportion to each other (Bernardo *et al.*, 2010). This leads to an improved cardiac function. There are many distinct mechanisms between pathological and physiological hypertrophy which will be discussed further.

1.8.1. *The effect of exercise on cardiovascular health in the elderly*

Multiple benefits have been observed in terms of cardiac health and performance in elderly patients undertaking endurance exercise programs. These benefits include reducing the ageing related decline of cardiovascular performance and actually improving cardiac function (Hollmann *et al.*, 2007). Some of the improved parameters observed in the elderly after exercise include an improvement in maximal oxygen consumption (Fujimoto *et al.*, 2010) and improvements in heart rate recovery and ventilator aerobic threshold (Giallauria *et al.*, 2005; Fujimoto *et al.*, 2010). These factors have important prognostic relevance.

The underlying causes of the positive effects observed in the hearts of the elderly in response to exercise are in part due to an increased cardiac output, positive remodelling of the left ventricle and changes in the sympathetic nervous system (Rengo *et al.*, 2012a; Rengo *et al.*, 2012b; Rengo *et al.*, 2012c; Femminella *et al.*, 2013; Salazar *et al.*, 2013). Other positive effects occurring outside of the heart may also contribute to the improvements observed in the heart, such as improvements in skeletal muscle perfusion and oxygen utilisation (Vigorito and Giallauria, 2014). In elderly patients with heart failure with reduced left ventricle ejection fraction there was a slower rate of left ventricular remodelling and an increase in left ventricle contractility at rest in patients who exercised (Giannuzzi *et al.*, 2003; Haykowsky *et al.*, 2007; Giallauria *et al.*, 2008; Haykowsky *et al.*, 2011; van der Meer *et al.*, 2012; Giallauria *et al.*, 2013).

1.8.2. *Exercise and fibrosis*

In response to exercise the composition and amount of collagen has often been reported to be altered in skeletal muscle (Gosselin *et al.*, 1998) and the heart (Thomas *et al.*, 2000; Thomas *et al.*, 2001). It has been reported that 10 weeks of exercise training on a treadmill reduced the increase of collagen deposited due to ageing in the septum of the left ventricle in rats when compared sedentary controls (Thomas *et al.*, 2000). The cross linking of collagen in the free wall of the left ventricle was also significantly lower in old exercise trained rats when compared to sedentary age matched controls (Thomas *et al.*, 2000; Thomas *et al.*, 2001). In other studies in rats it has been shown that an exercise training regime for a duration of 12 weeks blunted the increase in collagen positive tissue and extramyocyte space normally observed due to ageing (Kwak *et al.*, 2006; Kwak *et al.*, 2011). An increase in MMPs and reduction in TIMPs is normally observed in aged hearts, however exercise mitigated this change in rats

(Kwak *et al.*, 2006; Kwak *et al.*, 2011). The age associated increase in TGF-β1 was also ameliorated.

However, some studies have demonstrated that exercise training has no affect on the collagen composition of the heart. In one study, the total collagen content of the left ventricles of rats was not significantly change in response to 10 weeks of exercise training on a treadmill, with no significant alteration in the ratio of collagen I to III (Burgess *et al.*, 1996). Similarly voluntary wheel running in rats appeared to make no difference in the amount of cross linking and concentration of collagen in the left ventricle, although in the exercised rats there was a reduction in cardiac stiffness (Woodiwiss *et al.*, 1998). Another study also reported no significant changes in expression of collagen I or III in response to 13 weeks of enforced treadmill running in the hearts of rats (Jin *et al.*, 2000). In light of these studies it appears that exercise can reduce the amount of fibrosis deposited in the heart. This may be due to cellular changes in the heart.

1.8.3. *Cellular and molecular changes in the heart due to exercise*

After endurance exercise in the form of a swimming program cardiac hypertrophy was induced which consisted of a 45% increase in size of cardiomyocytes (Boström *et al.*, 2010). The proliferation markers PCNA, ki67 and PHH3 and also BrdU incorporation was significantly increased in hearts of exercised mice. Staining for Aurora B kinase showed that these cells were undergoing cytokinesis and not just becoming multinucleated. None of these features were detected in a murine model of pathological hypertrophy (Boström *et al.*, 2010). This suggests that cardiomyocytes respond to exercise by increasing in size and number.

Adaptations in coronary vasculature such as increased vessel number and size (White *et al.*, 1998), improved endothelial function (Black *et al.*, 2009), increased oxygen supply and blood flow (Laughlin, 1985; Laughlin *et al.*, 1989; Duncker and Bache, 2008) have been associated with endurance exercise. Proliferation of vascular smooth muscle cells and endothelial cells is requires for increased blood vessel size and number, which can be stimulated by growth factors and physical and hemodynamic forces (Brown, 2003). The amount of circulating bone marrow progenitors and endothelial cell progenitors increase in response to exercise, these cells may also contribute to neovascularisation (Thijssen *et al.*, 2009).

Endurance exercise does not increase the rate of apoptosis in cardiomyocytes (Jin *et al.,* 2000) and has actually been shown to attenuate increases in left ventricular

apoptosis and ratios of Bax/Bcl-2 which is normally associated with aging (Kwak *et al.,* 2006). This data is in agreement with previous findings that increased Bax and decrease Bcl-2 at the protein level is associated with ageing (Higami and Shimokawa, 2000; Liu *et al.*, 2012) illustrating that the Bcl-2 mitochondrial signalling is a vital cog in apoptosis due to ageing and exercise associated antiapoptotic affects. Inreases in cleaved caspase 3 and 9 and DNA fragmentation leading to cell death were the downstream affects of increased Bax to Bcl-2 which was also significantly decreased in the ageing heart due to exercise (Kwak et al, 2006). In this study it was noted that DNA fragmentation and cleaved caspase-3 were very closely correlated, suggesting that cleaved caspase 3 is an essential component of apoptosis (Kwak et al., 2006).

After endurance exercise Bcl-2 expression was decreased and expression of HSP70, an inhibitor of apoptosis, was increased in the ventricles of rats (Garrido *et al.*, 2001; Siu *et al.*, 2004). Hearts which overexpress HSP70 show a reduction in apoptosis after ischaemia reperfusion injury (Suzuki *et al.*, 2000).

Upregulation of oxidative stress is associated with ageing, especially in the heart (Beere *et al.*, 1999; Sastre *et al.*, 2000; Dinenno *et al.*, 2001; Soufi *et al.*, 2008) and is a major force in causing mitochondrial dysfunction and subsequent apoptosis (Sastre *et al.*, 2000; Dirks and Leeuwenburgh, 2005). It is thought that exercise may exert antiapoptotic effects by upregulating super oxide dismutase, heat shock proteins, NF-κB and ERK and Akt signalling which ameliorates some of the reactive oxygen species which cause the oxidative damage normally cumulating in apoptosis in the heart (Starnes *et al.*, 2003; Taylor and Starnes, 2003; Soufi *et al.*, 2008; Higashi *et al.*, 2012). This body of evidence suggests that the beneficial effects of exercise that occur in the heart maymay be due to an increase in cardiomyocyte proliferation and a reduction in apoposis.

1.8.3.1.Exercise and autophagy

Autophagy plays a huge role in the survival of cardiomyocytes. It is emerging that exercise training has a huge impact on regulation of autophagy in skeletal muscle. It has been demonstrated that exercise in conjunction with mild calorie restriction in rat prevented a age associated reduction in basal levels of autophagy in muscle (Wohlgemuth *et al.*, 2010). It has also been demonstrated that treadmill exercise in rats increased the expression of autophagy related genes Atg6 and Atg7 in the soleus muscle (Smuder *et al.*, 2011). A few studies have shown that autophagy is upregulated after exercise in the heart and skeletal muscle of mice after exercise (Grumati *et al.*, 2011;

Ogura *et al.*, 2011; He *et al.*, 2012). These results imply that exercise may prevent the age associated reduction in expression of autophagy related genes and may even promote the expression of autophagy genes in younger animals, which may mediate some of the beneficial effects of exercise.

On the other hand it has been reported that autophagy is decreased after exercise. After a single bout of treadmill running the skeletal muscle of mice showed a decrease in proteins associated with autophagy, such as Beclin-1 and Atg7 (Kim *et al.*, 2012b). This could be because a single bout of short duration exercise is not enough for physiological adaptions associated with exercise training to occur, whereas in other studies repeated bouts of exercise cause physiological changes to occur.

There appears to be conflicting results on the effects of exercise on autophagy. It has been demonstrated that in the heart autophagy is increased due to exercise and in the muscle both an increase and decrease in autophagy has been reported.

1.8.3.2. Changes in the fetal gene program due to exercise

In some aspects the process of pathological and physiological cardiac hypertrophy are similar in terms of some of the cellular mechanisms (Dorn, 2007). However, despite similarities evidence suggests that distinctive stimuli and subsequent signalling pathways are responsible for the two different processes (Bernardo *et al.*, 2010). Pathological hypertrophy is often associated with the reactivation or upregulation of genes associated with fetal cardiomyocytes, such as BNP, ANP, αskeletal actin and β-MHC (Izumo *et al.*, 1987; Izumo *et al.*, 1988; MacLellan and Schneider, 2000; McMullen *et al.*, 2003) which does not appear to occur in physiological hypertrophy in response to endurance exercise (McMullen *et al.*, 2003). In the human heart ANP serum levels have been reported to be raised during exercise and then reduced afterwards (Follenius and Brandenberger, 1988; Scharhag *et al.*, 2013). *ANP* expression has also been reported to be reduced in rat hearts with exercise induced hypertrophy (Diffee *et al.*, 2003). Similarly, in mouse it appears that exercise reduces the expression of *Anp* and *Bnp* in the heart after exercise (Boström *et al.*, 2010).

Transcription factors such as *MEF2C*, *GATA4, TBX5* and *NKX2.5* appear to be stimulated in pathways implicated with physiological hypertrophy (McMullen, 2008; Bernardo *et al.*, 2010). Indeed in swim trained mice expression of *Gata4* was significantly increased, however expression of *Anp* and *Bnp* remained unchanged when comparing exercised to sedentary animals (Xiao *et al.*, 2014). This increase in expression was also coupled with an increase in physiological hypertrophy. Similar

increases in *Gata4* expression have also been reported in a mouse model for diabetes after 8 weeks of treadmill running (Broderick *et al.*, 2012). In juvenile atlantic salmon it was observed that salmon that had experienced exercise training had increased cardiac expression of *gata4* and *mef2c* (Castro *et al.*, 2013). This was coupled with an increase in protein levels of pcna suggesting that the increased expression of *gata4* and *mef2c* may be driving cardiomyocyte proliferation after exercise.

C/EBPβ has been shown to be downregulated in response to endurance exercise in mice and may regulate *Gata4* expression (Boström *et al.*, 2010). Experimental decrease of C/EBPβ or exercise increases cardiomyocyte proliferation and size coupled with a change in expression of certain genes such as PGC1α, GATA4 and CITED-2. PGC1α is a transcriptional co-activator with roles in mitochondrial biogenesis and protecting the heart against pathological stress (Arany *et al.*, 2006; Finck and Kelly, 2007). C/EBPβ can control GATA4 expression by binding and sequestering SRF which would otherwise bind to GATA4 and α-MHC promoters. SRF is vital for the differentiation of cardiomyocytes and regulates the expression of *Tbx5* and *Nkx2.5* (Chai and Tarnawski, 2002; Akazawa and Komuro, 2003). The reduction of C/EBPβ being of benefit to the heart is also plausible as increased C/EBPβ has been reported in pathological models of cardiac remodelling (Oh *et al.*, 2010).

This body of data suggests that the fetal gene program is important in conveying the changes associated with exercise. Cardiac transcription factors appear necessary for cardiac hypertrophy and hyperplasia. Distinguishing this pathway from pathological remodelling includes the observation that ANP and BNP are downregulated in response to physiological hypertrophy but not pathological hypertrophy.

1.8.3.3.Models of enforced exercise in zebrafish

Very few studies look at enforced exercise in zebrafish, in which zebrafish swim against a current in a swim tunnel set at a predetermined speed (Van Raamsdonk *et al.*, 1998; Van der Meulen *et al.*, 2006; Wang *et al.*, 2011; Jean *et al.*, 2012; Palstra *et al.*, 2013) with a variety of different protocols used for enforced swimming.

Few studies have looked at the swimming performance of zebrafish. The maximum speed an adult zebrafish can swim (Ucrit) has been determined to be around 0.56m/s or 15.5BL/s (Chen *et al.*, 2002). The optimum swimming speed (Uopt) is the most energetically favourable speed of swimming. Swimming at Uopt requires burst and glide motions which may require both aerobic and anaerobic muscle action. Anaerobic muscle acion may occur during the burst part of swimming, whereas the

glide portion is exclusively aerobic (Wong *et al.*, 2011). Reported Uopt swimming speeds varies between studies, being reported as high as 18BL/s (Han and Ren, 2010) and as low as 8BL/s (Sciarretta *et al.*, 2014). Differences in reported Uopt speeds may be due to a variety of factors such as methodology, age of zebrafish, temperature and many more.

The different enforced swimming protocols used in fish can fall into three categories of exercise; acute, endurance and sustained (Wong *et al.*, 2011). Acute exercise consists of short duration higher intensity exercise, normally at increasing speed gradients until Ucrit is reached, and is mainly anaerobic, using predominantly fast twitch muscle fibres (Van Raamsdonk *et al.*, 1998; Wang *et al.*, 2011). Endurance exercise is longer in duration, usually around 1.5 to 6 hours per day, at a speed around that of Uopt (Van der Meulen *et al.*, 2006; Han and Ren, 2010; Jean *et al.*, 2012). Sustained swimming is aporximately 70% of the Uopt swimming speed and lasts for durations longer than 6 hours, often a few days (McClelland *et al.*, 2006; Ju *et al.*, 2009; Barth *et al.*, 2010).

Studies have shown that as humans and other mammals age, there is a decrease in physical performance (Carter *et al.*, 2002; Tanaka and Seals, 2003; Baker and Tang, 2010). A recent study has shown this to be true of zebrafish also (Gilbert *et al.*, 2014). Zebrafish were split into three different age groups; young, middle aged and old depending on the percentage of the average lifespan of 40 months (20-29%, 36-48% and 60-71% respectively). Zebrafish performed sprinting tests and were exercised until exhaustion once a week over 4 weeks. Performance in endurance and sprinting declined with age; prior to training young zebrafish had a Ucrit swimming speed of approximately 13BL/s, middle aged 12BL/s and old aged 8BL/s. Interestingly, only the old aged group showed no significant improvement in swimming speeds over the 4 weeks of training.

Similarly to mammals, exercise in fish has been demonstrated to have positive effects on the cardiovascular system. In trout, aerobic exercise induces growth of the ventricles (Farrell and Jones, 1992; O'Callaghan and Fenech, 2011). Aerobic exercise was also shown to increase the maximum cardiac output, oxygen content of the arteries, haematocrit, tissue oxygen extraction and capillaries in skeletal muscle in trout and salmon (Davie and Sparksman, 1986; Farrell and Jones, 1992; Gallaugher *et al.*, 2001; Gamperl and Farrell, 2004; O'Callaghan and Fenech, 2011; Wong *et al.*, 2011; Craig *et al.*, 2012; Lee *et al.*, 2012; Castro *et al.*, 2013).

Only one study to date specifically examines the effects of exercise on the cardiovascular system in adult zebrafish (Jean et al., 2013). In this study zebrafish were subjected to two 90 minute sessions of enforced swimming at 25cm/second (approximately 8BL/s) for 10 weeks. Hearts of exercised zebrafish were 33% larger, which they deduce was due to hyperplasia, rather than hypertrophy as the number of nuclei per cross sectional area of the heart increased by 8%. There were no changes in heart rate or shortening fraction. However the methods used to measure the number of nuclei per cross section does not appear very robust, the area counted was a small 0.5mm2 box on a section of heart rather than the whole section, the marker they used for counting cells was DAPI which is not cardiomyocyte specific so other cells may give a false positive and no further experiments were carried out to try and determine the mechanisms involved.

1.9. Heart Regeneration

Zebrafish and other teleost fish posses the remarkable ability to regenerate fins and many organs following trauma. For this reason zebrafish have long been used as a model to study tissue regeneration. the Following 20% ventricular resection heart regeneration in zebrafish follows a distinct pathway other a two month period; 1) regeneration is triggered by trauma, 2) quiescent tissue becomes activated, 3) there is localized development at the site of injury, 4) healing and growth of tissue, 5) morphogenesis to shape the regenerated adult tissue, 6) long term maintenance (Poss *et al.*, 2002) (Figure 1.12). Proliferating cardiomyocytes localized to the leading epicardial edge were shown to give rise to the newly regenerated myocardium via BrdU labelling. *nkx2.5*, *hand2* and *tbx20* were discovered to be expressed in the plane of regeneration via *in situ* hybridisation (ISH) (Lepilina *et al.*, 2006). This suggests that the mechanisms involved in cardiac regeneration may be related to cardiac development.

ISH also showed that *raldh2* is expressed by the epicardium globally at 24hpa which then becomes localised to the wound by 14dpa (Lepilina *et al.*, 2006). This expression pattern is similar to the expression of wnt in planaria during regeneration (De Robertis, 2010). Raldh2 is an enzyme that is pivotal for the synthesis of retinoic acid, which is a morphogenic factor. Retinoic acid signalling has been proven to be essential in cardiac development, it has roles in early patterning then as a mitogen (Sucov and Evans, 1995; Hoover *et al.*, 2008; Ryckebusch *et al.*, 2008; Waxman *et al.*, 2008). The importance of raldh2 in regeneration has been cemented when it was demonstrated that inhibiting retinoic acid receptors or overexpression of a retinoic acid

degrading enzyme, *cyp*26, prevented regenerative cardiomyocyte proliferation (Kikuchi *et al.*, 2011b).

A tomoxifen inducible cre-lox system was developed to label all cardiomyocytes with GFP within a zebrafish heart which was done at 48 hours post fertilisation (hpf) (Jopling *et al.*, 2010). At 7, 14 and 30dpa all of the regenerated cardiomyocytes were GFP positive suggesting they were the progeny of differentiated cardiomyocytes. This was reinforced by staining the heart for MF20; all the MF20 positive cells were also GFP positive. GFP positive cardiomyocytes were also shown to take up BrdU, showing DNA synthesis which would likely result in resumption of the cell cycle (Jopling *et al.*, 2010). By examining the sarcomeres of proliferating cardiomyocytes which were stained for the proliferation markers phosphorylated histone H3 (PHH3) or proliferating cell nuclear antigen (PCNA) it was determined that the proliferating cardiomyocytes have a disorganised sarcomeric structure (Jopling *et al.*, 2010). They concluded that the bulk, if not all of the regenerated myocardium was composed of cardiomyocytes which have undergone a limited amount of de-differentiation, involving the disassembly of sarcomeres, detachment from other cardiomyocytes and expression of cell cycle regulators in order to proliferate. Similar levels of dedifferentiation have also been observed in murine myocardial infarction models where cardiomyocytes were induces to proliferate via administration of neuregulin (Bersell *et al.*, 2009).

Interestingly, although zebrafish display age associated degeneration no impairment of regeneration has been observed in the heart and fins. Regeneration of the heart and caudal fin was shown to be indistinguishable between 6-12 months old zebrafish and 26-36 months old zebrafish (Itou *et al.*, 2012a). After ventricular resection cardiac regeneration and cardiomyocyte proliferation occurred at a similar rate in young and old zebrafish, with no difference in expression of raldh2 between the two age groups, suggesting that age does not affect the regenerative ability of zebrafish. Although repeated amputation of the caudal fin has demonstrated that there is no impairment of the regenerative response (Azevedo *et al.*, 2011), it would be interesting although unethical to see if the regenerative ability of the heart is exhausted after repeated resections

Following ventricular resection in 1 day old mice, regeneration occurred over the course of 21 days (Porello et al., 2002) in a similar process to that described in zebrafish (Poss et al., 2002). As neonatal murine cardiomyocytes are reported to be proliferative (Ahuja et al., 2004) markers for proliferation and cytokinesis such as PHH3 and aurora B kinase were examined and were shown to be co-localised with

cardiac troponin T (Porrello *et al.*, 2011). Levels of proliferation markers were significantly higher in resected hearts than sham operated hearts.

Cardiomyocyte proliferation in zebrafish is associated with sarcomere disassembly (Kikuchi *et al.*, 2010) which was also observed in resected 1 day old mouse hearts (Porrello *et al.*, 2011). BrdU labelling experiments show that the newly formed apex of the regenerated mouse heart was made up of newly proliferated cardiomyocytes and lacZ lineage tracing showed that these new cardiomyocytes arose from a cardiomyocyte lineage rather than a stem cell lineage (Porrello *et al.*, 2011), as similarly observed in zebrafish (Jopling *et al.*, 2010). Resection in 1 day old mouse hearts also elicits epicardial activation as observed in the upregulation of epicardial genes.

Ventricular resection was also performed on 7 day old mice, which failed to regenerate and instead formed scar tissue with no evidence of proliferation or sarcomere disassembly (Porrello *et al.*, 2011). This may be due to mouse cardiomyocytes becoming binucleated and withdrawing from the cell cycle at this time point (Li *et al.*, 1996).

These results show that mammalians do have similar heart regeneration potentials as zebrafish, however regenerative response is associated with proliferative potential, and as mammalian cardiomyocytes mature and become binucleated they begin to lose this response. Cardiomyocytes of adult zebrafish are similar in some ways to embryonic mammalian cardiomyocytes; they are mononucleated, small and have poorly developed sarcoplasmic reticulum (Poss, 2007). Examining factors that can increase cardiomyocyte proliferation would aid regeneration in mammalian hearts.

There is some reported evidence of a minimal amount of regeneration in human hearts after myocardial infarction (Beltrami *et al.*, 2001). Ki67 expression was reported in 4% of cardiomycytes in areas adjacent to infarcts and 1% of cardiomyocytes distal to the infarct region (Beltrami *et al.*, 2001). In these regions characteristics associated with cell division such as formation of mitotic spindles, karyokinesis and cytokinesis were also observed showing cardiomyocyte proliferation after myocardial infarction. Altogether this body of data suggests that although adult mammals cannot sufficiently regenerate their hearts following tissue damage, regeneration does occur in neonatal life and cardiomycotye proliferation does occur in adult life in response to trauma, however not enough to replace lost tissue. This suggests that the basic machinery is present in order for cardiomyocyte regeneration to occur, however the responsiveness of this machinery is lost with maturation of cardiomyocytes. Another source of potential cardiomyocytes could be stem cells.
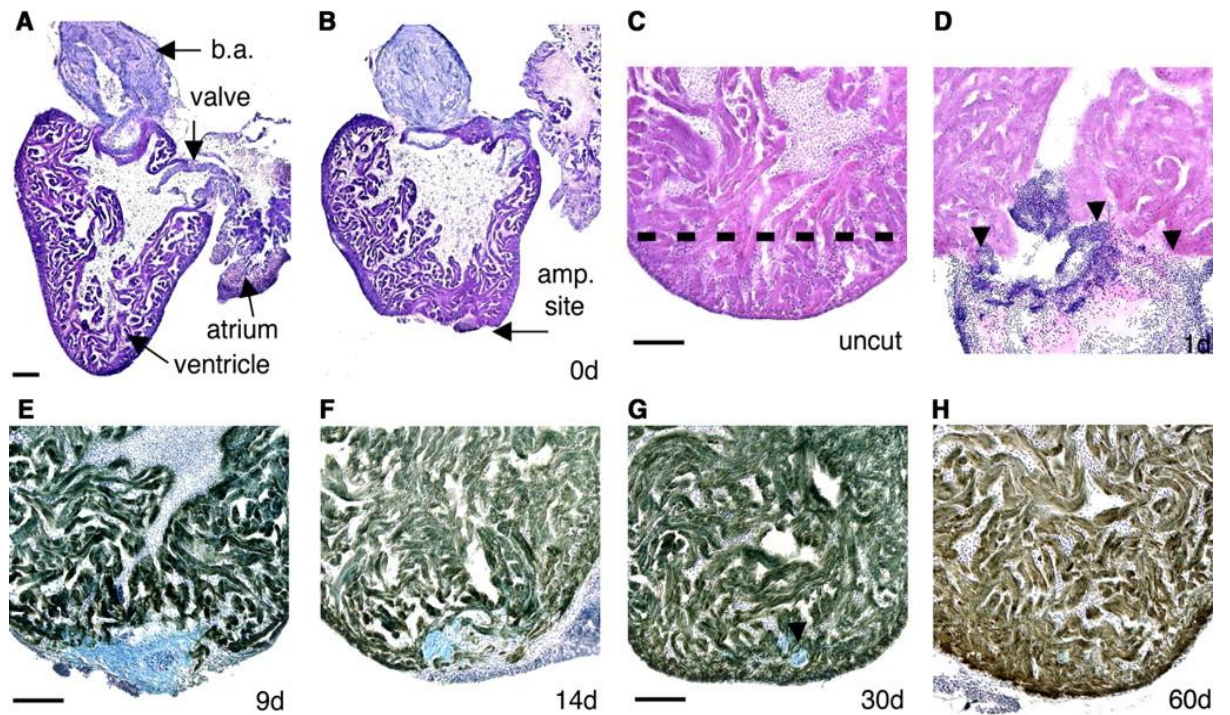
Figure 1.12: Heart regeneration in zebrafish
Haematoxylin and Eosin (H and E) staining of a midline sagital section of an intact zebrafish heart (A) and after approximately 20% ventricular resection (B). The plane of amputation in an intact zebrafish ventricle (C). 1 day after amputation a blood clot forms (D). 9 days after amputation fibrin (blue) develops (E). 15 days after amputation the fibrin (blue) is receding and is replaced by muscle (brown, F). 30 days after amputation the fibrin has almost disappeared and has been replaced by muscle (G). 60 days after amputation the ventricle has fully healed, showing no signs of injury and is indistinguishable from an uninjured ventricle (H).

Adapted from Poss *et al.,* 2002

1.10. Aims

In this thesis I aimed to find out if cardiomyocyte proliferation increases as zebrafish age. To do this I needed to establish a robust method for identifying proliferating cardiomyocytes. I then wanted to find out if cardiomyocyte apoptosis increases as zebrafish age. Knowing the rate of cardiomyocyte proliferation and apoptosis for different aged zebrafish would indicate if the number of cardiomyocytes in the zebrafish declines due to ageing, as has been described in humans and mammalian models. I then wanted to see if these changes in turnover were associated with other pathological changes and whether physiological or pharmacological intervention could improve cardiomyocyte turnover.

Chapter 2 : Methods

2.1. Zebrafish lines and maintenance

Wild-type AB zebrafish (Zebrafish International Resource Centre, ZIRC) were used for mapping cardiomyocyte proliferation and apoptosis at different ages and p38MAPK inhibition and exercise experiments. Zebrafish tanks were maintained at 26-27˚C, with a 14 hour light 10 hour dark cycle per day and fed tetramin flake food 3 times a day. The UK Home Office approved of all experimental interventions carried out on zebrafish.

2.2. Thymidine analogue labelling

Bromodeoxyuridine (BrdU, Sigma-Aldrich) Iododeoxyuridine (IdU, Sigma-Aldrich) or Chlorodeoxyuridine (CldU, Sigma-Aldrich) was dissolved at a concentration of 0.25mg/ml (unless otherwise stated) in 1% dimethyl sulfoxide (DMSO, Sigma-Aldrich) aquarium water. 80ml of thymidine analogue solution was poured into a glass tumbler, into which individual zebrafish are placed for 24 hours with a lid on top.

2.3. Movement tracking

2.3.1. Recording movement

A tank measuring 200mm X 150mm was filled with aquarium water to a depth of 50mm was placed on a white sheet of paper and placed underneath a high-speed digital image recording system (ImperX VGA 210 CCD camera, Supermicro workstation and Lynx GigE software, provided by Multipix Ltd, UK). The images (480 x 640 pixels) were acquired at 25 frames per second using a Cosmicar 12.5mm lens. Individual zebrafish were added to the tank for each recording. Zebrafish were allowed to adjust to the new tank for 5 minutes and then three 1 minute videos were captured as images at a rate of 25 frames per second.

2.3.2. Analysis of movement

Using FIJI (Schindelin *et al.*, 2012) the images for each 1 minute period were imported as image sequences (File>Import>Image sequence) (Figure 2.1A). This opened a sequence image dialogue box. For each 1 minute period there should be 1500 images with the starting image as '1'. The image was then tresholded to a black and white image so that all that was visible is the zebrafish and tank edges

(Image>Adjust>Threshold) (Figure 2.1B). The image was then converted to binary (Process>Binary>Make binary). Using the rectangle tool, the area inside the tank, excluding the tank wall, was selected. This area was cropped (Image>Crop) so that in all images all that was visible is the zebrafish and everything else in the image sequence was white (Figure 2.1C).

Next the 'MTrack2' plugin was used to track the movement of the zebrafish over the 1 minute period (Plugins>Tracking>MTrack2). This opened the 'object tracker' dialogue box. The minimum object size was increased to 10 pixels; at 1 pixel background 'noise' may be picked up and adult zebrafish were never smaller than 10 pixels. The 'maximum object size' and 'maximum velocity' was set at at 1000 to ensure the zebrafish was tracked. The minimum track length was increased to 50 frames (2 seconds). This prevented the tracking become distorted if the zebrafish 'disappeared' for a few frames, which occured if the zebrafish turned in a way that the scales reflected light. The 'display path lengths' and 'show paths' boxes were ticked. This resulted in an image showing the path that the zebrafish travelled over the 1 minute period (Figure 2.1D) and a results dialogue box (Figure 2.1E). The image of the path travelled was compared to the image sequence to see if they are in agreement.

The results dialogue box (Figure 2.1E) showed the position of the zebrafish for each frame and a summary of the whole track. 'length' showed how far the zebrafish moved, in pixels, 'distance travelled' was the distance between where the zebrafish started and ended over the minute and 'nr frames' was the number of frames which should always be 1500. This data was then copied into Excel and converted from pixels into 'mm' using an image of a ruler or object of known size captured at the same magnification (Figure 2.1F). When three repetitions were tracked for a zebrafish the average speed was then calculated (speed [mm/s]=distance [mm]/times[s]). Speed was also calculated as body lengths (BL) per second (speed [BL/s]=distance [BL]/times[s]).

2.3.3. Common problems and solutions

If there was more than one path visible it was either due to over or under cropping the image sequence. If the path was split in two this was due to over cropping; somewhere in the image sequence the zebrafish was lost outside the periphery of the image. If there was an extra path this was due to under cropping, some of the aquarium wall was included in the image sequence and a reflection of the zebrafish is observed. If either occurred, the image sequence was reviewed and thresholding and cropping performed again.
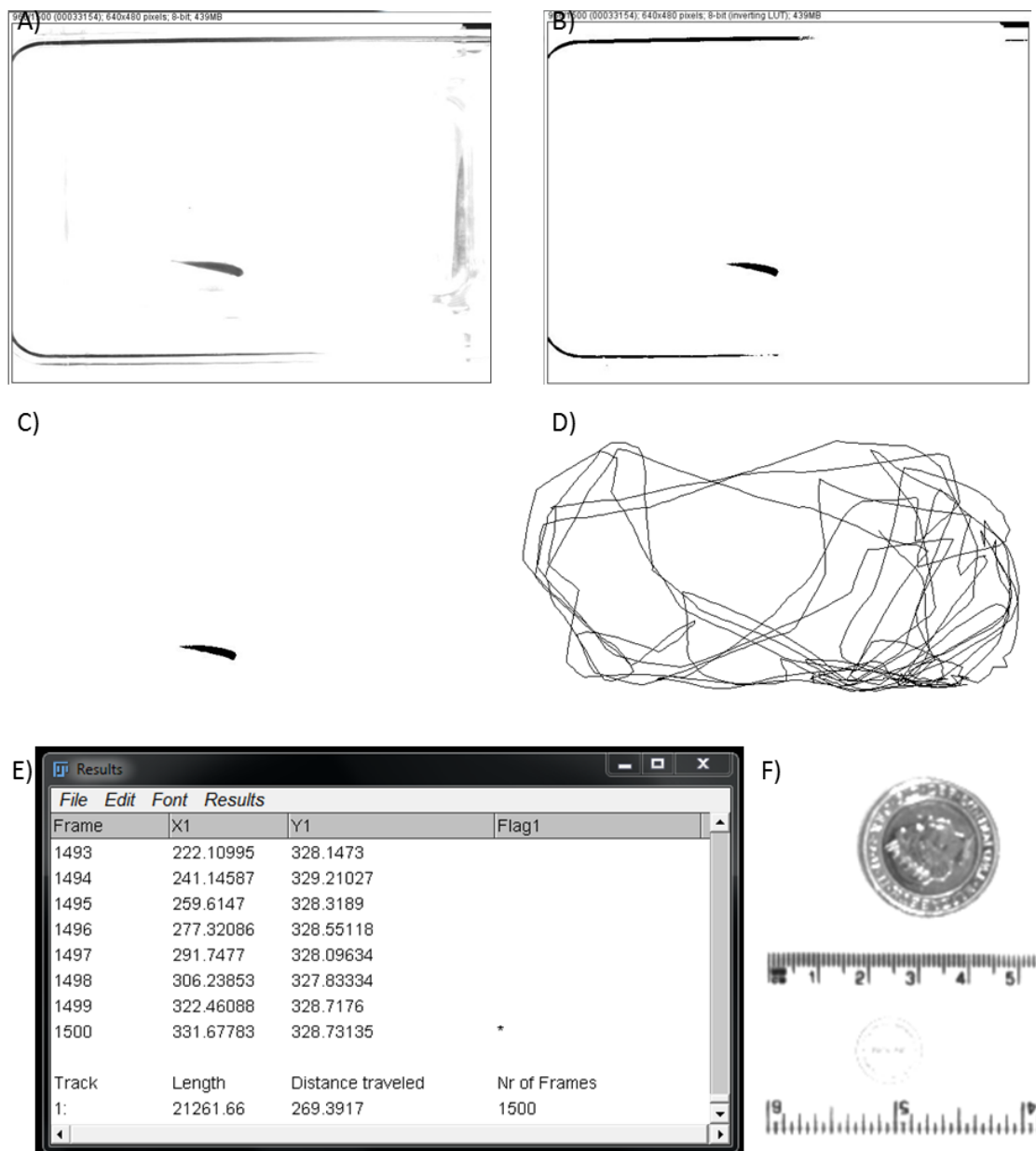
Figure 2.1: Tracking zebrafish movement
Raw images from an images sequence captured of a zebrafish swimming for 1 minute (A) were thresholded and converted to a binary image (B). The image was cropped to remove the aquarium walls (C). Using the MTrack2 plugin the path of movement over the course of 1 minute was mapped (D). The MTrack2 results dialogue box showed the position of the zebrafish in each frame (X1 and Y1) and the length and distance travelled (pixels) and total number of frames (E). A two pound coin and ruler were imaged at the same magnification as the zebrafish in order to convert the distances from pixels into mms (F)

2.4. Enforced swimming

A Fluval 406 canister filter (Hagen) was attached to a Perspex tank measuring 5cm X 95cm X 8cm to create swim tunnel with a constant but adjustable water flow. The speed of water flow was measured using a flow meter and adjusted to be a speed of 3 body lengths per second (BL/s). Up to 10 zebrafish of the same age and treatment group were placed in the swim tunnel for 72 hours. A 'sedentary' swim tunnel was used for sedentary control zebrafish. The sedentary tunnel was exactly the same in size as the swim tunnel, the only difference being the canister filter was not attached so there was no water flow.

2.5. SB203580 treatment

SB203580 hydrochloride (Tocris Bioscience) was dissolved in aquarium water with 1% DMSO at the desired concentration. 80ml of SB203580 solution was poured into a glass tumbler (Tesco) into which individual zebrafish were placed for 12 hours.

2.6. Dissections

Zebrafish were terminally anaesthetized in 100ml 1mg/ml tricaine (Sigma-Aldrich) dissolved in aquarium water for at least 5 minutes. Maximum standard length was measured from the tip of the snout to the posterior end of the last vertebrae using a ruler (Figure 2.2A). Zebrafish were weighed after drying on paper tissue to remove excess water.

Zebrafish were then pinned to a dissecting mat via the tail and eye socket. Under a light microscope the skin was cut from the anal fin to the base of the gill using iridectomy scissors (Figure 2.2A). The operculum and the pectoral girdle were removed using iridectomy scissors. The skin from the top of the operculum was cut to the initial incision by the anal fin. The skin overlying the heart was removed for better visualisation of the heart (Figure 2.2B). The heart and surrounding tissue was cut out and placed into a petri dish containing phosphate buffered saline (PBS, Fisher Scientific). If the heart was being extracted for RT-qPCR all solutions were made RNase-free by treating with 1ml diethylprocarbonate (DEPC, Sigma-Aldrich) per litre of solution, leaving overnight and autoclaving. The heart was then dissected from its surrounding tissue using watch maker forceps and iridectomy scissors then placed into ringer's solution for 10 minutes to allow the heart to beat out any blood inside it (Figure 2.2C). If the heart was being used for RT-qPCR analysis the heart was placed in a
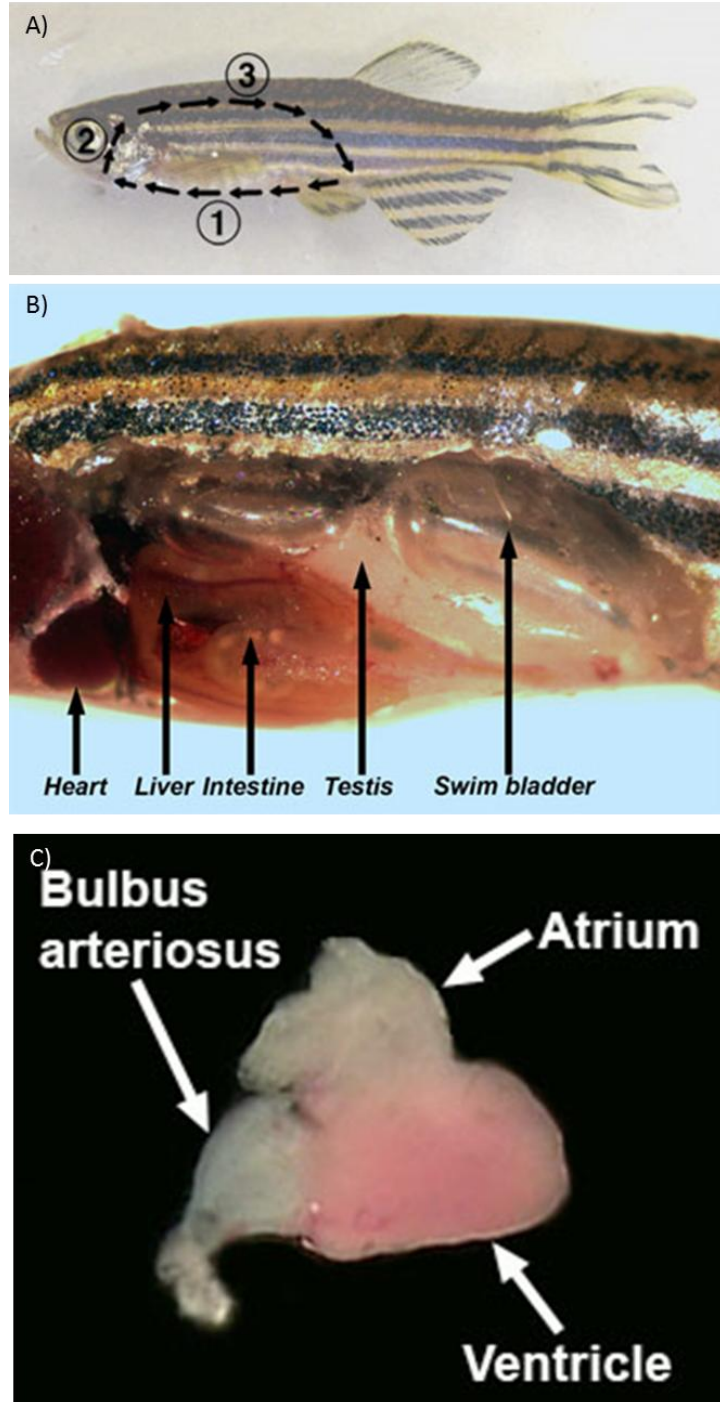
Figure 2.2:Dissecting zebrafish hearts
Incisions were made on a euthanised zebrafish (A, arrows), allowing better visability of the heart and other internal organs (B). The heart was then separated from the surrounding tissue (C).

Adapted from Gupta and Mullins 2010

1.5ml centrifuge tube (Fisher Scientific) with 1ml trizol (Life Technologies) and stored at -80°C until RNA extraction. If the heart was to be used for histological analysis the heart was placed in PBS for 10 minutes and then fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) overnight at 4°C.

2.7. Paraffin wax embedding

After being fixed overnight in 4% PFA hearts were then washed in PBS for 10 minutes, then dehydrated in 50% ethanol (Fisher Scientific) in dH2O for 30 minutes, then 70% ethanol in dH2O for at least 30 minutes or until they were processed for embedding at a later date. Next, hearts were transferred to glass vials and placed in 95% ethanol in dH2O for 30 minutes then in 100% ethanol for 30 minutes and again in fresh 100% ethanol overnight. 100% ethanol was then removed and 1:1 ethanol to histoclear was added for 30 minutes. Hearts were then processed into Histoclear (National Diagnostics) twice for 30 minutes at 65°C, then 1:1 histoclear to wax at 65°C then into wax twice at 65°C for an hour. Hearts were then removed from the glass vials and placed into a mould with paraffin wax. Hearts were orientated while the wax was still liquid with heated watchmaker forceps so the heart can be sectioned sagitally (Figure 2.2C).

2.8. Sectioning

Wax embedded zebrafish hearts were cut to a thickness of 8μm using a Leica RM2125 rotary microtome. Sections were floated on 1ml deionised water on slides heated to 37^{0}C on a slide warmer (Fisher Scientific). The sections from a whole heart was spread out over 6 slides in a way that each slide had approximately 3-4 sections in sequence so that each of the 6 slides had a fair representation of all of the regions of the zebrafish heart (Figure 2.3). After wax sections had spread out excess water was removed. Slides were placed in a rack and allowed to dry overnight in a 40^{0}C oven.

2.9. Masson's trichrome staining

Wax was removed from slides by steeping in histoclear for 2 10 minute washes in slide racks placed in plastic troughs. Slides were then rehydrated in decreasing concentrations of ethanol solutions; from 100% (twice) to 95%, 70% finishing in 50% ethanol. Slides were then washed in deionised water for 2 minutes and steeped in the mordant Bouin's solution (Sigma-Aldrich) overnight.

Figure 2.3: Arranging sections on slides

6 slides were placed next to each other on a slide warmer (1-6) with 1ml of deionised water. Sections (Pink numbered squares) were floated in order of sectioning on the slides so that each slide received 3-4 sections from different areas of the heart.

The following day the slides were rinsed under running tap water until the yellow colouration of Bouin's solution disappeared. Slides were then placed in Weigert's iron Haematoxylin (Sigma-Aldrich) for 10 minutes in order to stain nuclei and then rinsed under running tap water until the water ran clear. Slides were then stained with Biebrich scarlet-acid fucshin (Sigma-Aldrich) for 5 minutes, washed in deionised water for 2 minutes, soaked in a mixture of phosphotungstic acid:phosphomolybdic acid:water (1:1:2) for 5 minutes, stained with aniline blue (Sigma-Aldrich) for 5 minutes and then soaked in 1% acetic acid (Fisher) for 2 minutes. Slides were then washed in deionised water 3 times for 2 minutes each time and then dehydrated through an increasing concentration of ethanol solutions; from 50%, 70%, 95% up to 100%. Histoclear was then used to remove the ethanol from the slides by soaking for 10 minutes twice. Finally slides were mounted with a coverslip using Histomount (National Diagnostics).

2.10. Haematoxylin and eosin staining

Wax was removed from slides by steeping in histoclear for 2 10 minute washes. Slides were then rehydrated in decreasing concentrations of ethanol solutions; from 100% (twice) to 95%, 70% finishing in 50% ethanol. Slides were then washed in deionised water for 2 minutes and then nuclei was stained using Erhlich's aematoxylin (Sigma-Aldrich) for 10 minutes. The haematoxylin was then rinsed off with tap water for 5 minutes and then immersed in an acid/alcohol solution (1% HCl, Fisher Scientific, 70% ethanol) for a few seconds. Slides were then washed in running tap water for 5 minutes and then stained with eosin (Sigma-Aldrich) for 2 minutes). The eosin was rinsed of the slide with running tap water until it ran clear and then dehydrated through an increasing concentration of ethanol solutions; from 50%, 70%, 95% up to 100%. Histoclear was then used to remove the ethanol from the slides by soaking for 10 minutes twice. Finally slides were mounted with a coverslip using Histomount (National Diagnostics).

2.11. Immunofluorescence

2.11.3. Thymidine analogues and Mef2

Wax was removed from slides by steeping in histoclear (National Diagnostics) for 2 10 minute washes in slide racks in plastic troughs. Slides were then rehydrated in decreasing concentrations of ethanol solutions (Fisher Scientific); from 100% (twice) to 95%, 70% finishing in 50% ethanol. Slides were then washed in phosphate buffered saline (PBS, Fisher Scientific) for 2 minutes and then cells were permeabilised using a solution of PBS with 1% Tween 20 (Sigma-Aldrich) for 10 minutes. The slide rack was

removed from thee plastic troughs and the slides and slide rack placed in a glass 2 litre beaker. Antigen retrieval was then performed by heating the slides in a microwave for in a solution of citrate buffer (10mM Citric Acid, Sigma-Aldrich, 0.05% Tween 20, pH 6.0) until it is boiling and then for a further 15 minutes at a reduced power, ensuring the solution remains boiling and adding more solution if the slides begin to dry throughout. Slides were allowed to cool for approximately an hour, then transferred to a slide moisture chamber (Fisher Scientific) and washed twice in PBS for 5 minutes at a time. DNA in sections was denatured by putting 2N HCl (Fisher Scientific) on the slides for 30 minutes, HCl was then washed off the slides with PBS and then blocking solution added(10% fetal calf serum, Fisher Scientific, 1% DMSO in PBS) for 1 hour, to prevent any non-specific protein binding. After blocking the primary antibody for IdU (mouse anti-BrdU, BD Biosciences, 347580) was added to the slides at a concentration of 1/200 in blocking solution which was then incubated at 4°C overnight.

The following day slides were then washed in tris-buffered saline and tween 20 (TBST, 36mM tris, 50mM NaCl, 0.5% tween 20, pH6, Fisher Scientific) for 20 minutes at 37°C with constant agitation. The primary CldU (rat anti BrdU, Abcam, ab6326) and Mef2 (rabbit anti mef2, Santa-Cruz, sc-313) were added to the slides at concentrations of 1 in 250 and 1 in 50 respectively, in blocking solution and then incubated at 4°C overnight.

Slides were washed in PBS 4 times for 5 minutes at a time. Secondary antibodies were then applied to the slides (alexa fluor 488 anti mouse, alexa fluor 546 anti rat and alexa fluor 594 anti rabbit, Life Technologies) at a concentration of 1 in 400 in blocking solution and incubated at room temperature for 1 hour. Secondary antibodies were washed off the slides with 4 5 minutes washes with PBS. Slides were then mounted with a coverslip (Fisher Scientific) and hard set vectashield mounting medium (Vecta).

2.11.4. Cleaved caspase 3 and MF20

Wax was removed from slides by steeping in histoclear (National Diagnostics) for 2 10 minute washes in slide racks in plastic troughs. Slides were then rehydrated in decreasing concentrations of ethanol solutions (Fisher Scientific); from 100% (twice) to 95%, 70% finishing in 50% ethanol. Slides were then washed in phosphate buffered saline (PBS, Fisher Scientific) for 2 minutes and then cells were permeabilised using a solution of PBS with 1% Tween 20 (Sigma-Aldrich) for 10 minutes. The slide rack was removed from thee plastic troughs and the slides and slide rack placed in a glass 2 litre

beaker. Antigen retrieval was then performed by heating the slides in a microwave for in a solution of citrate buffer (10mM Citric Acid, Sigma-Aldrich, 0.05% Tween 20, pH 6.0) until it is boiling and then for a further 15 minutes at a reduced power, ensuring the solution remains boiling and adding more solution if the slides begin to dry throughout. Slides were allowed to cool for approximately an hour, then transferred to a slide moisture chamber (Fisher Scientific) and washed twice in PBS for 5 minutes at a time. Blocking solution was then added (10% fetal calf serum, Fisher Scientific, 1% DMSO in PBS) to slides for 1 hour, to prevent any non-specific protein binding. After blocking the primary antibody for MF20 (mouse anti MF20, Developmental Studies Hybridoma Bank) and cleaved caspase 3 (rabbit anti cleaved caspase 3, Cell Signalling, 9665) were added to the slides at a concentration of 1/200 then incubated at 4˚C overnight.

The following day slides were washed in PBS 4 times for 5 minutes at a time. Secondary antibodies were then applied to the slides (alexa fluor 488 anti mouse and alexa fluor 594 anti rabbit, Life Technologies) at a concentration of 1 in 400 in blocking solution and incubated at room temperature for 1 hour. Secondary antibodies were washed off the slides with 4 5 minutes washes with PBS. Slides were then mounted with a coverslip and hard set vectashield mounting medium (Vecta).

2.12. Microscopy and image analysis

Brightfield images of slides were captured using a Zeiss Axioplan microscope at 5x magnification. Fluorescent images were captured using a Zeiss Axioimager microscope. Between 3-6 whole sections were imaged fluorescently at 10x magnification for image analysis. The number of sections depended on how many positive cells there were in each section. To avoid counting the same cells on different section, sections used were never adjacent sections and were spaced at least three sections apart. Sections used all had a distinct lumen and were representative of the entire heart.

Image analysis was performed using FIJI as described in the results chapter 'Establishing and validating a suitable cardiomyocyte proliferation assay in zebrafish'.

2.13. RNA extraction

RNA extraction carried out as described by Lan et al, 2009 and all steps performed in an RNA and DNA free fume hood. Hearts dissected for RNA extraction were previously dissected and stored in 1ml of trizol in 1.5ml centrifuge tubes at -80°C. Samples were thawed on ice. Whilst still on ice samples were then homogenised using a 2ml syringe (Fisher Scientific) and 22g needle (Fisher Scientific) by rapid but controlled aspiration

and expulsion approximately 30 times per sample. A fresh needle and syringe was used for each sample. The process of homegenisation was then repeated with a smaller 24g needle (Fisher Scientific) to further homogenise and disrupt cells within the tissue.

200µl of chloroform was added to each sample which was then vigorously shaken for 15 seconds. Samples were then incubated for 3 minutes at room temperature then centrifuged at 10,000g for 15 minutes at 4°C in order to separate the aqueous phase (containing RNA) from the organic phase (containing proteins). 550µl of the aqueous phase was transferred to an RNase-free 1.5ml centrifuge tube. 1µl of GlycoBlue (Life Technologies) was added to each sample to facilitate precipitation and ease visualisation. 550µl of isopropanol (Fisher Scientific) was added to each sample and incubated at room temperature for 10 minutes. Samples were centrifuged at 10,000g for 10 minutes at 4°C. After centrifugation the supernatant was removed and discarded. The pellet, which was blue, was washed by adding 1 ml RNase-free 75% ethanol and centrifuging at 10,000g for 5 minutes at 4°C. The supernatant was removed and discarded. Excess ethanol was removed by centrifuging again at 10,000g for 5 minutes at 4°C. The remaining supernatant was carefully removed and discarded without disrupting the pellet. The pellet was then resuspended in 100µl DEPC treated deionised water. This crude RNA was then purified.

2.14. RNA purification

RNA purification was carried out using the RNeasy microkit (Qiagen). 350µl of the RLT buffer (Qiagen) was added to 100µl of the previously extracted RNA. 250µl of 100% ethanol was then added and pipetted up and down three times. Each sample (700µl) was added to a RNeasy MinElute spin column (Qiagen) inside the provided 2ml collection tube. The lid of the spin columns were closed and spun in a centrifuge at 8000g for 1 minute. The spin columns were transferred to new 2ml collection tubes. 700µl of RW1 buffer (Qiagen) was added to each spin column and then centrifuged at 8,000g for 1 minute. Spin columns were transferred to fresh 2ml collection tubes and 500µl of RPE buffer (Qiagen) was added to each spin column and allowed to incubate for 5 minutes. Spin columns were then centrfued at 8,000g for 1 minute in order to wash the column. The flow through was discarded and 500µl of 80% RNase-free ethanol was added to each column and centrifuged at 8,000g for 2 minutes. Spin columns were transferred to fresh 2ml collection tubes and centrifuged at 8,000g for 5 minutes with the lids open in order to ensure that the silica-gel membrane of the

columns were thoroughly dried. The spin columns were then transferred to 1.5ml RNase-free centrifuge tubes. 12µl of RNase-free deionised water was added to each column which were then centrifuged for 1 minute at 10,000g in order to elute the RNA. The quality of the RNA was then tested using a spectrophotometer.

2.15. RNA quality check

The quality of RNA was tested using a Nanodrop 8000 spectrophotometer (Thermo Scientific). The wells of the Nanodrop were polished and then blanked with RNase-free deionised water. 1µl of each sample was pipetted on to each well alongside a negative control (RNase-free deionised water). Absorbance was measured at 230, 260 and 280nm. The concentration of RNA was recorded in ng/µl, as too were the A_{260}/A_{280} and A_{260}/A_{230} ratios. RNA was only used if the A_{260}/A_{280} readings were between 1.9 to 2.1 and A_{260}/A_{230} between 2 to 2.2. Readings between these values are indicitative of purer RNA, with no, or very little, protein or salt contamination. RNA is diluted to a concentration of 10ng/µl in RNase-free deionised water and used for cDNA synthesis or stored at -80°C until used for cDNA synthesis.

2.16. Complimentary DNA (cDNA) synthesis

cDNA was synthesised using the high capacity cDNA reverse transcription kit (Life Technologies) following the supplied protocol. The reagents of the high capacity cDNA reverse transcription kit were allowed to defrost on ice, along with the RNA, if used from frozen.

2X RT master mix (Life Technologies) was made on ice in a 1.5ml RNase-free centrifuge tube, ensuring there was enough master mix for 10µl per sample. For each sample the 2X RT master mix should consist of 2µl RT Buffer (Life Technologies), 0.8µl 25X 100mM dNTP mix (Life Technologies), 2µl 10X random primers (Life Technologies), 1µl mulitscribe reverse transcriptase (Life Technologies), 1µl Rase inhibitor (Life Technologies) and 3.2µl nuclease free dH_2O (Life Technologies).

10µl of the 2X RT master mix was then added to a 0.2ml PCR tube (Fisher Scientific) for each sample, on ice. 10µl of each sample of the previously made 10ng/µl RNA (100ng of RNA in total) was pipetted into each reaction, pipetting up and down to mix. The reverse transcription was performed in an S1000 thermocycler (Bio-Rad) using the steps shown in Table 1. Upon completion of reverse transcription, cDNA was tested using polymerase chain reaction (PCR) or stored at -20°C until tested using PCR.

2.17. Primer design

Genes were identified from the literature and sequences found using ensembl (Flicek *et al.*, 2014). Primers were designed using primer blast (Ye *et al.*, 2012) (see Table 4 for a full list of promers and sequences). Properties of primers followed those outlined by Lan et al., 2009. The amplicon length of the PCR product had to be between 80-150bp, the length of the primers had to be between 18-25bp, with a GC content of 45-55% and a melting temperature between 59°C-62°C. All primers were purchased from Sigma-Aldrich.

2.18. Polymerase Chain Reaction (PCR)

The quality of cDNA was tested by conventional PCR and subsequent gel electrophoresis. Firstly, 5X green Go Taq reaction buffer (Promega), 10mM dNTP mix (Promega), forward and reverse primers for *β-actin*, and cDNA were allowed to defrost on ice.

One PCR master mix was made for all of the samples and a positive and negative control, in a 1.5ml centrifuge tube. For each single reaction 4μl of 5X green Go Taq reaction buffer, 1μl of 10mM dNTP mix, 0.2μl of Go Taq polymerase (Promega), 1μl of 10mM forward and reverse primer and 11.8μl of nuclease free water. The master mix was mixed by pipetting up and down a few times and then 19μl was pipetted into 0.2ml PCR tubes, 19μl per reaction. 1μl of cDNA for each sample was added to a PCR tube to give a final reaction volume of 20μl. The PCR was then performed in an S1000 thermocycler (Bio-Rad) using the steps shown in Table 2. After the PCR either gel electrophoresis was performed on the PCRs or PCRs were stored at 4°C until gel electrophoresis was performed.

2.19. Gel electrophoresis

Agarose gels were made using 1% agarose (SeaKem) dissolved in boiling TAE buffer (4.8g tris base, Fisher Scientific, 1.14ml of glacial acetic acid, Fisher Scientific, and 0.37g of EDTA, Fisher Scientific, per litre of buffer). 1ml of 10mg/ml ethidium bromide was added to the liquid gel which was then poured into a gel tray with combs (Fisher Scientific) and allowed to set for at least 20 minutes.

Once set, the gel was transferred to a gel running tank (Fisher Scientific), connected to a power pack (Fisher Scientific). 6μl of 100bp ladder (NEB) was pipetted

into the first well and then 10µl of each sample into the following wells. Once all samples were loaded, a current of 100 volts was applied to the gel for 20 minutes.

Gels were visualised using a UVP GelDoc-it 310 imaging system (Fisher Scientific). Images of the gel were printed and saved. If the negative control did not produce a band and the positive control produced a band the PCR was deemed successful. If there was a band in the negative control this was indicative of some sort of contaminiation and the PCR was repeated with fresh reagents. If there were no bands for any of the samples, including the positive control, the PCR was repeated using fresh reagents and a different positive control sample. Only samples which produced a clear band were used for subsequent real time quantitative polymerase chain reaction (RT-qPCR) analysis.

2.20. Real time quantitative polymerase chain reaction (RT-qPCR)

All methods and analysis involved in RT-qPCR adhered to the minimum information for publication of quantitative real time PCR experiements (MIQE) guidelines (Derveaux *et al.*, 2010; Taylor *et al.*, 2010).

2.20.3. Setting up the reaction

Real time quantitative polymerase chain reaction (RT-qPCR) was conducted using Fast SYBR Green Master Mix (Life Technologies), following the manufacturers instructions. Reactions were set up on a 96 well PCR plate (Life Technologies) using 10µl of 2X SYBR Green Master Mix, 0.8µl each of 10mM forward and reverse primers, 7.4µl of nuclease free water and 1µl of cDNA, giving a total reaction volume of 20µl. A 7500 Real-Time PCR system (Life Technologies) was used with the SDS software (Life Technologies), using the run settings in Table 3, finishing with a melt curve.

2.20.4. RT-qPCR analysis

Raw fluorescence data was copied from the SDS software into Microsoft Excel and analysed using LinRegPCR (Lan *et al.,* 2009). Cycle threshold (Ct) values, primer efficiencies and R^2 values were calculated using the program LinRegPCR . ΔCt and subsequent ΔΔCt values were calculated using the ΔCt value of the geometric mean of three reference genes. Relative expression levels were then compared using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where:

ΔCt= Ct (target gene) – Ct (reference gene)

ΔΔCt = ΔCt (sample heart) – ΔCt (control heart)

Selection of the most stable reference genes will be assessed and discussed in chapter 3.

	Temperature (^{0}C)	Time (minutes)
Step 1	25	10
Step 2	37	120
Step 3	85	5
Step 4	4	∞

Table 1: Steps used for programming the thermocycler for cDNA synthesis

Step	Temperature (^{0}C)	Time (minutes)	Repetitions
Initial denaturation	95	2	1
Denaturation	95	1	20
Annealing	60	1	20
Extension	72	1	20
Soak	4	∞	1

Table 2: Steps used for programming the thermocycler to peform regular PCR

Step	Temperature (^{0}C)	Time (seconds)	Repetitions
Initial denaturation	95	20	1
Denaturation	95	3	40
Annealing/extension	60	30	40

Table 3: Steps used for programming the 7500 Real-Time PCR system for performing RT-qPCR

Gene	Forward primer	Reverse Primer
β-actin	TCCATTGTTGGACGACCCAG	TGGGCCTCATCTCCCACATA
s18	AAGAAGATCAGGGCTCACCG	GCGACGACCAGTTGTTTTGG
usp5	TACCGAGGACAGGAGACAGG	CTCTCCACGTGTTCTCTTCCA
β-2M	AAACGGCCACAATGAGAGCA	AGCAGATCAGGGTGTTTGGT
slc25a5	GCCCCCATTGAGAGAGTCAA	CTCCAGAACGACAGGAAGCC
gapdh	TCACACCAAGTGTCAGGACG	TCAAGAAAGCAGCACGGGTC
rpl13a	TCCTCGCTCTTAAAACCGCC	GAGCCATGTGGGGACACTTC
elfa	ACGGTGACAACATGCTGGA	CCGCTAGCATTACCCTCC
gata4	CTCGACTTCTCCGGTGTACG	ATAGGAGGTGGTTTCCACGC
gata5	GGAAGAGAGGGTTCCCGACT	CCAAATGCGATGGTTTCCTGA
nkx2.5	TTCAGTGCTTCAGGCTTTTACG	GGGTCTTCCTGACAACAGCC
anp	GCAACATGGCCAAGCTCAAG	CTGTCCCAGGATGTGGAAGG
bnp	CATTCCCGCTTCAAAGCACA	CTTCTCTTTCCGCCGGTGTT
tbx5a	AATGCAGCCGTCAGCAAATC	TTTGTCCACAGCTCTCGCTC
tbx5b	AGGCTTAAACCCCAAAGCCA	AGTCTGGGTGCACATACAGG
mef2c	CCTGGAGCCAAGAACACCAT	GGTATTGATGGCAGACGGGT
tert	GTAAACTCGGGCGTCTCTGT	AGGCTGTAGCGCAGTGATAG
becn1	ATGGAGAACTTGAGTCGCAGA	GCACTCCTCACAAAGTGGGT
sqstm	CATCTGGAGCGCAGCAAAAC	CCACATCTATGCCAAGGGGG
aldh1a2	GAGCAAACCCCTCTCACCTG	CGCCACTTTGTCTATGCCCA

Table 4:Primers used for RT-qPCR analysis

2.21. Statistical analysis

Statistical analysis was carried out using Statistics Package for the Social Sciences (SPSS). The two statistical tests used were two sampled t-tests for comparing two data sets or one-way ANOVA for multiple data sets. When one-way ANOVA tests proved a significant difference a Tukey test was used *post hoc* in order to ascertain which groups were significantly different from each other. Two sampled t-tests was the most suitable for normally distributed data comparing two measurements (Ennos, 2007). One-way ANOVA was the most suitable for comparing multiple data sets when the data was normally distributed (Ennos, 2007). All data was examined using a Kolgomorov-Smirnov test to ensure data was not significantly different from normal distribution.

2.22. Experimental design and sample size

When working with animals selecting the optimum number for each experiment was vital. Not using enough animals would lead to misleading data wherein significant differences may have been missed which may otherwise exist. Selecting too many animals may have lead to wastage of resources and questions research ethics. The statistical analysis used on experimental data had a huge impact on the number of animals used. For simple analysis like a Students t-test preliminary experiments suggested 3-4 animals were optimum (Chow *et al.*, 2007; Rosner, 2010). For more complex analysis, such as a one-way ANOVA, preliminary experiments suggested 7-10 animals were optimum (Chow *et al.*, 2007; Rosner, 2010).

Chapter 3 : Ageing in the zebrafish heart

3.1.Introduction

The pathological changes which occur in the human heart have been well documented as previously discussed in section 1.3, as too have the gross changes in the zebrafish body as they age. However the changes that occur in zebrafish hearts due to ageing have not been described. In humans and animal models the main pathological effect of ageing on the heart can be summarised as the net reduction of cardiomyocytes, which which represents a very advanced stage of cardiac hypertrophy and stiffness which may lead to heart failure. (Kajstura *et al.*, 2010a; Fiechter *et al.*, 2013). Zebrafish have become an important tool for studying many human diseases, if the zebrafish heart does display pathological changes similar to that of humans due to ageing, zebrafish could be a valuable model to add to existing knowledge of cardiac ageing and ways to slow down ageing and reduce pathological features.

3.1.1. *Zebrafish growth*

Unlike most animals including mammals, which have a predetermined growth potential in which there is a limit to the size the organism achieves, many fish species exhibit indeterminate growth where size is ultimately determined by external factors, such as stocking density and food availability, and growth can persist throughout the lifespan of the organism (Mommsen, 1998).

Zebrafish do not exhibit true indeterminate growth (Biga and Goetz, 2006). Although zebrafish have been reported to continue growing after achieving sexual maturity (Rowlerson *et al.*, 1997; Gerhard *et al.*, 2002) and can regenerate many organs including the muscle and heart (Rowlerson *et al.*, 1997; Poss *et al.*, 2002) different studies have reported that when zebrafish are treated with exogenous growth hormone or have a constitutively active growth hormone transgene there is only 20% increase in growth which is short term (Simpson *et al.*, 2000; Morales *et al.*, 2001). Compare this with species of fish with true indeterminate growth, such as the coho salmon or giant danio which exhibit an 11-fold gain in weight or 129% increase in growth with elevated growth hormone levels, respectively (Devlin *et al.*, 1994; Simpson *et al.*, 2000).

Although zebrafish growth is not completely indeterminate, stocking density has been shown to affect the rate of growth in zebrafish; in one study body mass was shown to triple 14 days after zebrafish were transferred from a 'standard' aquarium stocking density, 5 zebrafish per litre, to a lower stocking density, 0.3 zebrafish per litre (Wills

et al., 2008). Importantly, this increase in body mass also resulted in an increase in cardiac growth via increased cardiomyocyte proliferation, as opposed to hypertrophy (Wills *et al.*, 2008).

It appears that 'indeterminate growth' is a spectrum rather than a clear cut property as some species appear to have a more indeterminate growth potential than others, for example coho salmon appears to possess a greater potential for growth than the giant danio, which in turn has a greater potential for growth than zebrafish. Zebrafish do appear to have indeterminate growth although the potential for growth is not as big as other species of fish. Care must therefore be taken in standardising zebrafish to be compared.

3.2. Morphological changes in zebrafish hearts due to ageing

3.2.1. Aims

The aims of the first section of this chapter are to describe changes that occur in the zebrafish heart due to ageing. This will determine whether zebrafish are a suitable model for cardiac ageing.

3.2.2. Results

3.2.2.1. Choosing zebrafish

Due to reported gender differences in the incidence of heart disease and cardiomyocyte turnover in humans (Kajstura *et al.*, 2010a), I will focus only on male zebrafish throughout this thesis. In order to compare data obtained from zebrafish to humans and choose the most appropriate ages to examine I converted the age of zebrafish (months) into the percentage of average life span elapsed (Figure 3.1A and B), assuming the average life span of zebrafish to be 36-42 months with some zebrafish living to 66 months (Gerhard *et al.*, 2002; Conn, 2011). The increased incidence of heart disease appears to occur at around 65 years old in humans (Lloyd-Jones *et al.*, 2002), which is approximately 65% of the average lifespan, using 70 years as the average lifespan (Figure 3.1C).

I decided that it would be important to map the changes occurring in the zebrafish heart before this point, as this is when subtle pathologic changes must occur. I decided to examine zebrafish that were 6, 12, 15 and 24 months old. This is the equivalent of 15%, 31%, 38% and 62% (Figure 3.1C), respectively and would give a range of different time points during adult life before excessive pathological changes would be expected to occur due to ageing. Due to difficulties in obtaining zebrafish at the correct ages when required for experiments the zebrafish intended to be 6 months

old and 24 months old were in fact 7 months and 23 months old respectively. Preliminary experiments and the literature suggested that for conducting multiple comparisons, using 7-10 animals for each group would be optimum (Chow *et al.*, 2007; Rosner, 2010). In each of the age groups 10 male zebrafish were processed. Erring on the side of caution and processing the maximum number of optimum samples allowed sufficient numbers of zebrafish to be available for statistical analysis when problems were encountered during processing. This was a justified decision, as indeed, although 10 zebrafish were processed for each age used for this section and again in sections 3.4, 3.6 and 3.4, problems encountered during the processing and sectioning of hearts, staining, immunofluorescence or running out of sections meant that it was not always possible to have 10 zebrafish in each age group for analysis. Altough many precautions were taken possible problems could occur at any stage of processing the hearts rendering the heart unusable. Hearts could be damaged during dissections. Hearts were so small that they could be lost in wax during embedding. Hearts could be damaged during sectionting or sectioned in the wrong plane. During immunofluorescence hearts could be damaged by becoming too dry or being left in solutions too long.

(A) Age (months)	Age (percentage of average lifespan)
0	0
6	15
9	23
12	31
15	38
18	46
21	54
24	62
27	69
30	77
33	85
36	92
42	108
48	123
54	138
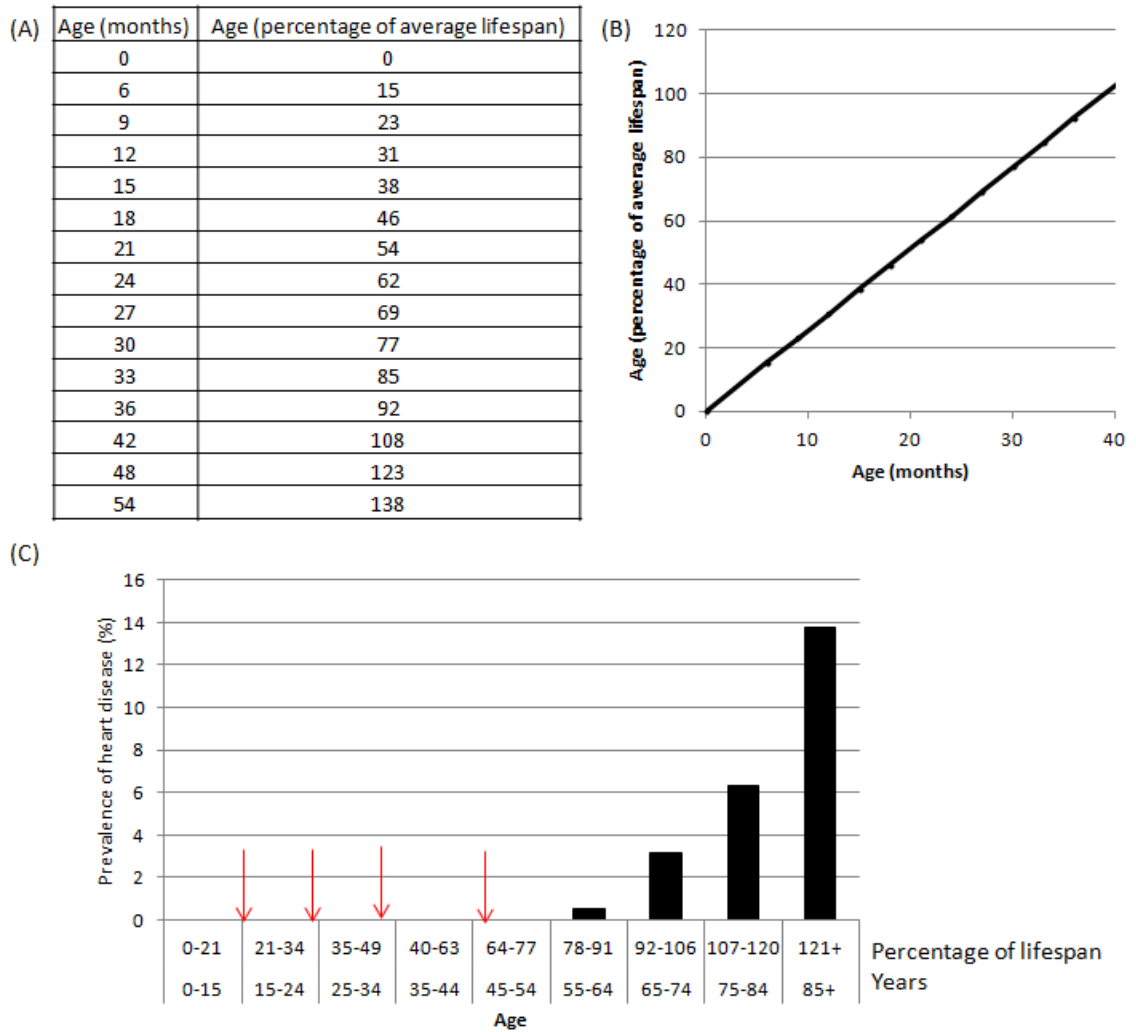
Figure 3.1: Choosing the age of zebrafish to examine
The average lifespan of a zebrafish is 36-42 months. Converting months into the percentage of average lifespan at three monthly intervals starting at 6 months (A) helps visualise the progression of time for a zebrafish lifespan (B). When human data (Lloyd Jones *et al.,* 2002) was converted to percentage of average lifespan (100 years=100%) showing prevalence of heart failure, an increase in incidence appears to occur between 65-74% (C, no data on prevalence of heart disease shown before 55 years old). I chose to study zebrafish hearts at 7 months, 12 months, 15 months and 23 months old (red arrows). I chose these ages as I wanted to examine hearts at spread of different ages, which included a point in which cardiac remodelling may occur.

103

3.2.2.2.Zebrafish possess indeterminate growth

It has been reported that zebrafish possess some degree of indeterminate growth therefore continue to grow into adulthood (Rowlerson *et al.*, 1997; Gerhard *et al.*, 2002). In order to confirm that the zebrafish I used did also possess indeterminate growth zebrafish were measured and weighed before dissections were performed. There was a significant difference increase in the length (Figure 3.2A) and weight (Figure 3.2B) of 7 months old zebrafish when compared to 23 months old zebrafish. This suggests that the zebrafish I used for this study did continue to grow during the periods studied. Although there appears to be a decrease in length and weight when comparing the 15 months old zebrafish to the 12 months old zebrafish this is not statistically significant.

(C)

Age (months)	Number of zebrafish	Length (mm)	STD	Weight (g)	STD
7	10	25.20	1.81	0.36	0.05
12	10	30.00	2.40	0.61	0.10
15	10	27.30	1.25	0.49	0.10
23	10	33.10	2.60	0.70	0.14
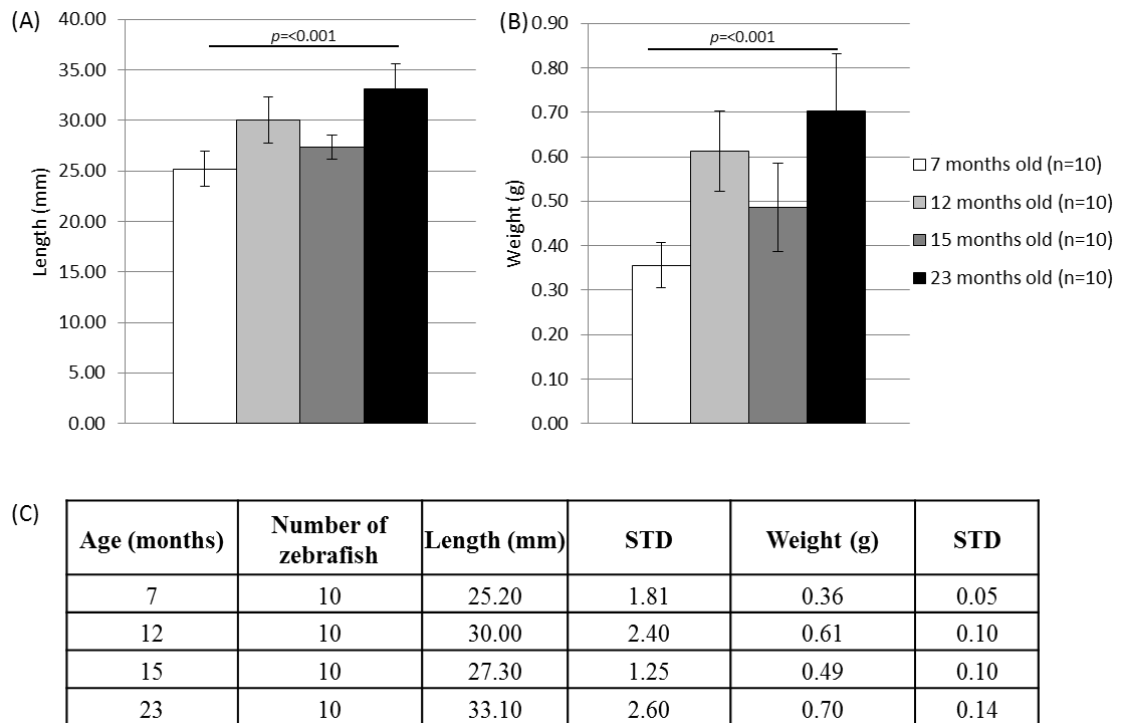
Figure 3.2: Zebrafish possess indeterminate growth
Zebrafish appear to have indeterminate growth. The length (A) and weight (B) increase significantly as zebrafish age. Error bars are standard deviation. Statistical analysis was performed using a one-way ANOVA. The mean of the length and weight along with standard deviations (STDs) are show in (C).

3.2.2.3.Fibrosis develops as zebrafish age

As hearts of humans and animals change in morphology and often develop fibrosis in later life, the hearts of zebrafish were examined histologically for changes in morphology and signs of fibrosis. Heart sections were then stained for the presence of collagen using Masson's trichrome, which stains collagen blue (Figure 3.3). Hearts from zebrafish aged 7 months (Figure 3.3A) had collagen in the bulbous arteriosus (Figure 3.4A) and in the atrioventricular and bulboventricular valves (Figure 3.4B) which has been reported elsewhere (Hu *et al.,* 2001). Hearts from zebrafish aged 12 months (Figure 3.3B) and 15 months (Figure 3.3C) only had collagen observed in the bulbous arteriosus, atrioventricular valve and bulboventricular valve. No abnormal collagen was present. In the 23 months old zebrafish hearts collagen could be observed in the previously described locations and in 5/9 hearts collagen was found outside of the bulbous arteriosus and the valves was observed (Figure 3.3D). Collagen deposits varied in terms of magnitude and location within the hearts and were observed within atrial and ventricular walls (Figure 3.4C) and on trabeculae (Figure 3.4D). Fibrosis in these areas of the zebrafish heart has not previously been reported in aging zebrafish. The incidence of fibrosis in 23 months old zebrafish hearts was significantly higher than the other age groups and occurred in 55.5% of hearts examined (Figure 3.3E). In later experiments (Chapter 4) cardiac fibrosis was found in one out of ten 21 months old zebrafish hearts. This suggests that the frequency of cardiac fibrosis increases as zebrafish age.

3.2.2.4.The ventricular wall thickens with ageing

One property of aged human hearts is the increase in thickness of the ventricular wall (Hees *et al.*, 2002). In order to measure ventricular wall thickness in zebrafish in a robust manner heart sections were stained using Mason's trichrome method (Figure 3.3). Six different points on the ventricle were selected on a section stained with Masson's trichrome, starting horizontally below the atrioventricular junction and then at 45˚ intervals (Figure 3.5). The mean of these 6 measurements was calculated for each zebrafish heart.

The 23 months old ventricular walls did indeed appear to be thicker than all of the other age groups; the 23 months old zebrafish appeared to possess ventricular walls almost twice as thick as the other age groups at around 40μm thick compared to 20μm respectively (Figure 3.6B). As older zebrafish are larger and would therefore be expected to have larger hearts, the thickness of the ventricle wall was calculated as a

ratio of length (Figure 3.6A). Even when expressed as a ratio of body length to normalize for differences in body size, zebrafish aged 23 months still had significantly thicker ventricular walls (Figure 3.6A).
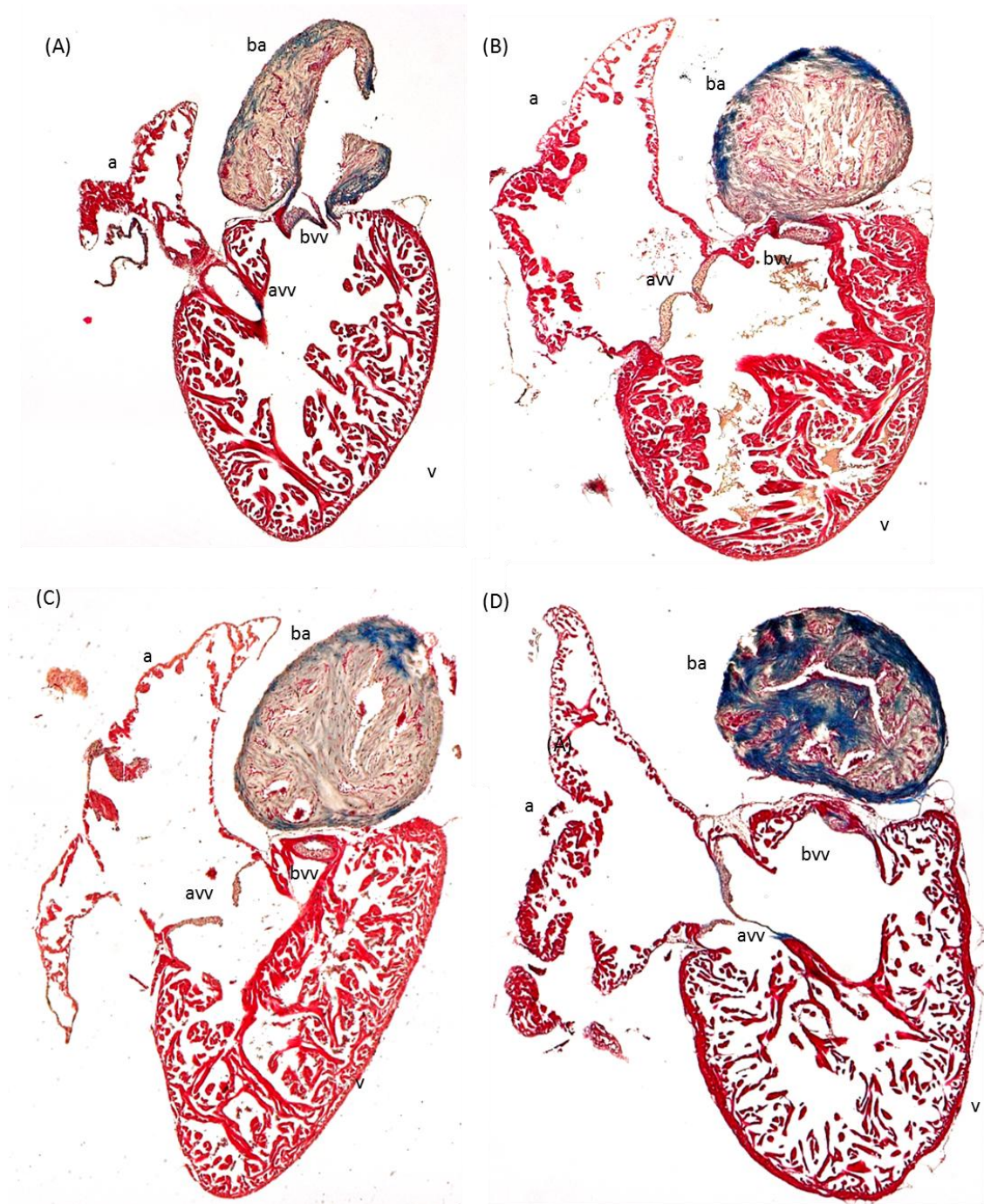
Figure 3.3:Zebrafish hearts appear morphologically different at different ages
Zebrafish heart sections from 7 months old zebrafish (A), 12 months old zebrafish (B), 15 months old zebrafish (C) and 23 months old zebrafish (D). Sections are stained using the Mason's trichrome method. This stains collagen blue and muscle red. Hearts from zebrafish aged 3, 12 and 15 months appeared to have thinner ventricular walls and denser trabeculae when compared to the 23 months old zebrafish. The 23 months old zebrafish heart shown here is a representation of one of the non-fibrotic 23 months old hearts.
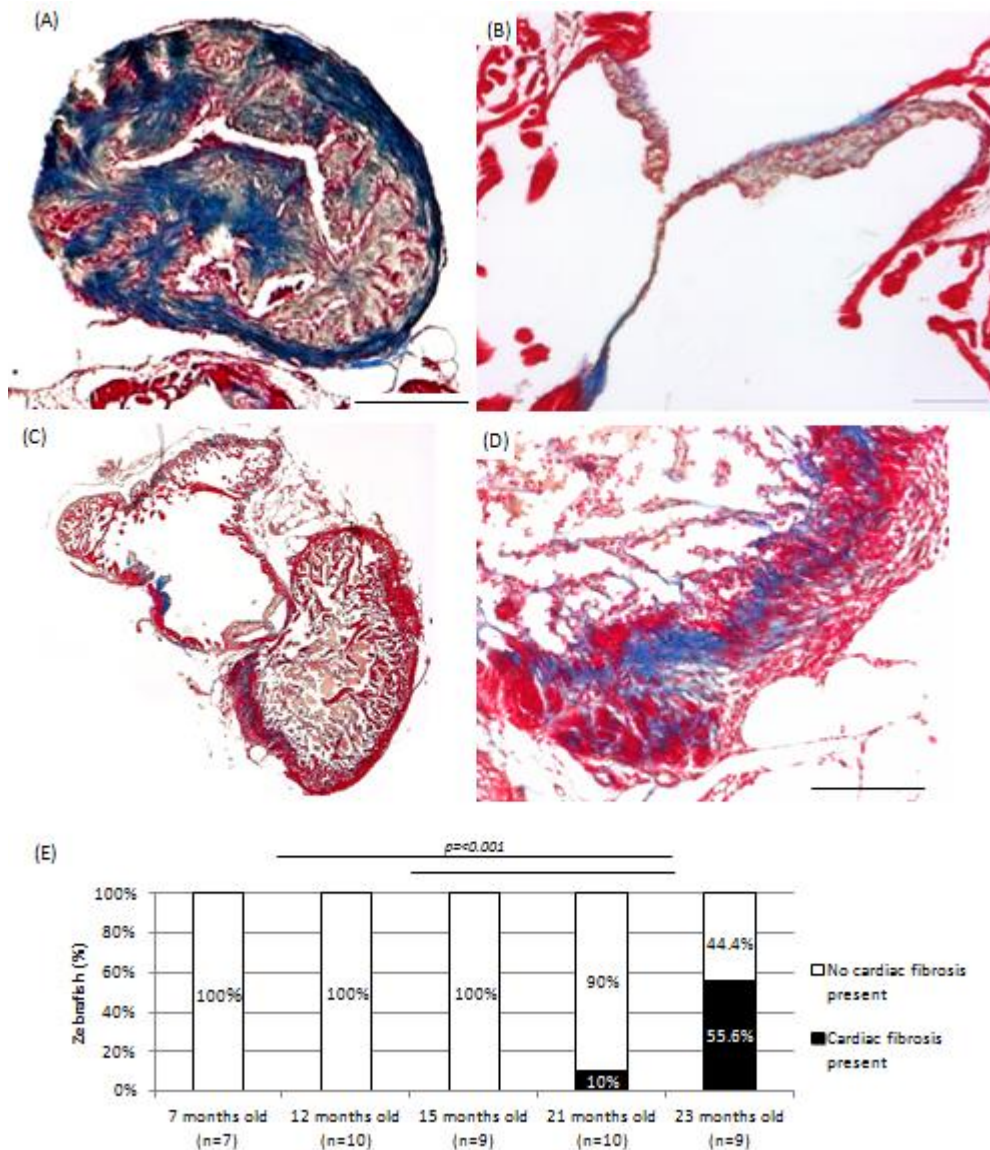
Figure 3.4:The incidence of cardiac fibrosis increases with age

Tissues in the zebrafish heart which have been reported to naturally contain collagen include the bulbous arteriosus (A) and the bulboarterial and atrioventricular valve (B). Abnormal collagen deposits, otherwise unreported, were observed in the atrial and ventricular walls (C and DD) in one out of ten 21 months old zebrafish (examined in the next chapter) and 5 out of 9 hearts from 23 months old zebrafish, but none of the other age groups (E). Fibrosis was scored as being present or completely absent. The frequency of fibrosis in 23 months old zebrafish was significant greater when analysed using a one-way ANOVA.
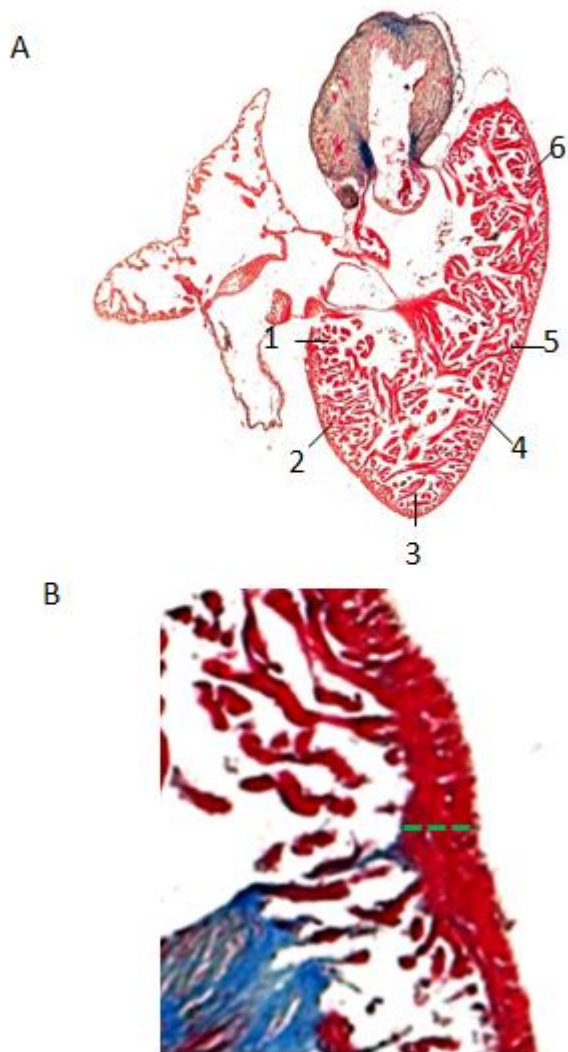
Figure 3.5:Measuring the thickness of the ventricular wall
The different points of the ventricle wall measured (A). Starting below the atrium at every 45° degrees the wall was measured for 6 points and the average was calculated from these values. The area measured was from the external wall of the myocardium to the spngy myocardium, including the compact myocardium (green dashed line, B).

(A)

(B)
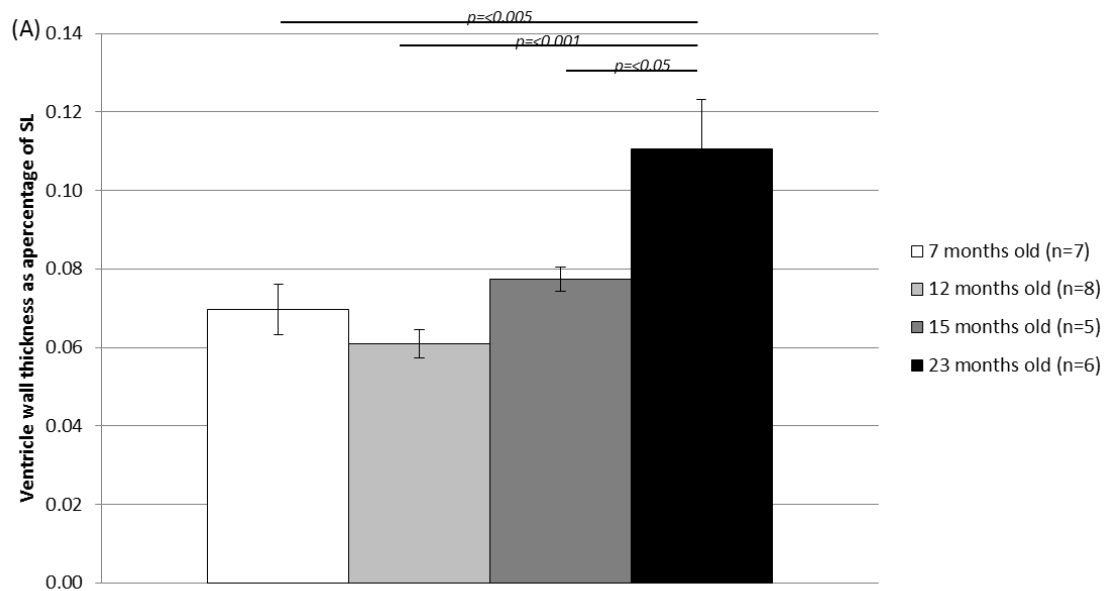
Age (months)	Number of zebrafish	Mean ventricle wall thickness (μm)	SEM	Ventricle wall thickness (percentage of SL)	SEM
7	7	17.25	1.59	0.0696	0.0064
12	8	18.23	1.00	0.0610	0.0035
15	5	24.28	1.25	0.0775	0.0031
23	6	37.84	5.18	0.1107	0.0124

Figure 3.6: The ventricular wall thickens with age

The ventricle wall thickens as zebrafish age (A). Thickness is plotted as a percentage of standard length (SL) for each zebrafish in order to normalise the data and give a fair comparison between zebrafish of different lengths. The mean values, including the ventricle wall thickness (μm), is included in table (B), along with SEM. Statistical significance was observed when using a one-way ANOVA. Error bars are SEM.

3.2.2.5. Ventricular lumen area increases due to ageing

To indicate whether ventricular dilation may occur as zebrafish age the binary images of zebrafish ventricles were inverted (Figure 3.7C). This allowed for the measurement of the lumen area (Figure 3.7D). From these images the area of the lumen can be calculated as a percentage of the total ventricular area (area of ventricular tissue + lumen area). Plotting the lumen area as a percentage of the total ventricular area standardizes the measurement, as heart size may be variable between individual zebrafish.

The lumen area as a percentage of total ventricular area is higher in the 23 months old zebrafish than the 12 months old zebrafish and 15 months zebrafish (Figure 3.8). There is an increase in ventricular wall thickness due to ageing, which would increase the amount of ventricular tissue. However the lumen area as a percentage of the total ventricular area increases, suggesting that there is a reduction in trabeculae and an increased dilation of the ventricle. This fact appears obvious when looking at the Masson's trichrome stained images of zebrafish heart sections; the 7 months old zebrafish appear to have thinner, sparser trabeculae which become denser and thicker up until 23 months when trabeculae appear thinner and there are fewer (Figure 3.3). This suggests that there is a gradual change in cardiac morphology between 15 to 23 months of age in zebrafish.
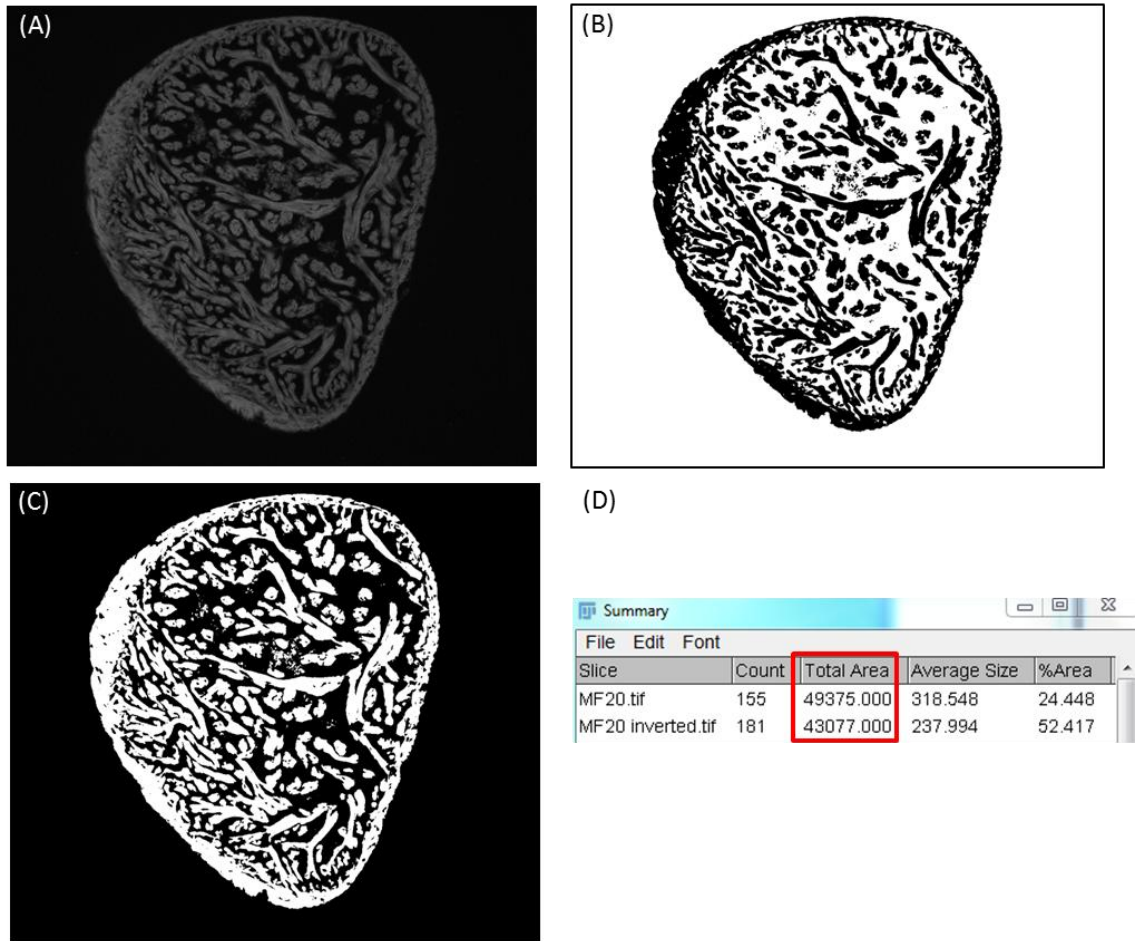
Figure 3.7:Measuring lumen and ventricle tissue area
The typical appearance of an immunofluorescent image of a sagital section of a zebrafish ventricle when using an anti-MF20 antibody (A).   This was thresholded and converted into a binary image using FIJI (B) in order to measure the tissue area. This image was then inverted (C) so that the lumen area could be measured in pixels (D) and then converted into millimetres. The average measurements from 5-6 sections were used for each zebrafish heart.

Figure 3.8:**Ventricular lumen area increases as zebrafish age**
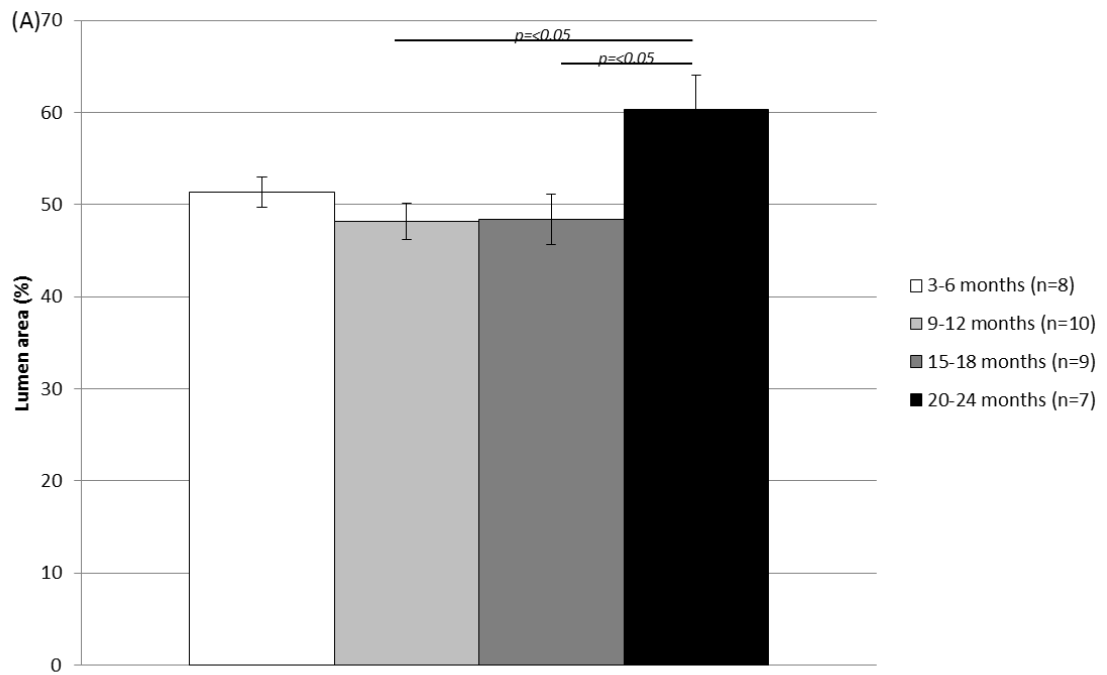
Age (months)	Number of zebrafish	Ventricular tissue area (mm2)	SEM	Lumen area (mm2)	SEM	Lumen area as a percentage of ventricular tissue	SEM
7	8	21.97	1.44	19.47	1.49	51.33	1.64
12	10	26.79	2.27	21.97	1.29	48.20	1.98
15	9	22.93	2.78	19.42	2.97	48.35	2.74
23	7	18.40	2.42	21.58	4.03	60.30	3.77

Figure 3.8:Ventricular lumen area increases as zebrafish age
There is an increase in the lumen area of the ventricle as zebrafish age, when lumen area is expressed as a percentage of the total area occupied by the ventricle in mid sagittal sections (A). Error bars are SEM, significant difference was observed when analysed with a one-way ANOVA. The lumen area as a percentage of ventricular area is included in table (B). The average measurements from 5-6 sections were used for each zebrafish heart.

3.2.2.6.Adipose tissue around the heart increases due to ageing

When humans reach older ages, the amount of adipose tissue around the heart increases (Waller, 1988; Montani *et al.*, 2004; Bertaso *et al.*, 2013). Increased epicardial fat deposits have been associated with increased cardiac risk (Bertaso *et al.*, 2013). To determine if this is also true of zebrafish a robust and objective method for measuring the level of adipose tissue deposited on the ventricular wall in Masson's trichrome stained sections of zebrafish hearts. Firstly the zebrafish heart was divided into five geographical regions; the blulboarterial cleft, posterior, apex, anterior and bulboventricular cleft (Figure 3.9A). The severity of adipose deposits was scored for each region from 0-3; 0=no adipose deposits, 1=few adiopose deposits, 2= the majority of the region covered by adipose deposits and 3=the majority of the region covered in multi-layered adipose deposits (Figure 3.9B-E). Each area was scored and added together for each heart, giving an adipose score out of 15 for each individual.

In the zebrafish aged 7 months, 12 months and 15 months there appeared to be minimal adipose deposits, each of these age groups scoring on average between 1 and 3 for the entire heart (Figure 3.10). However in the 23 months old hearts there was a significant increase in adipose deposits, this age group scoring on average around 9, thus suggesting that as zebrafish reach advanced ages the amount of adipose tissue deposited around the heart increases. This is similar to what happens in human hearts. Next I wanted to see if changes in cardiomyocyte turnover due to ageing may have been the underlying cause of the pathological features I have reported.

3.3.Establishing and verifying a suitable assay for cardiomyocyte proliferation in zebrafish

3.3.1. Aims

In order to determine the level of proliferation in zebrafish cardiomyocytes I needed to develop a suitable proliferation assay which could accurately measure cardiomyocyte proliferation. After reviewing different markers of proliferation in chapter 1, I decided that thymidine analogues would be most suitable. In this section I initially aimed to optimise the treatment of zebrafish with multiple thymidine analogues. The task of manually counting sufficient numbers of cardiomyocytes appeared to be too time consuming to conduct in many zebrafish. My next aim was to therefore make the process of counting cardiomyocytes more automated to allow for a higher throughput of zebrafish. This entailed using the program FIJI (Schindelin *et al.*, 2012) to establish a

novel program which could be used to rapidly to robustly count zebrafish cardiomyocytes in immunofluorescent images.
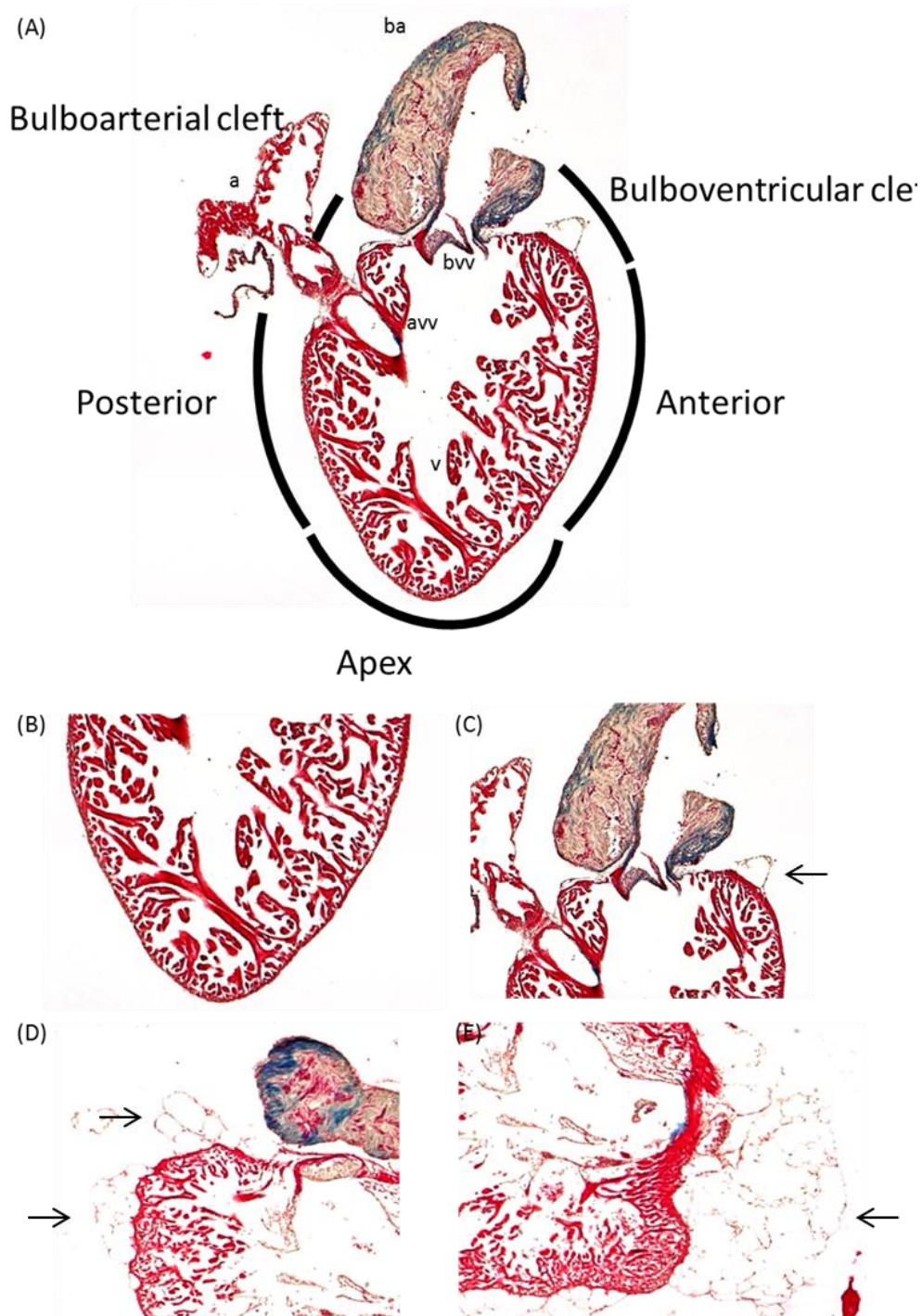
Figure 3.9:Scoring cardiac adipose tissue deposits
The surface of the heart was scored at 5 different regions which I called the 'bulboarterial cleft', the 'posterior surface', the 'apex', the 'anterior surface' and the 'bulboventricular cleft' (A). Areas with no adipose deposits were scored '0' (B). Areas with a few adipose deposits were scored '1' (Black arrows, C). Areas with few, multilayer adipose deposits were scored '2' (D). Areas with multiple, multilayered adipose deposits were scored '3' (E).

(A)

p=>0.001
p=>0.001
p=>0.001

Legend:
- □ 3-6 months (n=7)
- ▨ 9-12 months (n=10)
- ▨ 15-18 months (n=9)
- ■ 20-24 months (n=8)

(B)

Age (months)	Number of zebrafish	Mean adipocyte score	SEM
7	8	1.17	0.28
12	10	1.50	0.22
15	9	2.56	0.56
23	8	8.88	0.74
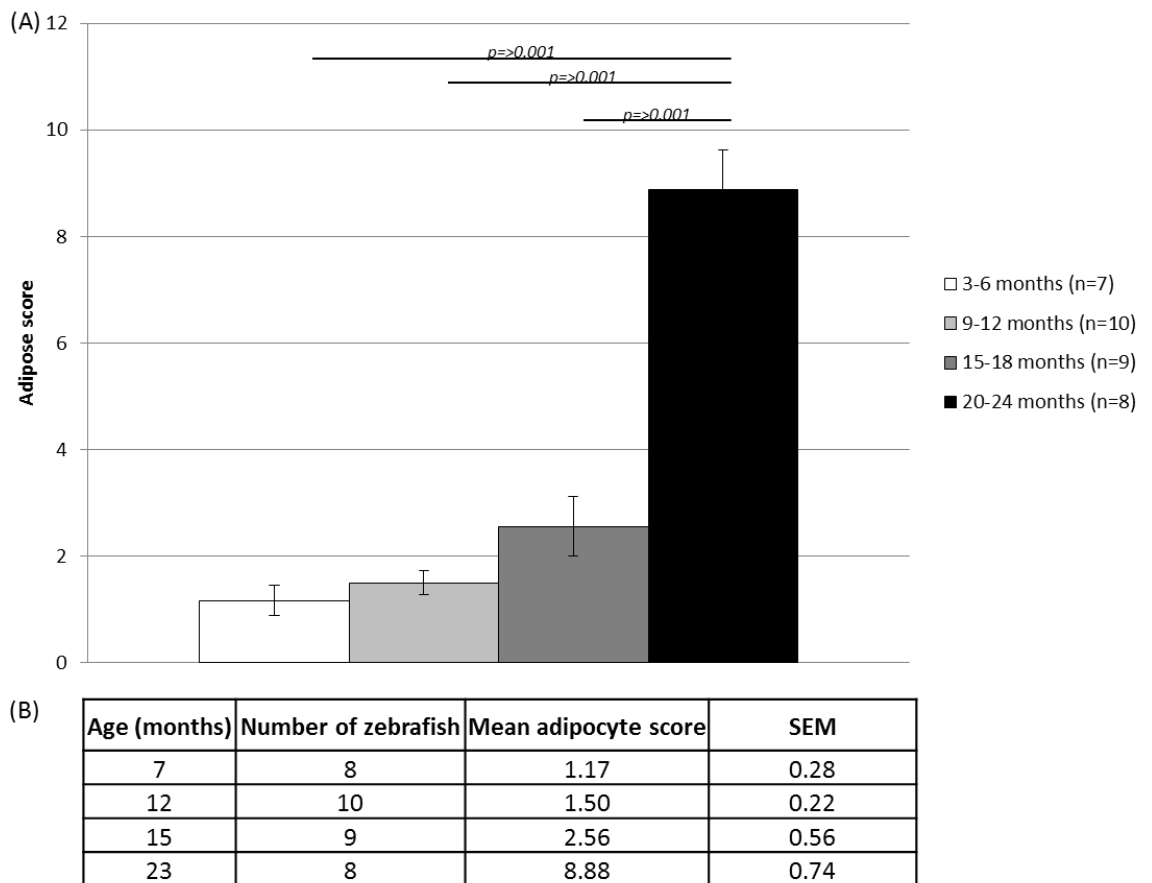
Figure 3.10: Adipose deposits increase as zebrafish age

As zebrafish reach an advanced age the adipose deposit score increases (A). The mean adipocyte deposit score for each age group, along with SEM is shown in table (B). Error bars are SEM, statistical significance was observed when a one-way ANOVA was performed.

3.3.2. Results

3.3.2.1. BrdU optimisation

I decided to initially try using BrdU to label proliferating cardiomyocytes in zebrafish hearts. BrdU pulsing has been used successfully in adult zebrafish in various studies and tissues (Poss *et al*., 2002; Rowlerson *et al*., 1997; Her *et al*., 2011). I therefore wanted to see if this method worked and this work would form preliminary data for more complex multiplexing of thymidine analogues. The two reported methods of administration in zebrafish are via intraperitoneal injection (Poss *et al.*, 2002)or immersion in a solution (Rowlerson *et al.*, 1997). As injections are relatively stressful and the pulsing time cannot be user determined (i.e. choosing to immerse a zebrafish for a predetermined length of time) I decided to use the immersion technique. In order to establish a robust method to measure cardiomyocyte proliferation, the correct dose of BrdU would need to be administered. To date there are no studies which use the immersion technique to label proliferating cardiomyocytes with BrdU. Rowlerson *et al*., 1997 'generally' (their description) used 150μg/ml BrdU to label muscle cells. I decided to use approximately 3-6x higher doses of 500μg/ml and 1000μg/ml to ensure all proliferating cardiomyocytes are labelled. If the BrdU dose is too low, BrdU may be more difficult to detect and if cells proliferate more than once the BrdU would become more dilute.

Three 6 months old male zebrafish were immersed in either 0.5mg/ml or 1mg/ml of BrdU dissolved in aquarium water for 24 hours then immediately euthanized. Hearts were dissected, embedded in paraffin wax and sectioned 8μm thick. Sections were processed for immunofluorescence using antibodies for myosin heavy chain (MF20) and BrdU. Sections were also counter stained with DAPI to label cell nuclei. Images were captured of three sections for each zebrafish (Figure 3.11). Cardiomyocytes were then counted by hand. MF20 marks heart muscle so any DAPI staining in these areas should label cardiomyocytes. Any of these DAPI and MF20 positive cells which are also BrdU positive should be a cardiomyocyte that is in the process of proliferating or has proliferated. After processing and analysis there was no significant difference in the percentage of BrdU positive cardiomyocytes between the two different doses (Figure 3.12). This suggests that at both doses BrdU was readily incorporated into proliferating cardiomyocytes implying that no proliferating cardiomyocytes were 'missed' and not labelled by BrdU. Subsequently, 500μg/ml of thymidine analogues would therefore be the preferable dose due to cost and reducing the chance of any potential side effects.
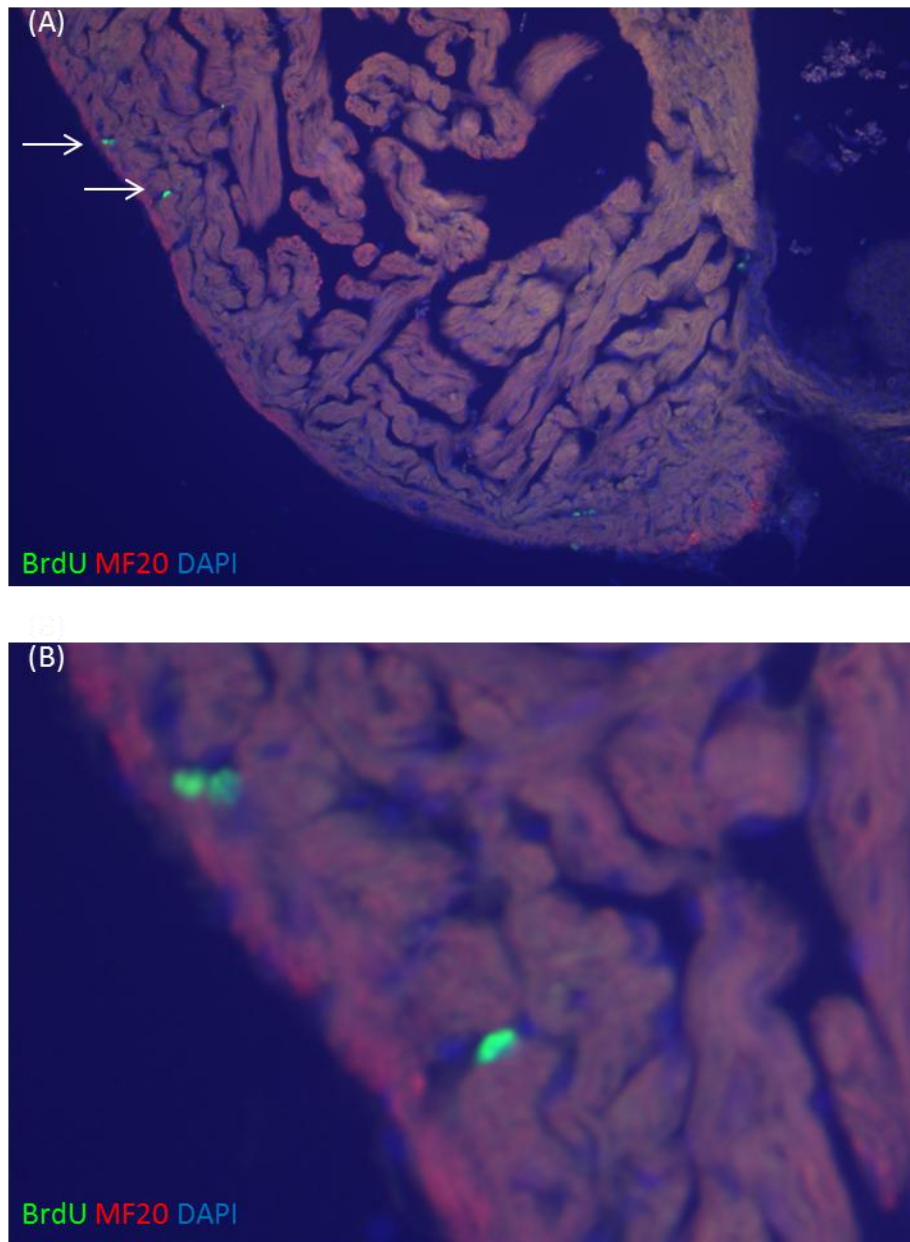
Figure 3.11:Immunofluorescent detection of BrdU in zebrafish heart sections
Immunofluorescence was used to detect BrdU (green) and MF20 (red) in sections of zebrafish hearts (A and B). Sections were counterstained with DAPI to mark the nuclei of cells. White arrows show BrdU positive cardiomyocytes. In B, the BrdU positive cardiomyocytes at the top were doublets, a cardiomyocyte which had gone through mitosis. The single BrdU positive cardiomyocyte in B would have gone through S-phase but not cytokinesis. This is because the zebrafish would have been euthanized before this had occurred.

(A)
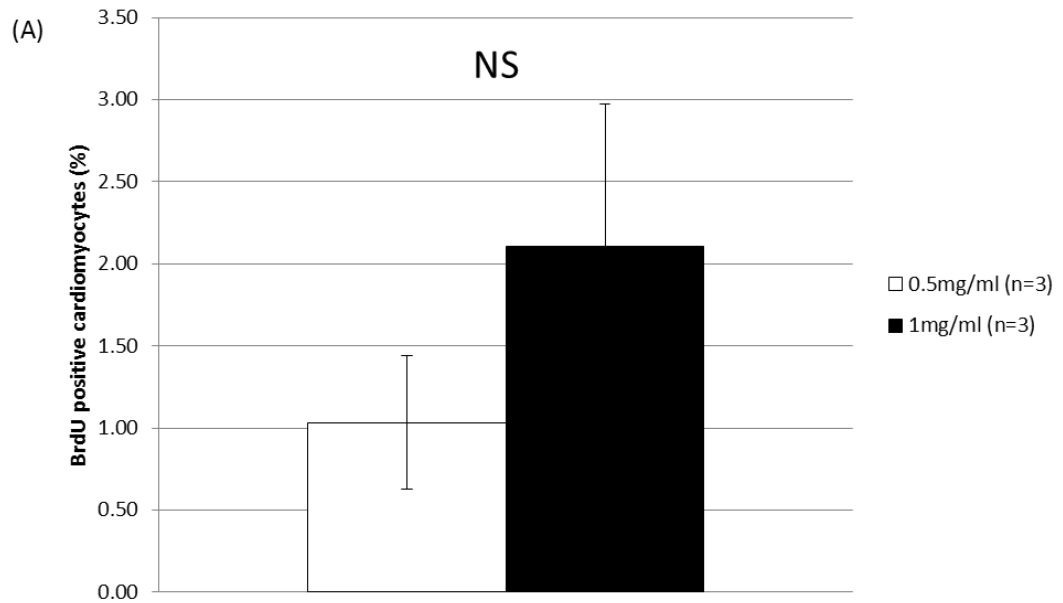
(B)

BrdU concentration	BrdU positive cardiomyocytes counted per zebrafish	Total cardiomyocytes counted per zebrafish	BrdU positive cardiomyocytes (%)	SEM
0.5mg/ml	23.22	1853.79	1.03	0.87
1mg/ml	30.89	1522.44	2.11	0.57

Figure 3.12: Optimising BrdU dosing

BrdU positive cardiomyocytes were counted in mid-sagittal sections of zebrafish immersed in either 0.5mg/ml or 1mg/ml BrdU for 24 hours (A). No significant differences were observed in the percentage of BrdU positive cardiomyocytes between the two concentrations of BrdU. Error bars are SEM. No statistical significance was observed when analysed using a student's t-test. The mean number of BrdU positive cardiomyocytes counted and total number of cardiomyocytes counted for each zebrafish are in table (B).

3.3.2.2. Using multiple thymidine analogues

Other than being able to dictate the labelling time of cells undergoing proliferation, another advantage of using thymidine analogues is the ability to multiplex thymidine analogues (Tuttle *et al*., 2010). This means that proliferating populations of cells can be identified at two different points in time, giving the ability to measure the length of the cell cycle or the level of proliferation before and after different interventions. Using a nuclear specific marker of cardiomyocytes such as mef2 enabled me to specifically measure proliferation in cardiomyocytes. The thymidine analogues used to do this are IdU and CldU, which can be identified by different BrdU antibodies (Tuttle *et al*., 2010).

As the antibodies for IdU and CldU are both marketed as anti-BrdU antibodies I decided it was important to ensure that there was no cross reactivity between the antibodies. To ensure there was no cross reactivity between the antibodies 6 months old zebrafish males immersed in IdU alone or CldU alone for 24 hours were processed and imaged with both of the anti-BrdU antibodies which specifically detect either IdU or CldU (Figure 3.13). No cross reactivity was observed when immunofluorescence for each primer was performed alone on tissue treated with only one thymidine analogue. In the tissue treated with IdU the anti-IdU antibody detected proliferating cells positive for IdU. In tissue treated with IdU, the anti-CldU antibody did not detect proliferation. In the CldU treated the anti-CldU antibody detected proliferating cells positive for CldU. In the CldU treated tissue the anti-IdU antibody did not detect proliferation. If cross reactivity occurred it would be expected that the antibodies would indiscriminately bind to IdU and CldU and cellular proliferation would be detected in all the images. This shows that there is no cross reactivity and multiplexing thymidine analogues are a suitable assay in the zebrafish heart. This experiment also demonstrates that there is spectral separation of the fluorochromes. This means there is no spectral bleed through between the channels, further ensuring the suitability of using two thymidine analogues.

3.3.2.3. Automatic counting

As zebrafish hearts contain thousands of cardiomyocytes, manually counting cardiomyocytes in paraffin wax sections would be a very time consuming and cumbersome task. FIJI (Schindelin *et al.*, 2012), an open source image processing program designed for use in the life sciences and based on ImageJ (Abramoff, 2004), has the ability to automatically count cells making the task rapid and suitable for a relatively higher throughput. However ensuring that the use of FIJI for counting

zebrafish cardiomyocytes is robust and accurate requires a thorough understanding of the methodology involved and high quality images of a fixed exposure and magnification in order to derive meaningful results from the data.
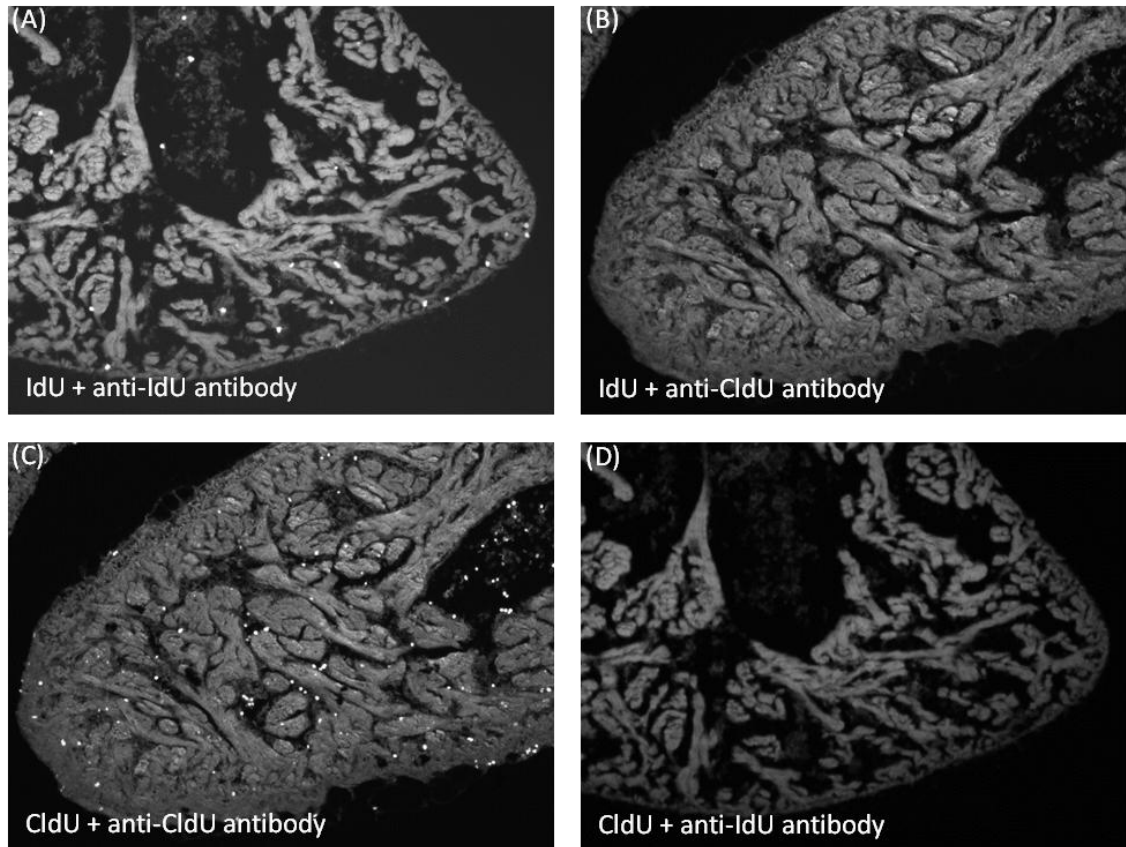
Figure 3.13: Testing the specificity of antibodies and spectral separation of fluorochromes
To test the specificity of the antibody used to detect IdU, immunofluorescence was performed on cardiac sections from a zebrafish treated with 0.5mg/ml IdU using the antibody for detecting IdU (A) or the antibody used to detect CldU (B). Fluorescence was observed in (A) but not (B) indicating that the antibody used for detecting IdU does bind to IdU and The CldU antibody does not bind to IdU. To test the specificity of the antibody used to detect CldU, immunofluorescence was performed on cardiac sections from a zebrafish treated with 0.5mg/ml CldU using the antibody for detecting CldU (C) or the antibody used to detect CIdU (D). Fluorescence was observed in (C) but not (D) indicating that the antibody used for detecting CldU does bind to CldU and the IdU antibody does not bind to CldU. Images (A) and (D) are the same section and (B) and (C) are the same section. However they were imaged with a different fluorescent filter. This demonstrates that spectral separation has occurred.

3.3.2.3.1. *Thresholding and counting*

In order to use FIJI to automatically count cells the image needed to be a binary image, in which only the cells to be counted were visible. In order to do this image thresholding needed to be carried out.

An image is essentially a series of pixels with varying numerical values arranged into a two dimensional grid. A pixel is the smallest addressable element in an image and has physical coordinates as well as intensity values. In a binary image each pixel will have a value (bit-depth) of '0' or '1'. In an 8-bit image the range of bit depth is 0-255 (256 different values). Thresholding an image is the process of partitioning a digital image into sets of pixels (segments) which usually makes the image easier to analyse (Figure 3.14). In order to find the appropriate level of thresholding I used a histogram (Figure 3.14B). In terms of imaging, a histogram represents graphically the tonal distribution (bit depth) of an image by plotting the number of pixels for each value. The horizontal axis represented tonal variations and the vertical axis the number of pixels. A surface plot, the three dimensional representation of the histogram, was used for visual confirmation (Figure 3.14C).

To threshold an image using FIJI I selected Image>Adjust>Threshold. The threshold was set so that only pixels with a relatively higher pixel value could be observed in order to distinguish real signal from background noise. Initially the range of pixel values was determined by manually counting cells by hand (Figure 3.14A) and then thresholding until only the counted cells were visible (Figure 3.14D). The image was then made into a mask (Process>Binary>Convert to Mask) and the number of particles (cell nuclei) were counted (Analyse>Analyze particles) (Figure 3.14E). The images were then merged to ensure that the cells FIJI counted matched the cells that were manually counted (Image>Colour>Merge Channels) (Figure 3.14F).

In images there were sometimes dead pixels or artefacts which were significantly smaller than the nuclei which gave false positive counts. In order to eliminate these, a minimum pixel size was determined for particles to be counted. In order to do this the minimum particle size was increased by 1 pixel until the number of cells counted by FIJI was equal to the number of cells counted by hand. In this instance the minimum pixel size used was 2. Once this was determined the level of thresholding and minimum pixel size for particles to be counted was used as a constant for subsequent images captured on the same microscope, using the same magnification and the same exposure but was still reviewed periodically with different batches of images as a form of quality control.
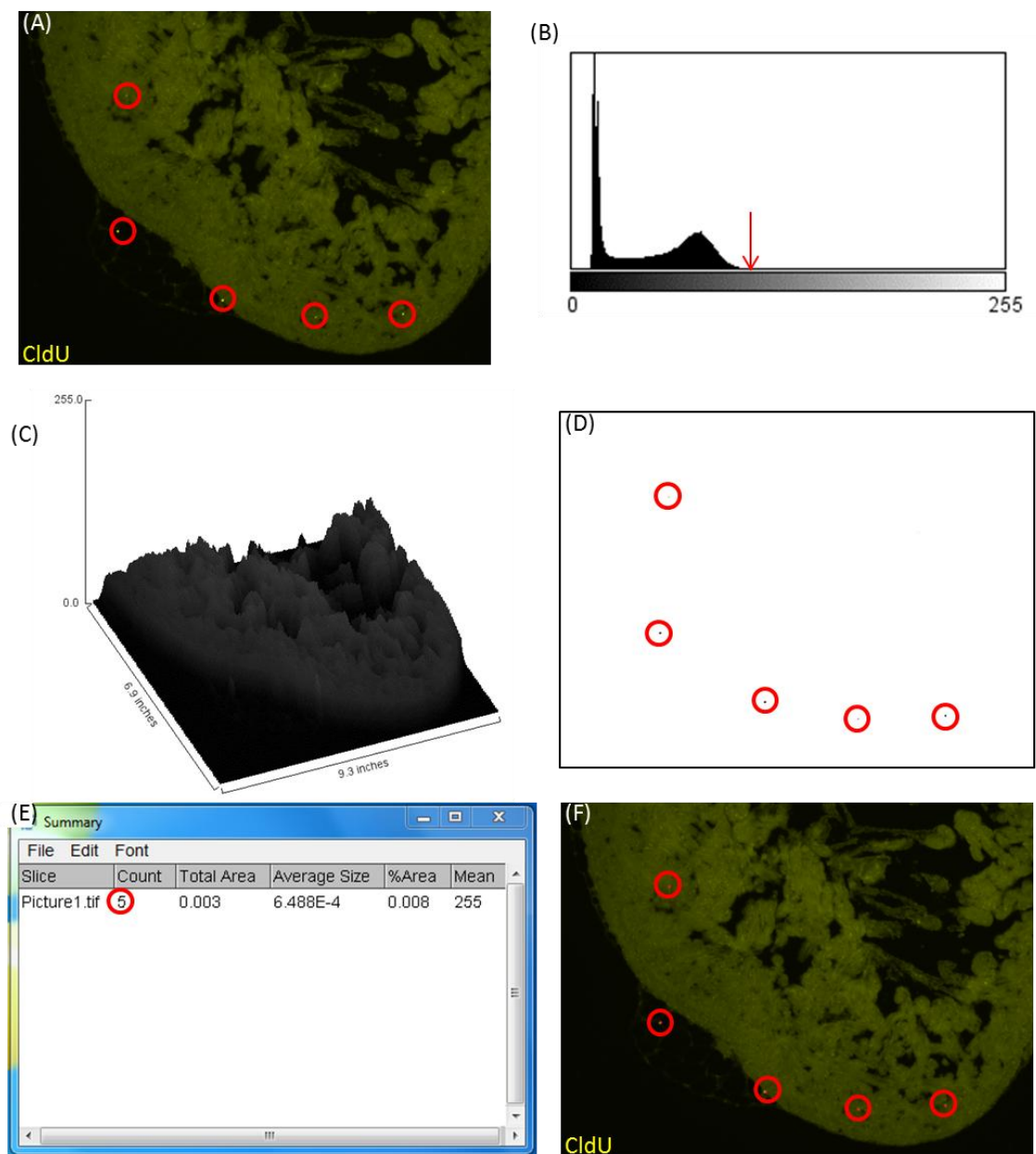
Figure 3.14:Thresholding and counting (image 1)
Immunofluorescence for the detection of CldU was carried out on cardiac sections (A). Positive cells are circled in red (5). A histogram was used to show the spread of pixel values in the image (B). The red arrow shows the pixel value where background fluorescence disappears and only the CldU positive cells remain visible. This information can also be depicted in a 3D histogram (C). The image is thresholded to the pixel value where only the CldU positive cells remain visible (D). Automatic counting can then be performed using FIJI (E), which also gives a count of 5 (circled in red). overlayed onto the original image showing that the CldU positive cells overlap in both images (red circles, overlay of thresholded image in red), confirming the robustness of this method of thresholding (F).

Figure 3.15Thresholding and counting (image 2)
Immunofluorescence for the detection of CldU was carried out on cardiac sections (A). Positive cells are circled in red (5). A histogram was used to show the spread of pixel values in the image (B). The red arrow shows the pixel value where background fluorescence disappears and only the CldU positive cells remain visible. This information can also be depicted in a 3D histogram (C). The image is thresholded to the pixel value where only the CldU positive cells remain visible (D). Automatic counting can then be performed using FIJI (E), which also gives a count of 5 (circled in red). overlaid onto the original image showing that the CldU positive cells overlap in both images (red circles, overlay of thresholded image in red), confirming the robustness of this method of thresholding (F).

123

In order to confirm the reproducibility of automatic counting by FIJI, 6 typical images of mef2 positive cells were counted by hand and then using the FIJI method I devised, described previously. There was a statistically significant positive correlation between manual counting and counting with FIJI (Figure 3.16), as determined by a Pearson's correlation coefficient and an R^2 value of 0.998. This confirms the reliability of using automated counting to increase the throughput, rather than counting manually.
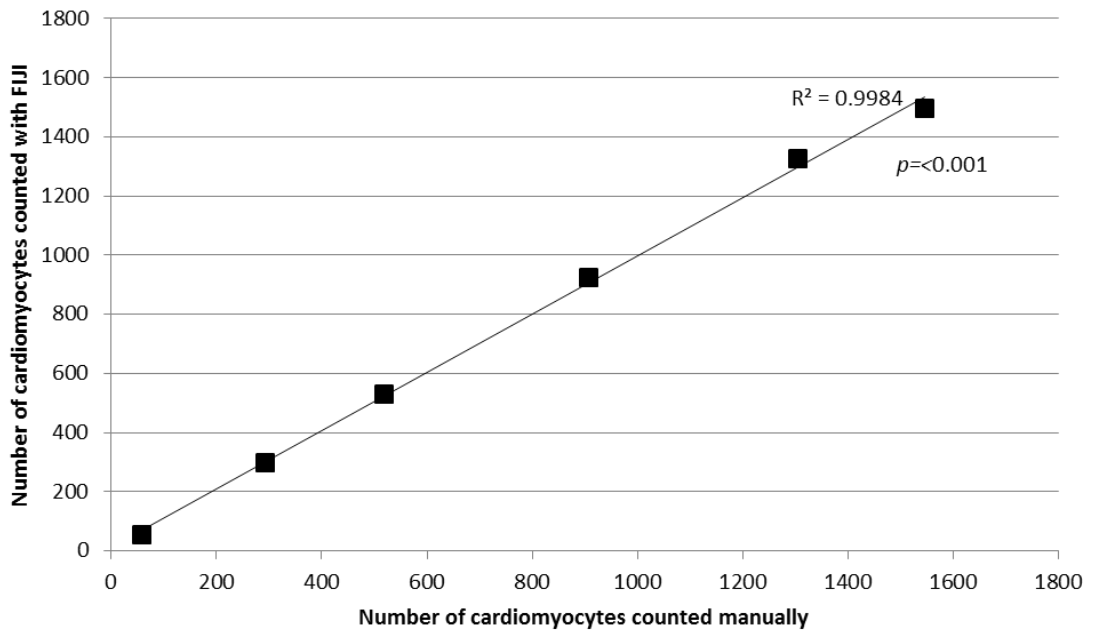
Figure 3.16: Confirming automatic counting with FIJI by counting manually
6 typical images were captured showing the immunofluorescent detection of mef2 positive cells (cardiomyocytes) in sagittal cardiac sections from zebrafish. Each image was counted manually and then counted using the method I have developed using FIJI. A Pearson's correlation coefficient showed a positive correlation and R2 values demonstrate the reproducibility between automatic counting using FIJI and counting manually.

3.3.2.2.1. *Image stitching*

In order to give a meaningful representation of cardiomyocyte proliferation throughout the entire zebrafish heart full mid sagittal sections of individual hearts were analysed. Zebrafish hearts were, however, too large to be able to be captured in one image at a magnification suitable to resolve individual cardiomyocyte nuclei on most compound microscopes. In order to overcome this, separate images of the same section at different locations were captured and stitched together *post hoc* to give a 'patchwork' image of the entire heart section.

In FIJI the stitching plugin uses the Fourier shift theorem to compute all possible translations (x, y[, z]) in different images giving the optimum overlap in relation to the cross correlation measure (Preibisch *et al.*, 2009). When more than two images are stitched together a global optimization is used to determine the correct placement. At the borders where there are overlapping areas a linear intensity blending can be applied to remove any differences in brightness, however this was not used as I decided keeping images as raw as possible would give the most accurate results. This meant that any changes in thresholding were manual and the extent of which were known.

In order to demonstrate image stitching an image adjacent to that in Figure 14 was thresholded in exactly the same way in order to count the number of positive cells and verify automatic counting in the resultant stitched patchwork (Figure 3.15). To stitch images using FIJI the images to be stitched were placed in the same folder. In FIJI I selected Plugins>Stitching>Deprecated>Stitch Directory with Images. FIJI then used the Fourier shift theorem to find the translation which gives the optimum overlap and produce a patchwork consisting of the stitched images (Figure 3.17).

In order to verify that the stitching had worked appropriately and not affected the number of cells counted using FIJI the number of cells in the overlapping area of the patchwork and the non-overlapping areas was ensured to be equal to the number of cells in the patchwork (Figure 3.17). Automatic counting was then verified in conjunction with image stitching by using thresholded images (Figure 3.18), which was the same as counting by hand.
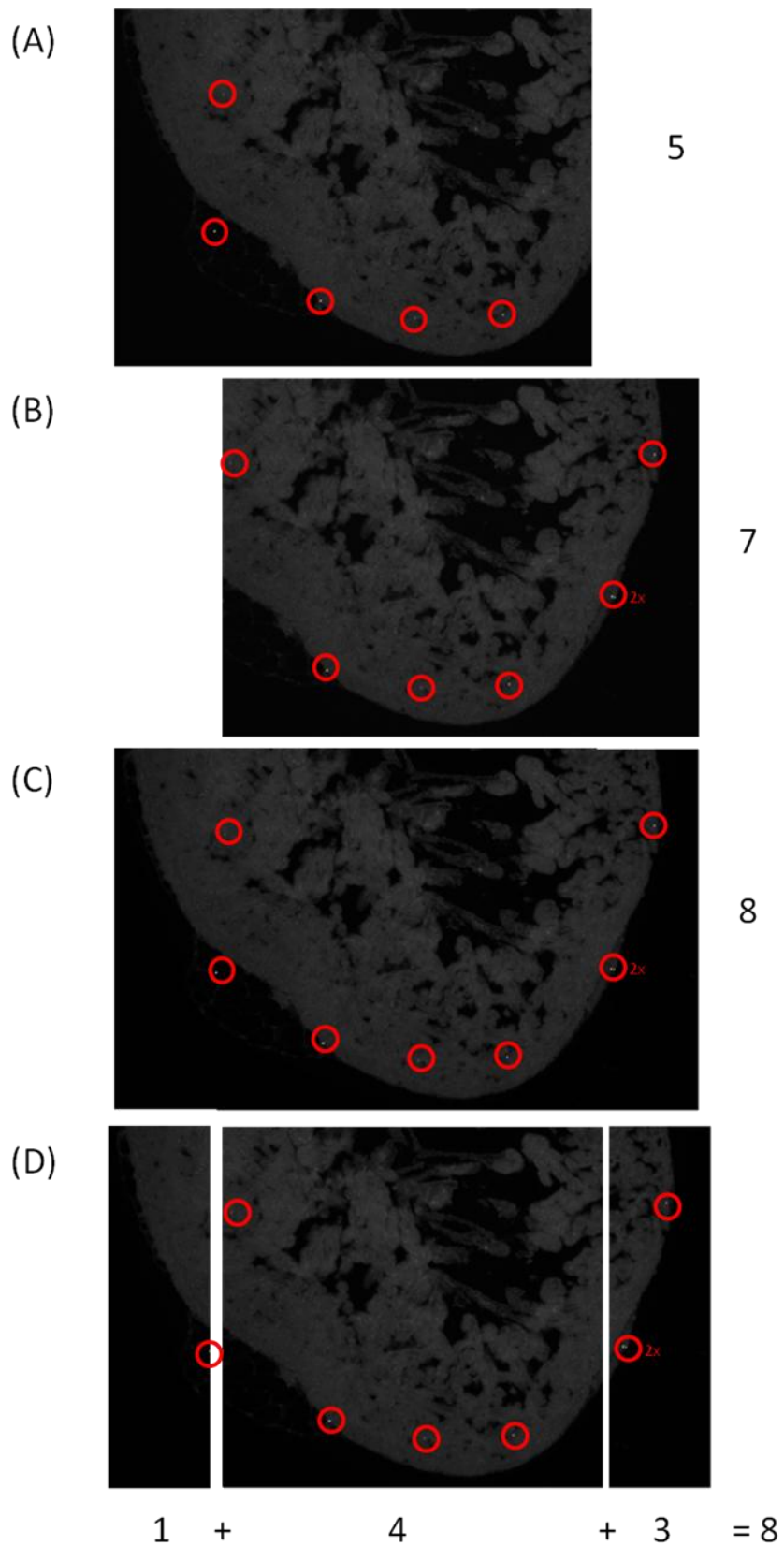
Figure 3.17:Image stitching
Image 1 (A) and image 2 (B) are stitched together using FIJI (C). The stitched image can then be split into the non-overlapping regions, left and right side, and the overlapping region, centre, showing that no cells are lost during the image stitching process (D). CldU positive cells are circled in red.
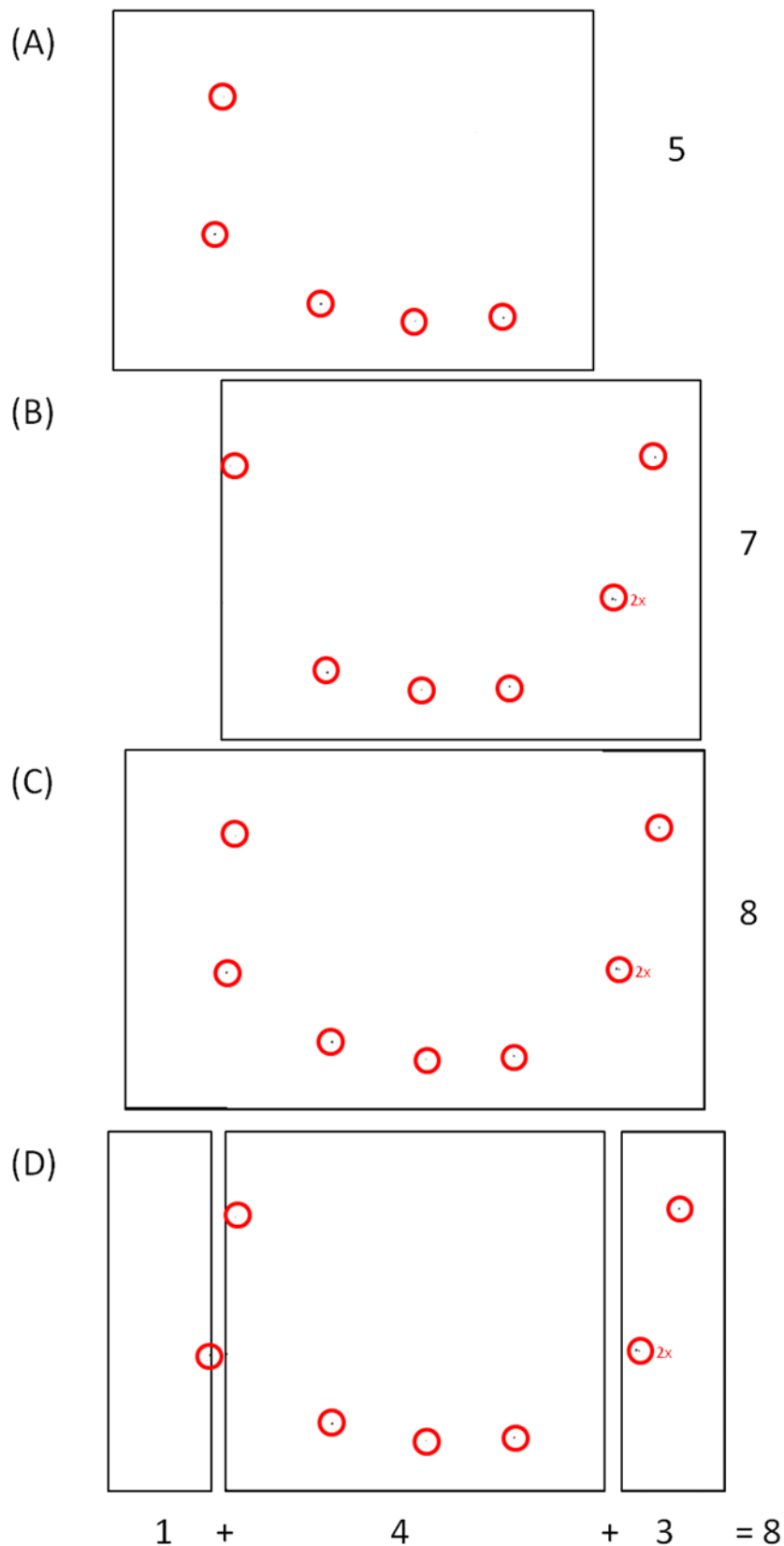
Figure 3.18:Image stitching with thresholding and automatic counting
Thresholded image 1 (A) and image 2 (B) are stitched together using FIJI (C). The stitched image can then be split into the non-overlapping regions, left and right side, and the overlapping region, centre, showing that no cells are lost during the image stitching process (D). FIJI was also used to count the number of cells, showing that stitching and automatic counting work together. CldU positive cells are circled in red.

3.3.2.2.2. *Counting double positive cells*

When counting cells it is important to know which cells are cardiomyocytes (Mef2+ve), as there are many other cell types in the heart, and which cells have proliferated (IdU or CldU+ve). It is also necessary to count cardiomyocytes which have proliferated more than once (Mef2+ve and IdU and CldU+ve). In order to do this the masks which were used to count cells were multiplied (Process>Image calculator). A model of this process is shown in Figure 3.19.

As masks are binary, if there was a nuclei in one image the value of the pixels for that area of the image was '1' and if there was not, the pixel value was '0'. Therefore '1 X 0 = 0' and thus the pixel value in the resultant image was '0'. If there was a double positive cell the pixel values on both images were '1'. As '1 X 1 = 1' the pixel value was '1' in the resultant image. The cells were then counted in the resultant image as previously described.

Patchworks were made for the mef2, IdU and CldU channels and thresholding and automatic counting conducted using the methods previously described (Figure 3.20). The channels were then multiplied together in a worked example of counting double positive cells to give the various permutations occurring in the multiplexed thymidine analogue zebrafish heart (Figure 3.21). By multiplying the IdU (Figure 3.21A) and CldU (Figure 3.21B) channels this resulted in the cells which have proliferated in IdU and CldU (Figure 3.21C). When the IdU (Figure 3.21D) channel was multiplied by the Mef2 (Figure 3.21E) channel this resulted in the cardiomyocytes which proliferated in IdU (Figure 3.21F). When the CldU (Figure 3.21G) channel was multiplied by the Mef2 channel (Figure 3.21H) this gives the cardiomyocytes which proliferated in CldU (Figure 3.21I). When the results of the IdU X CldU channels were multiplied by the Mef2 channel this gave the cardiomyocytes which proliferated in both IdU and CldU. A model of this process is show in (Figure 3.19) showing how this process works in relation to other, non-cardiomyocytes within the heart.

To ensure false positive counts did not occur, in every batch of immunofluorescence a negative control was performed in which sections of IdU and CldU positive zebrafish heart were treated in exactly the same way, except primary antibodies were omitted (

Figure **3.22**). The images were processed in exactly the same way; image stitching was possible due to the low amount of autofluorescence in zebrafish cardiac tissue (Figure 21A,C and E). However when thresholding was conducted at the same level as other

hearts in the batch there were never any cells to count (Figure 21B,D,F and E). This shows that the secondary antibodies are specific to the primary antibodies used and there is no autofluorescence at the same level of fluorescence as the secondary antibodies. This process acted as an added step of quality control for each batch of immunofluorescence, imaging and image processing which reduces any chance of false positive counts.
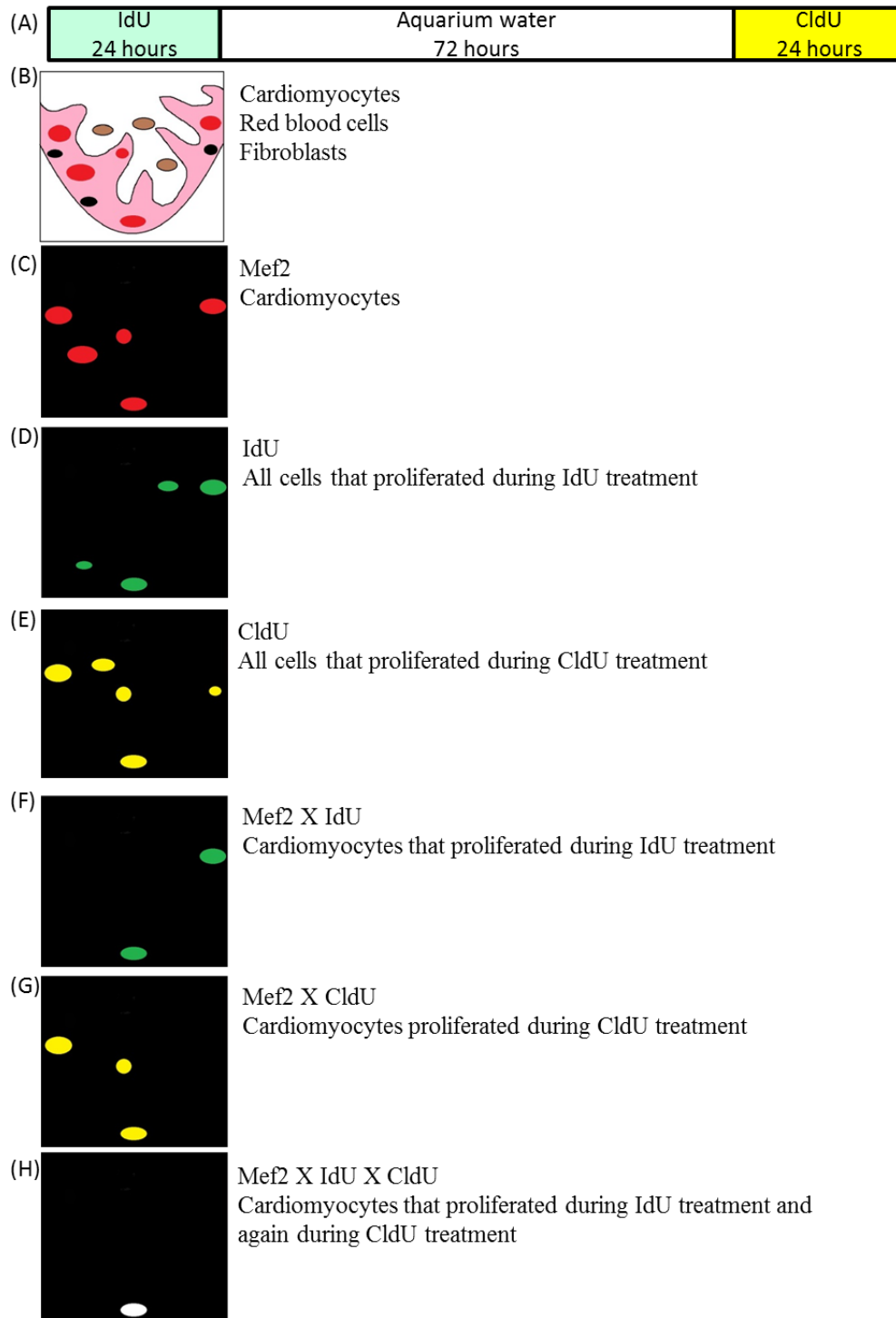
Figure 3.19:A model for multiplexing thymidine analogues and cardiomyocyte counting

If zebrafish were immersed in an IdU solution for 24 hours, had a 'washout' period for 72 hours and then immersed in a CldU solution for 24 hours (A) allowed me to identify proliferative cells. The zebrafish heart contains nucleated red blood cells (black) within the lumen and a layer of cardiac fibroblasts (brown) amongst cardiomyocytes (red) (B). Using immunofluorescence cardiomyocytes (C), IdU positive cells (green, D) and CldU positive cells (yellow, E) can be detected. Using FIJI these images were then multiplied to give IdU positive cardiomyocytes (F), CldU positive cardiomyocytes (G) and IdU and CldU double positive cardiomyocytes (H).

130
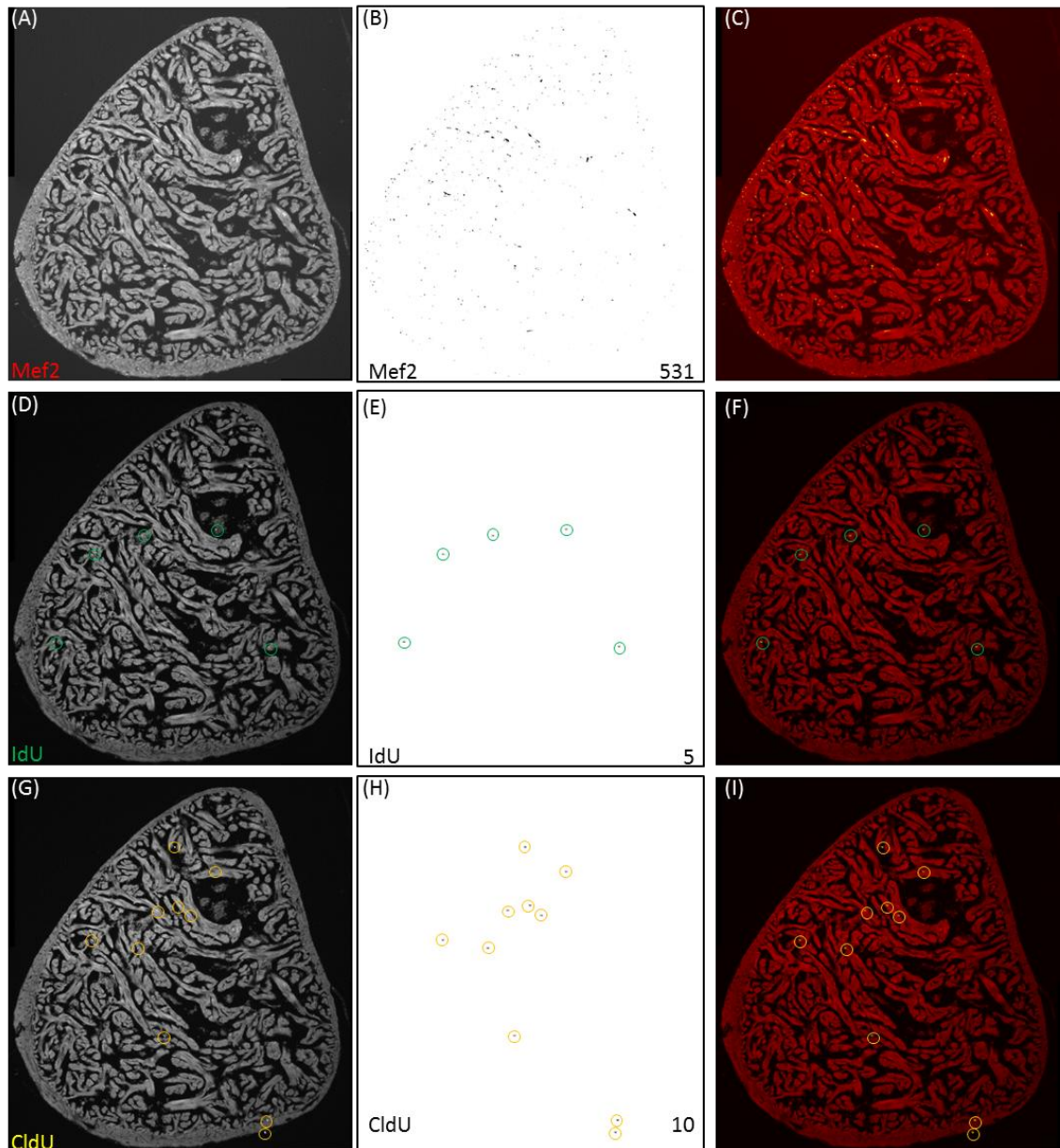
Figure 3.20: Counting multiple channels in patchwork images

Imunofluorescent detection of mef2 positive cells (cardiomyocytes) was carried out on sections of zebrafish hearts (A). Images were thresholded and made binary as previously described (B). Automatic counting was then performed on this image (531). Overlaying (A) and (B) gives (C) which shows that the thresholded image overlaps the raw image. The same process was carried out to detect IdU positive cells using the IdU specific antibody. (D) is the raw image (positive cells circled green, 5), (E) is the binary counted image which was counted and then overlayed onto the raw image (F). This was also carried out to detect CldU positive cells. (G) is the raw image (positive cells circled yellow, 10), (H) is the binary counted image which was counted and then overlayed onto the raw image (I). This demonstrates that the protocol I developed can accurately count cells in patchwork images using multiple fluorescent channels. Numbers in the bottom right corner of binary images were the number of cells counted using FIJI.
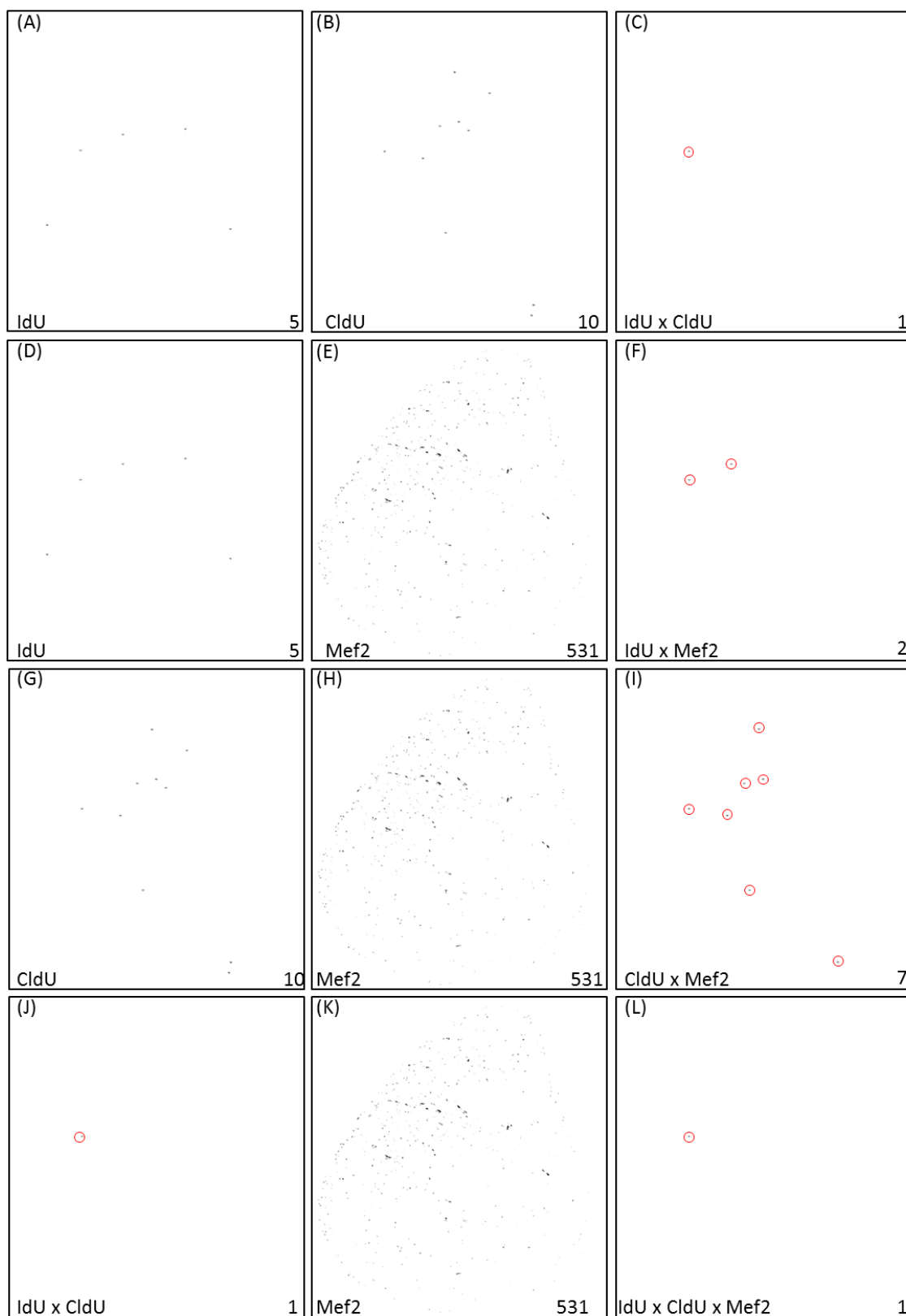
Figure 3.21:Counting double positive cells in patchwork images
This is a working example of the model outlined in Figure 3.19. FIJI was used to make binary images from patchworks of each channel. Multiplying the binary image of IdU positive cells (A) with the binary image of CldU positive cells (B) gives an image of cells which are both IdU and CldU double positive (C), circled in red. Multiplying the binary image of IdU positive cells (D) with the binary image of mef2 positive cells (E) gives an image of cells that are both IdU and mef2 positive (F), circled in red. Multiplying the binary image of CldU positive cells (F) with the binary image of mef2 positive cells (G) gives an image of cells that are both CldU and Mef2 positive (H), circled in red. Multiplying the binary image of IdU and CldU double positive cells (I) with the binary image of mef2 positive cells (K) gives an image of cells that are only positive for IdU, CldU and Mef2 (L), circled in red.

Figure 3.22:Specificity of primary antibodies and image processing
To ensure no background fluorescence was giving false positive results, immunofluorescence was carried out as described in the methods section, except primary antibodies were omitted. Images were captured using the channels usually used to detect the secondary fluorescent antibodies used to normally detect their respective primary antibodies for mef2 (A), IdU (C) and CldU (E). These images were processed as previously described into binary images (B,D,F) which were counted using FIJI (G). FIJI did not count anything on these images showing that the primary antibodies are indeed needed to detect their respective antigen and that there is no non-specific binding from the fluorescent secondary antibodies or faults causing false positive in the image processing methodology.

3.3.1.2.IdU and CldU optimisation

Although optimisation of the dose of BrdU was carried out and the literature suggests that the labelling efficacy of halogenated thymidine analogues is the same (Tuttle *et al.*, 2010) it was decided that it would be prudent to ensure this to be true in zebrafish hearts as this is the main tool that is to be used to measure cardiomyocyte proliferation robustness and accuracy is fundamental.

Five male 6 months old zebrafish were immersed in either 0.5mg/ml or 0.25mg/ml IdU for 24 hours followed by the same concentration CldU for 24 hours and then immediately sacrificed (Figure 3.23A). Doses of 0.5mg/ml and 0.25mg/ml were used as BrdU pulsing showed 0.5mg/ml BrdU to be more than sufficient. To reduce costs and potential side effects, a lower dosage of 0.25mg/ml was also tested. Hearts were processed for immunofluorescence and the images were processed and counted using FIJI. There was no significant difference in the amount of IdU positive cardiomyocytes or the amount of CldU positive cardiomyocytes (Figure 3.23B and C) with either dose of IdU and CldU. This suggests that there is no difference in the efficacy of either 0.5mg/ml or 0.25mg/ml of thymidine analogue at labelling DNA in proliferating. 0.25mg/ml would therefore be the preferable dose due to cost and reducing the chance of any potential side effects.

To ensure that IdU and CldU incorporation is correlated in the same zebrafish the percentage of IdU positive cardiomyocytes was plotted against CldU positive cardiomyocytes (Figure 3.24). A Pearson's correlation coefficient shows that there is indeed a positive correlation and that although IdU incorporation appears lower than CldU incorporation it is still positively correlated. The reason why IdU incorporation was lower was due to the cumulative stress of successive treatments. Stressing zebrafish by placing them in tanks shaped differently to what they are accustomed to appears to increase cardiomyocyte proliferation, this will be discussed in more detail in the next chapter. In hindsight it may have been prudent to reverse the order of thymidine analogue treatment, treating zebrafish with CldU followed by IdU. This would determine if the efficacies of IdU and CldU are the same. If the IdU incorporation was higher in this reversed scenario it would almost certainly rule out any doubt that one analogue is more likely to be incorporated into proliferating cells than another. Differences in efficacies of different thymidine analogues have not been reported in the literature (Tuttle *et al.*, 2010). My laboratory plan on performing this experiment prior to publishing some of this data.

(A) IdU 24 hours | CldU 24 hours

(B) IdU positive cardiomyocytes (%)
NS
p=0.38

(C) CldU positive cardiomyocytes (%)
NS
p=0.74

☐ 0.25mg/ml (n=5)
■ 0.5mg/ml (n=5)

(D)

Thymidine analogue	Concentration	Number of zebrafish	Total positive cardiomyocytes counted	Total cardiomyocytes counted	Thymidine analogue positive cardiomyocytes (%)	SEM
IdU	0.25mg/ml	5	48.2	8280.8	1.05	0.12
IdU	0.5mg/ml	5	70.2	8878.8	1.02	0.21
CldU	0.25mg/ml	5	132.6	8280.8	1.83	0.50
CldU	0.5mg/ml	5	175.2	8878.8	2.04	0.37

Figure 3.23: Thymidine analogue dosing
Zebrafish were immersed in either 0.25mg/ml IdU for 24 hours followed by 24 hours in 0.25mg/ml CldU dissolved in aquarium water or 0.5mg/ml IdU for 24 hours followed by 24 hours in 0.5mg/ml CldU (A). Immunofluorescence was performed on sagittal cardiac sections and the percentage of IdU positive cardiomyocytes (B) and CldU positive cardiomyocytes (C) was compared between zebrafish receiving either 0.25mg/ml thymidine analogues or 0.5mg/ml thymidine analogues. No significant differences were observed between the different concentrations when compared using a student's t-test. Error bars are SEM. The number of cells counted and percentage of thymidine positive cardiomyocytes are found in table (D).
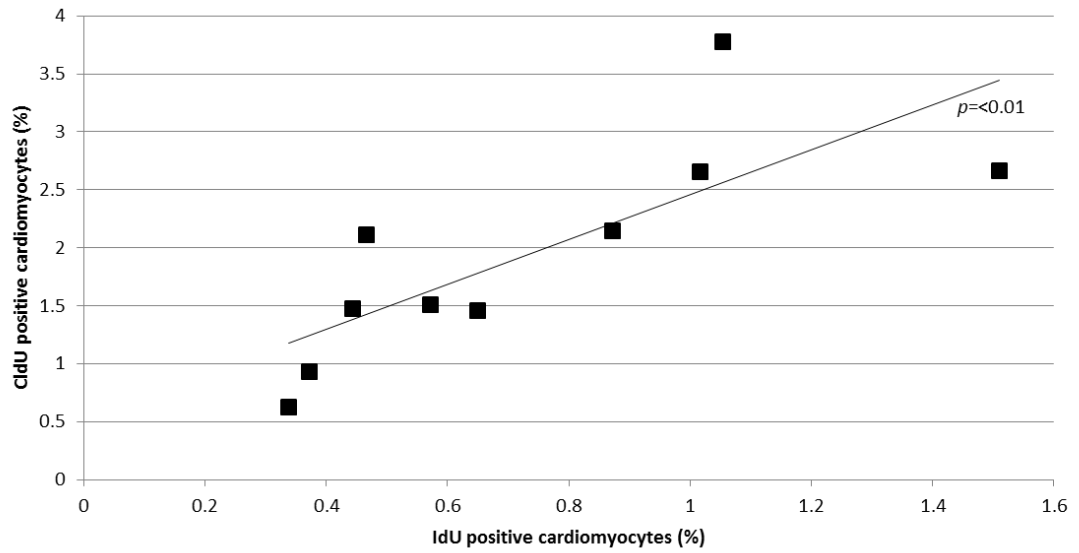
Figure 3.24: Correlations between IdU and CldU incorporation
The percentage of IdU positive cardiomyocytes plotted against the percentage of CldU positive cardiomyocytes shows a positive gradient that is significant when analysed using a Pearson's correlation coefficient illustrating the percentage of IdU and CldU positive cardiomyocytes are positively correlated.

3.4.   Changes in cardiomyocyte proliferation due to ageing

3.4.1.   Aims

As I developed a novel assay to measure cardiomyocyte proliferation in zebrafish the aim of this next section was to put that assay to the test. I aimed to discover if there is a change in the rate of cardiomyocyte proliferation in zebrafish due to ageing. Changes in cardiomyocyte turnover may be the underlying cause for the pathological changes I have previously observed. These changes include an increased frequency of fibrosis, increases in ventricular lumen area and increasing thickness of the ventricular wall.

3.4.2.   Results

3.4.1.1.Cardiomyocyte proliferation remains constant at different ages

Zebrafish were immersed in IdU for 24 hours to label proliferating cells, allowed a period of 72 hours in aquarium water and then immersed in CldU for 24 hours and euthanized immediately (Figure 3.25A).

The percentage of cardiomyocytes that were IdU positive was not significantly different in the different aged zebrafish, ranging from 0.58% to 1.01% (Figure 3.25B). CldU positive cardiomyocytes were not significantly different either, ranging from 0.73% to 1.33% of cardiomyocytes (Figure 3.25C). Double thymidine analogue positive did not significantly change throughout the lifespan of a zebrafish, ranging from 0.15% to 0.39% (Figure 3.25D). One benefit of using two thymidine analogues is that they can label rapidly cycling cells which would present as being IdU and CldU double positive. This would demonstrate whether newly formed cardiomyocytes originate from a subset of cardiomyocytes which rapidly proliferate and would therefore be double thymidine analogue positive, or if cardiomyocytes all have a similar proliferative ability. When the number total number of cardiomyocytes that are double thymidine analogue positive are compared as a percentage of cardiomyocytes that have proliferated (either IdU or CldU positive) there is no significant difference in different aged zebrafish. This implies there is no difference in the proportion of recently proliferated cardiomyocytes that re-enter the cell cycle in different aged zebrafish (Figure 3.25E). Throughout the ages approximately 15% of cardiomyocytes re-enter the cell cycle within the duration of the immersing schedule (5days).
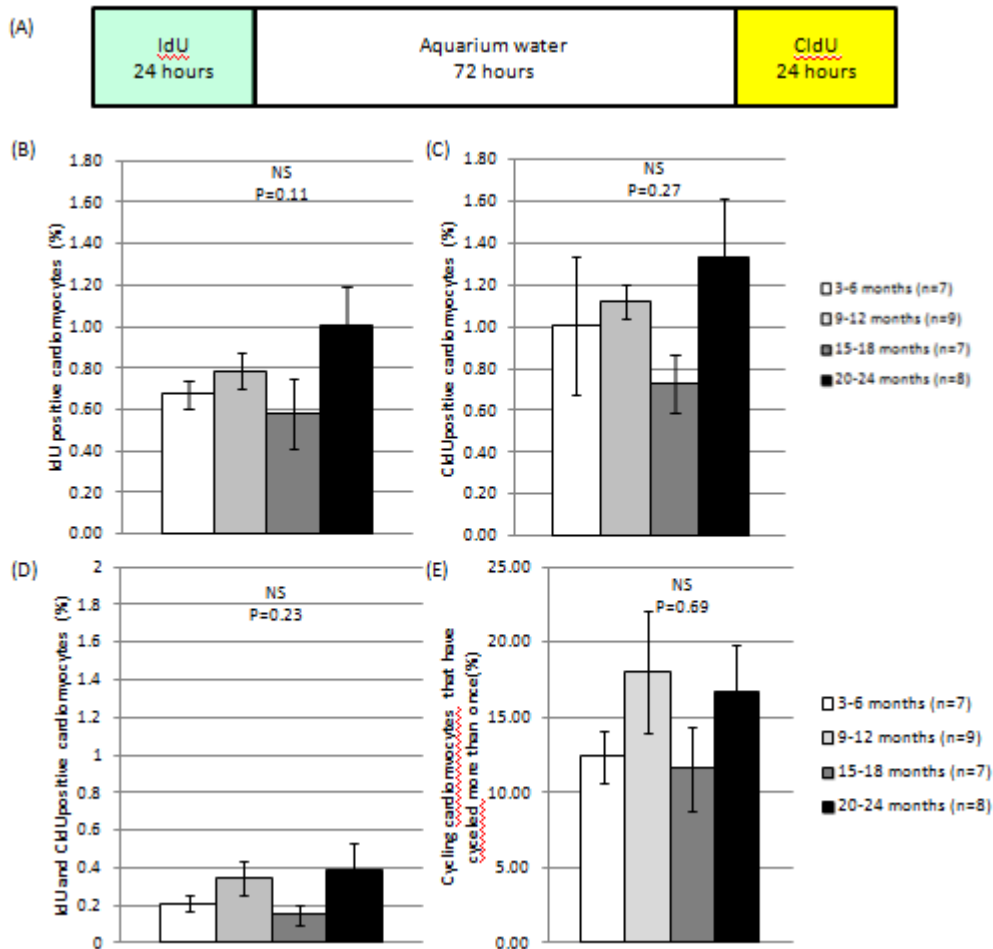
Figure 3.25: Cardiomyocyte proliferation does not change due to ageing
Zebrafish were immersed in IdU for 24 hours, aquarium water for 72 hours and then CldU for 24 hours (A). Immediately after the CldU dip hearts were dissected and processed for immunofluorescence. The number of IdU positive cardiomyocytes (B), CldU positive cardiomyocytes (C) and IdU and CldU double positive cardiomyocytes (D) were counted for each age group. A one-way ANOVA determined that there was no significant difference in cardiomyocyte proliferation between the age different age groups(B and C), or the number of cardiomyocytes re-entering the cell cycle after initial proliferation (D). The percentage of cardiomyocytes that were IdU and CldU positive and had therefore cycle more than once, was calculated as a percentage of cardiomyocytes that were either IdU or CldU positive and had therefore cycled only once whilst in a thymidine analogue (E). This shows how likely a cardiomyocyte is to re-enter the cell cycle in the 72-120 period after initially dividing, of which there was no significant difference between age groups when analysed using a one-way ANOVA. Error bars are SEM. Figure 3.26 shows the mean counts for zebrafish of each age group.

Age (months)	7	12	15	23
Number of zebrafish	8	10	9	8
Mef2 +ve	3430.57	4003.44	3867.00	3754.63
Mef2 and IdU+ve	23.14	31.44	22.43	37.75
Mef2 and CldU+ve	34.57	44.89	28.14	49.88
Mef2, CldU and IdU+ve	7.14	13.78	5.86	14.63
IdU+ve cardiomyocytes (%)	0.67	0.79	0.58	1.01
SEM	0.07	0.09	0.17	0.18
CldU+ve cardiomyocytes (%)	1.01	1.12	0.73	1.33
SEM	0.33	0.08	0.14	0.28
CldU and IdU+ve cardiomyocytes (%)	0.21	0.34	0.15	0.39
SEM	0.04	0.09	0.05	0.14

Figure 3.26:Cardiomyocyte proliferation does not change at different ages
The mean counts for zebrafish of each age group used for Figure 3.25. Number of zebrafish used, number of cardiomyocytes (Mef2+ve), number of IdU positive cardiomyocytes (Mef2 and IdU+ve), number of CldU positive cardiomyocytes (Mef2 and CldU+ve) and double thymidine analogue positive cardiomyocytes (Mef2, CldU and IdU+ve) are shown with corresponding percentages and standard error of mean (SEM).

3.4.1.2. Mitotic events present as single cells or doublets

Next, I aimed to examine how the IdU positive cardiomyocytes appear on the sections. If cardiomyocytes had undergone s-phase whilst IdU was present but not gone through cytokinesis, the cardiomyocyte would appear as a single cell. If a cardiomyocyte had gone through s-phase and cytokinesis whilst IdU was present then there would be two daughter cardiomyocytes next to each other. If cardiomyocytes had gone through another round of proliferation then there would be a cluster of more than two IdU positive cardiomyocytes. This would give a further indication of whether certain cardiomyocytes are more 'stem like' than others and have an increased likelihood of proliferating multiple times.

Sections from seven zebrafish hearts were analysed to see if IdU positive cardiomyocytes were single, doublets or formed clusters (Figure 3.27). Single positive IdU cardiomyocytes were observed in 51.69% of mitotic events. Doublets were observed in 46.02% of mitotic events. Clusters of IdU positive occurred in 2.29% of mitotic events.
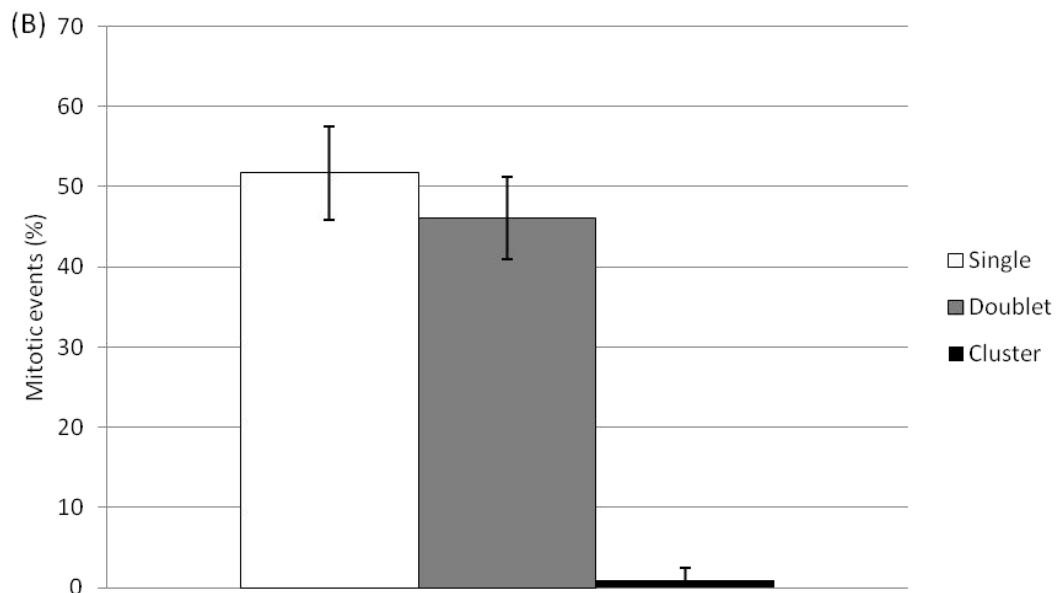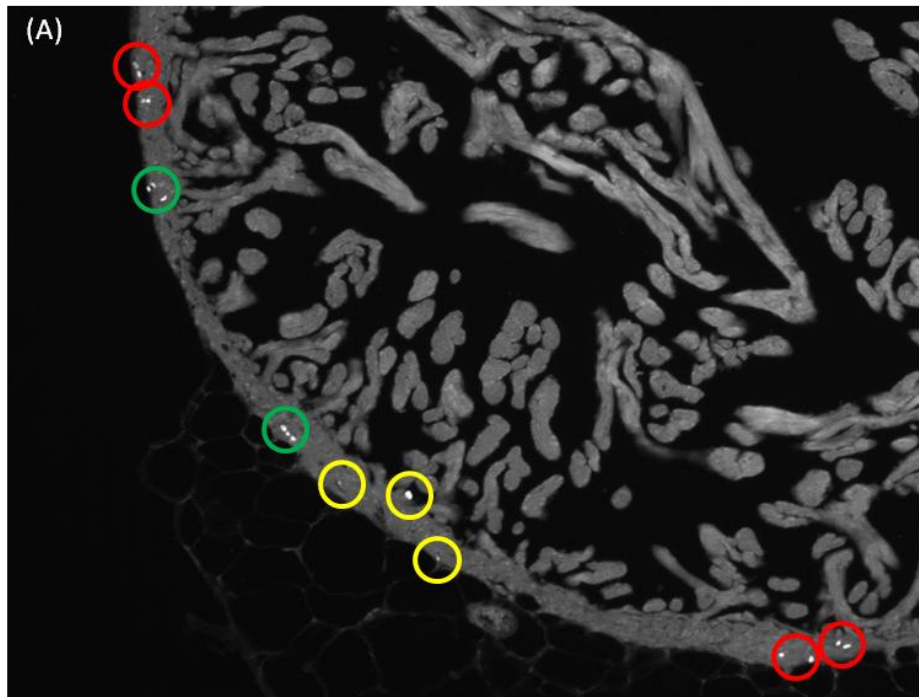
Figure 3.27: IdU positive cells are the result of one mitotic event
Sections from zebrafish hearts were analysed (n=7) to see if IdU positive cardiomyocytes were single (yellow circles), doublets (red circles) or formed clusters (green circles, A). If they were singular it would suggest that either the cardiomyocyte was in the process of mitosis, or that the other daughter cell was on an adjacent section. This was observed in 51.69% of mitotic events (B). If there were two IdU positive cardiomyocytes next to each other this would suggest that mitosis had been completed. This was observed in 46.02% of mitotic events. If there was a cluster of cardiomyocytes together, this would suggest that cardiomyocytes had undergone more than one mitotic event. This was a very rare event. Most proliferating cardiomyocytes were observed appeared within the compact myocardium. The compact myocardium has previously been proposed to be an area of higher cardiomyocyte proliferation in zebrafish (Gupta and Poss 2012).

3.5.   Establishing an assay to measure cardiomyocyte apoptosis in zebrafish

3.5.1.   Aims

As there was no difference in cardiomyocyte proliferation I wanted to see if there was a change in the frequency of apoptosis due to ageing. In order to do this I aimed to establish and verify a suitably robust assay in a similar way to that of the proliferation assay I previously developed.

3.5.2.   Results

3.5.2.1. Image processing cleaved caspase 3 images

Images were captured, as previously described, using anti-MF20 and anti-cleaved caspase 3 antibodies, whilst counter staining DAPI (Figure 3.28). MF20 was used instead of Mef2 to mark cardiac tissue as the anti-cleaved caspase 3 antibody and anti-Mef2 antibody were both raised in rabbits. This meant that the anti-cleaved caspase 3 antibody and anti-Mef2 antibody could not be used together. As MF20 is not nuclei specific, unlike Mef2, a different method needed to be developed in order to count cardiomyocytes.

Patchwork hearts were made for each fluorescent channel and thresholded accordingly, making the images binary, as described previously (Figure 3.29). The MF20 channel was multiplied with the DAPI channel, meaning that only cells within the MF20 positive (cardiac) tissue remained and was counted (Figure 3.30A). The cells that were MF20 positive were cardiomyocytes. In order to determine of any cardiomyocytes were undergoing apoptosis at the time the zebrafish was euthanized, the binary cleaved caspase 3 image was multiplied with the binary MF20 image, thus giving cells that were cleaved caspase 3 and MF20 positive which were therefore apoptotic cardiomyocytes (Figure 3.30B).

In order to test the specificity of the antibodies a negative control was performed, in which a slide was treated in exactly the same way, except no primary antibodies were added. The images from the slides were processed in the same way with the same level of thresholding showing no signal was present (Figure 3.31) ensuring the binding of antibodies was specific and not background fluorescence or non-specific binding of secondary antibodies.
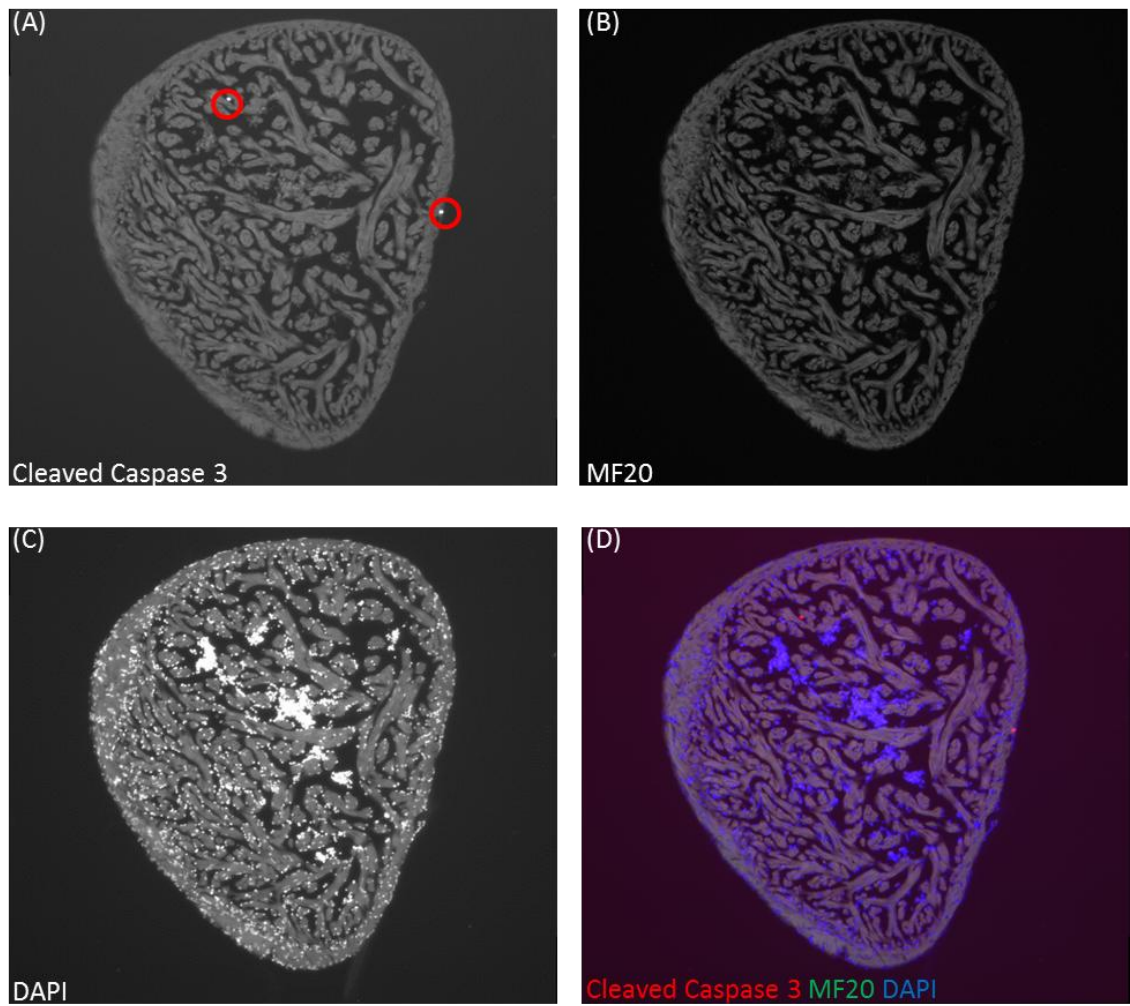
Figure 3.28: Cleaved caspase 3 immunofluorescence in zebrafish hearts
Immunofluorescence was used on cardiac sections to detect cleaved caspase 3 (A), red circles, MF20 (B), and DAPI (C) in the same section (D).
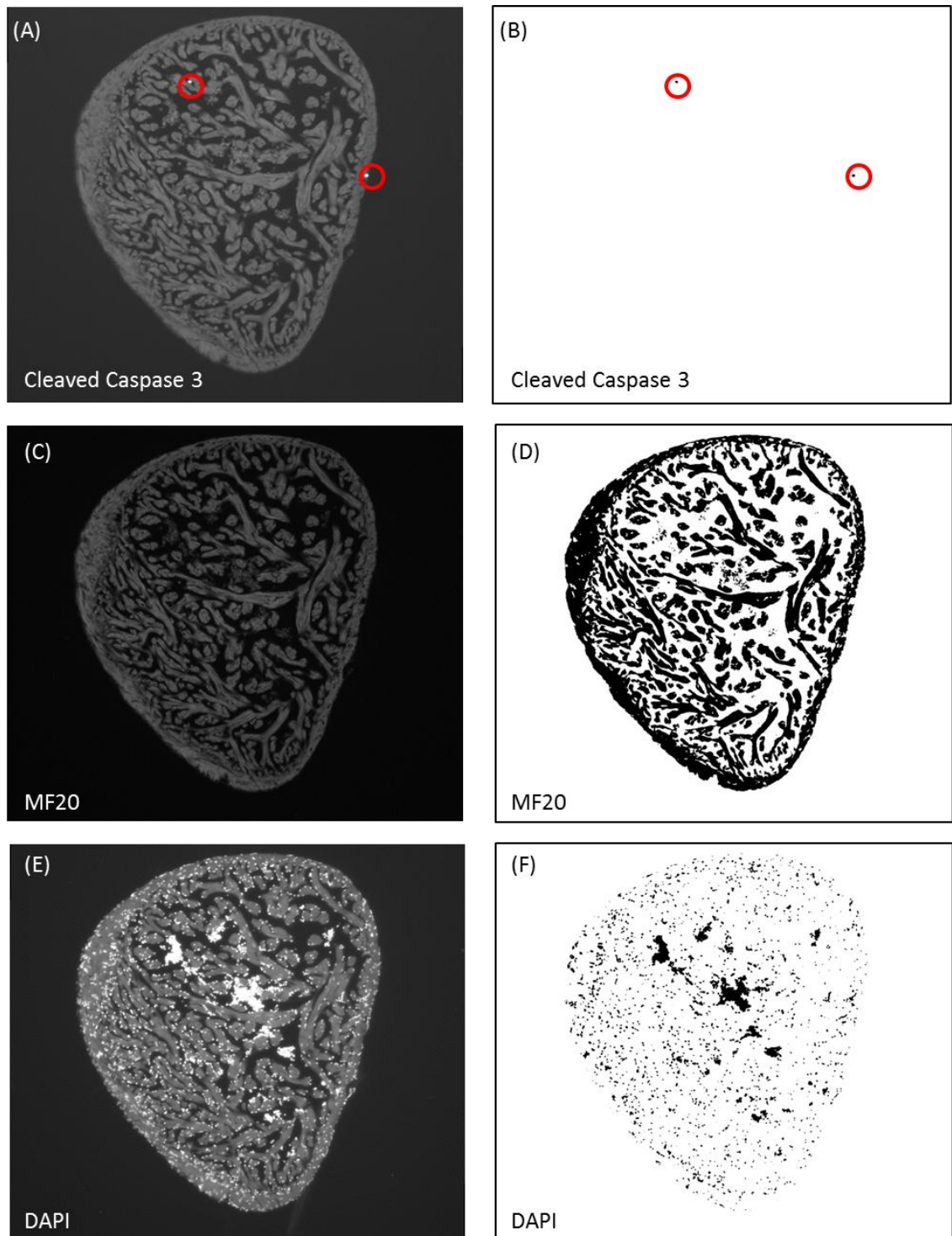
Figure 3.29:Thresholding cleaved caspase 3 images
Cleaved caspase 3 (A), MF20 (C) and DAPI images were thresholded and converted to binary images (B,D and E) as previously described, in order to count cells using FIJI.
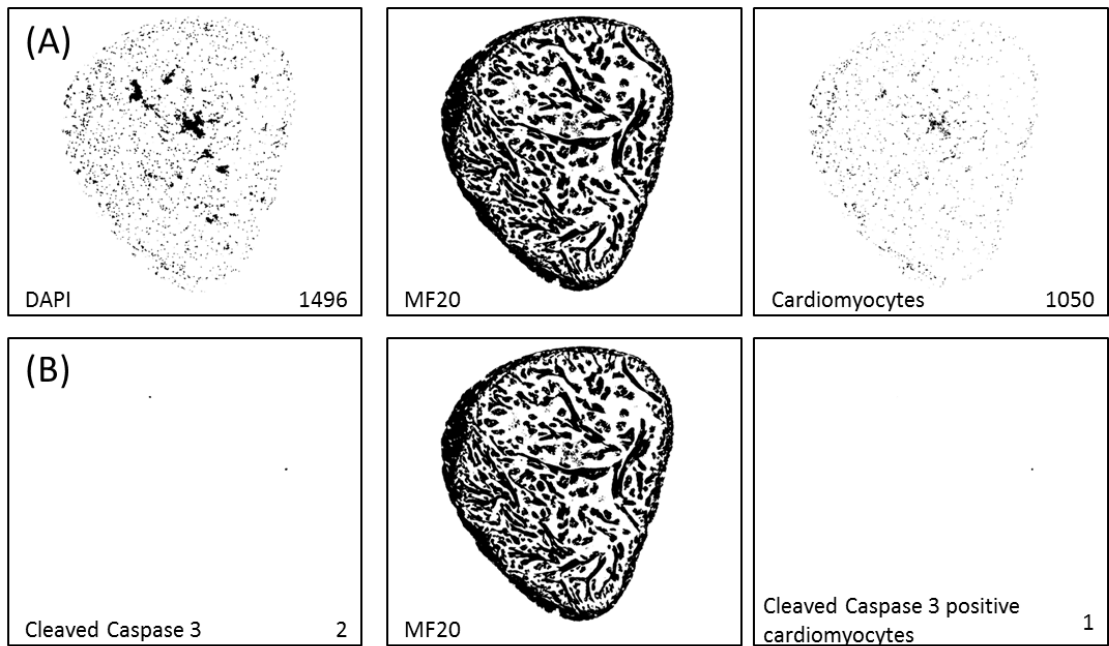
Figure 3.30:Multiplying cleaved caspase 3 images
DAPI multiplied by MF20 identifies the MF20 positive cells in the section (1050 cardiomyocytes) (A). Cleaved caspase 3 multiplied by MF20 identifies the caspase 3 and MF20 positive cells (1) (B).
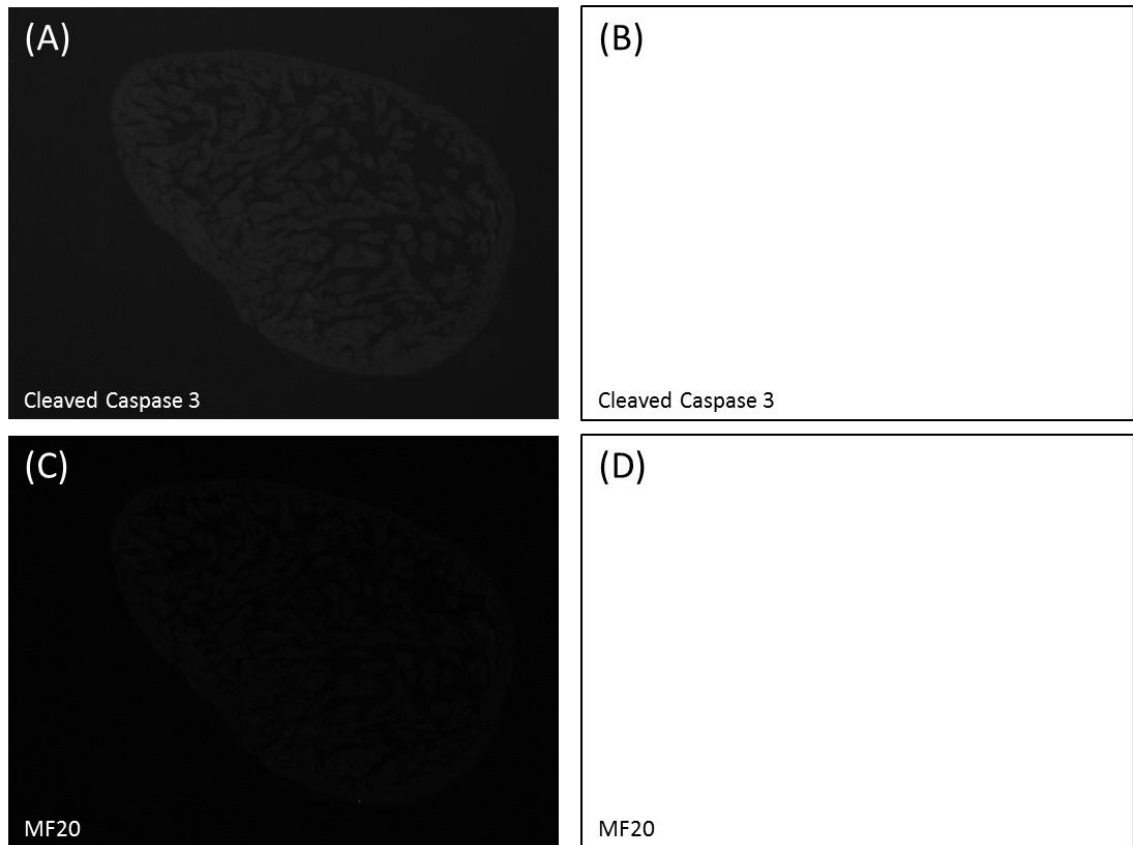
Figure 3.31:Specificity of primary antibodies and image processing
To ensure no background fluorescence was giving false positive results, immunofluorescence was carried out as described in the methods section, except primary antibodies were omitted. Images were captured using the channels usually used to detect the secondary fluorescent antibodies used to normally detect their respective primary antibodies for cleaved caspase 3 (A) and MF20. These images were processed as previously described into binary images (B and D) which were counted using FIJI. FIJI did not count anything on these images showing that the primary antibodies are indeed needed to detect their respective antigen and that there is no non-specific binding from the fluorescent secondary antibodies or faults causing false positive in the image processing methodology.

3.6. Changes in cardiomyocyte apoptosis due to ageing in zebrafish

3.6.1. *Aims*

As I have previously developed an assay for measuring apoptosis of zebrafish cardiomyocytes I aimed to use this assay to see if apoptosis changes as zebrafish age. There was no difference in cardiomyocyte proliferation in different aged zebrafish so it may have been that apoptosis caused a net reduction in the number of cardiomyocytes which resulted in the observed age related pathological changes in the zebrafish heart.

3.6.2. *Results*

3.6.2.1. *Cardiomyocyte apoptosis increases in zebrafish hearts due to ageing*

Cardiomyocyte apoptosis has been associated with ageing in humans (Kajstura *et al.*, 2010a). Decreases in cardiomyocyte proliferation or increases in apoptosis would reduce the number of cardiomyocytes present in the heart. This may account for the changes I have observed in zebrafish cardiac morphology as they age. To see if an increase in cardiomyocyte apoptosis also occurs in zebrafish as they age the cardiomyocyte apoptosis assay that I previously developed was used on zebrafish aged 7 months, 12 months, 15 months and 23 months (Figure 3.32). Zebrafish aged 23 months old had a significantly higher percentage of cardiomyocytes undergoing apoptosis, averaging 0.83% compared to 0.13%, 0.18% and 0.26% in 7 months old, 12 months old and 15 months old zebrafish, respectively. This demonstrates that the rate of cardiomyocyte apoptosis increases in zebrafish from the age of 15 to 23 months.

(A)

(B)

Age (months)	Number of zebrafish	Cardiomyocytes counted	Caspase 3 positive cardiomyocytes counted	Caspase 3 positive cardiomyocytes (%)	SEM
7	8	11571.25	14.50	0.13	0.02
12	10	12637.70	22.40	0.18	0.02
15	9	12803.00	32.56	0.25	0.02
23	7	13629.14	110.43	0.81	0.13
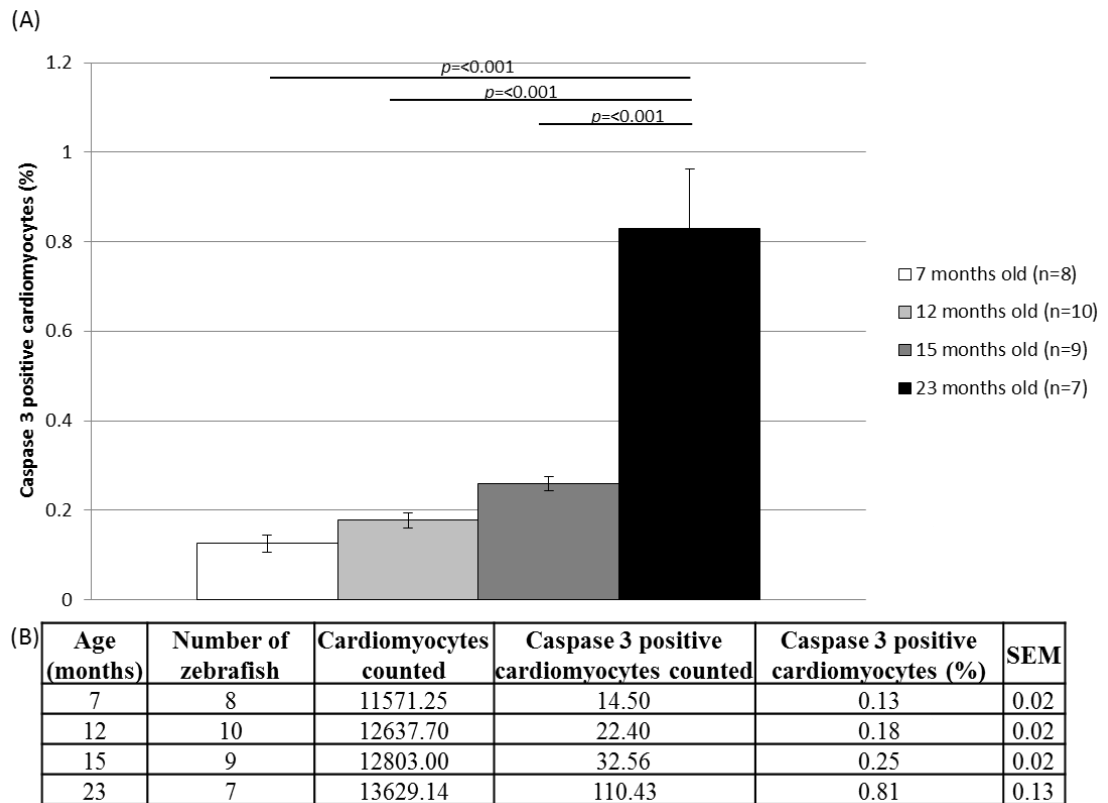
Figure 3.32: Cardiomyocyte apoptosis increases in elderly zebrafish
The amount of caspase 3 positive cardiomyocytes increases in elderly zebrafish (23 months old) when compared to all other younger age groups (A). Error bars are SEM. Statistical significance was observed when analysed using a one way ANOVA. The number of cells counted are included in table (B).

3.4. Changes in cardiomyocyte turnover in aged hearts with cardiac fibrosis

3.4.1. Aims

It has been demonstrated that cardiac fibrosis can occur in zebrafish when the cell cycle of cardiomyocytes is impeded (Poss et al., 2002; Wills et al., 2008). I therefore aimed to see if cardiac fibrosis was associated with a decrease in proliferation and, or, an increase in apoptosis of cardiomyocytes.

3.4.2. Results

3.4.2.1. There is no difference in cardiomyocyte proliferation in hearts with cardiac fibrosis

To determine if cardiac fibrosis occurs due to changes in cardiomyocyte turnover the 23 months old age group was reanalysed by subdividing this age group it into a 'no cardiac fibrosis' control group, in which no cardiac fibrosis was evident and a 'cardiac fibrosis' group in which hearts had fibrosis. Hearts within the 'no cardiac fibrosis' group showed no signs of fibrotic tissue other than the bulbous arteriosus and the valves. Hearts in the 'cardiac fibrosis' group showed signs of fibrosis in the myocardium.

In the hearts of 23 months old zebrafish with no cardiac fibrosis 1.21% of cardiomyocytes were IdU positive compared to 0.81% in the hearts with fibrosis (Figure 3.33A). This was not significantly different when analysed using a students t-test. There was also no significant difference in the frequency of CldU positive cardiomyocytes between hearts with no cardiac fibrosis and hearts with fibrosis, 1.11% compared to 1.54% respectively (Figure 3.33B). There was also no significant difference in the frequency of double thymidine analogue positive cardiomyocytes or the frequency of cells re-entering the cell cycle between the hearts with fibrosis and without fibrosis (Figure 3.33C and D). This implies that changes in cardiomyocyte proliferation is neither affected nor the cause of cardiac fibrosis.

3.4.2.2. There is an increase in cardiomyocyte apoptosis in hearts with cardiac fibrosis

Next, the percentage of cleaved caspase 3 positive cardiomyocytes was compared between the healthy and fibrosis groups. It appears that there was more than double the amount of cleaved caspase 3 cardiomyocytes in the hearts of zebrafish which also had fibrosis compared to those without fibrosis; 1.13% compared to 0.55%, respectively (Figure **3.34**). This suggests that fibrosis is associated with increased cardiomyocyte apoptosis.
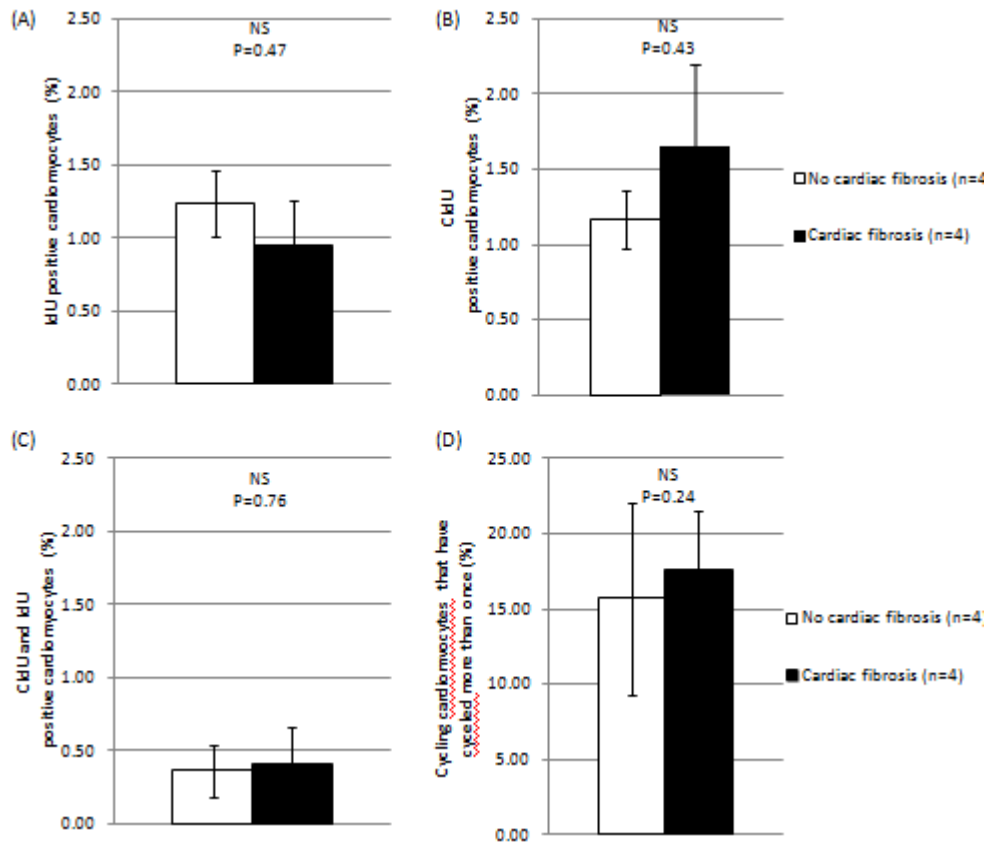
Figure 3.33 Fibrotic hearts do not have increased cardiomyocyte proliferation
The data collected for 23 months old zebrafish was split into two groups, one for
zebrafish without cardiac fibrosis and one for zebrafish with cardiac fibrosis. There was
no significant difference in the percentage of IdU positive cardiomyocytes (A), the
percentage of CldU positive cardiomyocytes (B) or the number of CldU and IdU double
positive cardiomyocytes (C), indicating that there is no difference in cardiomyocyte
proliferation. There was also no difference in the rate of cardiomyocytes re-entering the
cell cycle (D). Statistical analysis was performed using a student's t-test. Error bars are
SEM. Table (E) includes the average number of different cells counted for each
zebrafish heart in the different groups.

(A)

(B)

Experimental group	Number of zebrafish	Caspase 3 positive cardiomyocytes (%)	SEM
No cardiac fibrosis	4	0.55	0.08
Cardiac fibrosis	3	1.13	0.18
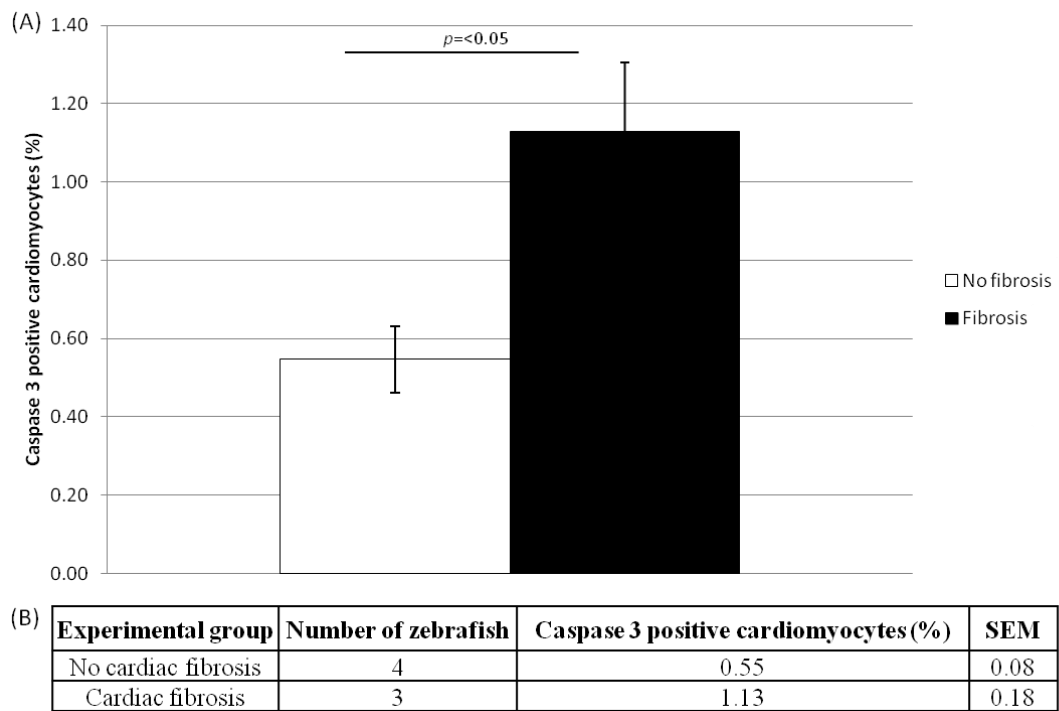
Figure 3.34: Fibrotic zebrafish hearts have increased cardiomyocyte apoptosis
Cardiomyocyte apoptosis is increased in zebrafish hearts with fibrosis when compared to age matched zebrafish with no cardiac fibrosis (A). Error bars are SEM, statistical significance was observed when a one-way ANOVA was performed. Table (B) includes the average counts for each zebrafish in the different experimental groups.

3.5. Discussion

3.5.1. *Morphological changes in zebrafish hearts due to ageing*

It is widely reported that zebrafish possess indeterminate growth (Rowlerson *et al.*, 1997; Gerhard *et al.*, 2002). This means that zebrafish grow continuously throughout their lifespan, provided there is enough space and nutrients available. The zebrafish that I examined did indeed appear to have indeterminate growth as the 23 months old zebrafish had a significantly longer standard length and larger mass than that of 6 months old zebrafish (Figure 3.2). Confirming that these zebrafish possessed indeterminate growth.

Next I wanted to find out if zebrafish succumbed to pathological changes in the heart that have been associated with ageing in humans and animal models. As zebrafish age there appears to be changes in cardiac morphology that have been associated in humans and animals with pathology, such as cardiac fibrosis, increases in ventricular wall thickness and an increase in adipose tissue deposits on the epicardium (Lakatta, 1993; Fleg *et al.*, 1995; Lakatta, 1999; Chang *et al.*, 2000; Schwartz, 2007). The oldest age I examined was 23 months old. This is not extremely old for zebrafish, 23 months represents approximately 62% of their elapsed life span. In humans a comparable age is approximately 44 years. This coincides with the age prior to increase in the frequency of heart disease. At this equivalent age in humans it may be expected that there is some pathogenic cardiac remodelling occurring which increases the risk of heart disease in later life (Figure 3.1). Although the reason I choose to examine 23 months old zebrafish my reasons were based on reports in humans and converted to lifespan in zebrafish. As pathological changes were observed in 23 months old zebrafish hearts justifies my decision to choose this age group.

Fibrosis was found in the oldest age groups of zebrafish examined; aged 21 and 23 months (Figure 3.4). The incidence of fibrosis appears to increase as zebrafish age, from 0% at 15 months old, 10% at 21 months old and 56% at 23 months old. Zebrafish beyond these groups may even have a higher incidence of cardiac fibrosis. Due to the difficulty in obtaining aged animals older age groups of zebrafish were not examined. At 23 months of age there appears to be a sufficient amount of remodelling being initiated. I decided therefore it was more beneficial to focus on an age where it may be possible to reverse or slow the rate of remodelling. Fibrosis is associated with ventricular stiffening which makes cardiac relaxation inefficient causing diastolic dysfunction (Burlew, 2004). This suggests that the observed cardiac fibrosis may affect cardiac performance in zebrafish and therefore be pathogenic.

It was observed that the wall of the ventricle was significantly thicker in the 23 months old zebrafish when compared with the other age groups (Figure 3.6). This infers that ventricle remodelling occurs, resulting in an increase in wall thickness as zebrafish age, which has also been reported in humans (Gerstenblith *et al.*, 1977; Hees *et al.*, 2002). Thickening of the ventricular wall reduces the volume of the heart and may cause obstructions to blood flow in some instances. Ventricular remodelling in response to ageing causes an overall change in heart shape, as the heart becomes more spherical than ellipsoid (Hees *et al.*, 2002). Changes in cardiac shape have been associated with an increase incidence of aortic and mitral regurgitation, myocardial infarction and coronary artery disease (Vandenbossche *et al.*, 1988; Dawson and Gibson, 1989; Lamas *et al.*, 1989; Van Dantzig *et al.*, 1996).

Zebrafish hearts may also become more dilated in response to ageing as the lumen area as a percentage of total ventricular area increased significantly in zebrafish aged 23 months old (Figure 3.8). Dilation of the ventricles due to age has been reported in human hearts (Hees *et al.*, 2002). Ventricular dilation is a characteristic of ventricular remodelling and is implicated in contributing to a variety of pathologies (Vandenbossche *et al.*, 1988; Dawson and Gibson, 1989; Lamas *et al.*, 1989; Van Dantzig *et al.*, 1996). The increased lumen area in zebrafish may also be due to a reduction in trabeculae. Trabeculae appear thicker in the 7 months old, 12 months old and 15 months old zebrafish compared to the 23 months old zebrafish. A reduction in trabeculae may be specific to zebrafish ageing as zebrafish hearts have much more elongated trabeculae when compared to mammals. A reduction in trabeculae may have negative consequences on cardiac function as trabeculae in zebrafish are important for transfusion of oxygen and nutrients from the blood into the heart and force generation (Hu *et al.*, 2001). Changes in trabeculae may also cause disruptions in conduction velocity and electrical resistance (Hu *et al.*, 2001).

Adipose tissue around the heart appears to increase as zebrafish age; zebrafish aged 23 months old had a much higher adipose score than younger zebrafish (Figure 3.10). Pericardial adipose tissue also increased with age in humans (Waller, 1988; Montani *et al.*, 2004; Bertaso *et al.*, 2013). Accumulation of adipose tissue has been associated with changes in the structure of the left ventricle and subsequent functional changes in humans (Kim *et al.*, 2011; Konishi *et al.*, 2012). Pericardial fat has also been associated with ischemia and atherosclerosis (Rosito *et al.*, 2008; Nakanishi *et al.*, 2011; Bertaso *et al.*, 2013). These pathogenic changes may also occur in zebrafish hearts due to an accumulation of fatty deposits around the zebrafish heart. Although

Masson's trichrome staining is not a recognised method for identifying adipose tissue. However, upon dissecting the zebrafish hearts, it was obvious that the areas identified as adipose tissue in the Masson's trichrome stained sections was adipose tissue. A way to confirm that this tissue was definitely adipose tissue could have been to carry out oil red staining on sections. However, there were not enough sections to do this.

Morphological changes occur in the zebrafish heart due to age. These changes are similar to morphological changes which occur in human hearts that are associated with pathogenesis. These include an increase in fibrosis, accumulation of fatty deposits and ventricular wall thickening. These pathological changes result in a reduced cardiac output in humans and are detrimental to the whole organism. It would be interesting to see if these changes also cause a reduced output in zebrafish hearts and impair the quality of life. However, this is currently out of reach due to technical difficulties, such as the size of zebrafish and expense and availability of echocardiography equipment. As I have characterised the changes in the whole organ, the next step was to characterize changes at the cellular level in the zebrafish heart due to ageing.

3.5.2. *Establishing a method to measure cardiomyocyte turnover*

Whilst markers of cell proliferation such as PCNA and PHH3 are commonly used in assays examining proliferation of cardiomyocytes in zebrafish (Poss *et al.*, 2002; Sun *et al.*, 2009; Jopling *et al.*, 2010), halogenated thymidine analogues appeared most suitable for a variety of reasons to fulfil the aims of future experiments. Using the halogenated thymidine analogues IdU and CldU allowed me to determine the length of time that cells are pulsed, and thus increase the numbers of positive cells to count reducing the total number of cells to count. The likes of PCNA and PHH3 are only transiently present so the number of cells needed to count in order to get a significant number of proliferating cells is higher. Multiplexing thymidine analogues, as done with IdU and CldU, allowed cells to be labelled at two different time points allowing proliferation before and after different interventions and treatments to be measured. This also allowed me to examine if cardiomyocytes are likely to re-enter the cell cycle. This means that changes in proliferation can be measured in the same zebrafish but at different points in time, rather than comparing proliferation rates in different zebrafish which could be variable due to their indeterminate growth.

When using endogenous markers of cell turnover to measure proliferation only cells undergoing proliferation at the time of death will be measurable, even this varies depending on the marker of proliferation used. For example Ki67 is detectable at all

stages of the cell cycle, except G0 (Forones *et al*., 2005), whereas PCNA is only expressed during S-phase (Motiwale *et al*., 2005). This would mean that if PCNA was used there would be fewer cells that were positive for proliferation compared to if Ki67 was used, even though there is no actual difference in the proliferation rate. Using thymidine analogues is analogous to tally counting proliferating cells in a given time period, whereas markers of proliferation are more like a snap shot of what happens to be occurring at the time of death.

Another benefit of using both CldU and IdU is that information can be garnered about cell cycle length and source of proliferating cardiomyocytes. For example, it may be that under certain circumstances all cardiomyocytes have a similar proliferative potential, whereas under other conditions it may be that there is a sub population of cardiomyocytes that are more likely to proliferate, which would present as there being more IdU and CldU positive cardiomyocytes. This did not appear to occur in zebrafish hearts due to ageing as there was no significant difference in the number of double IdU and CldU positive cardiomyocytes present.

Thymidine analogues are, however, not without their problems, such as potential toxicity and complications which may arise from administering the thymidine analogues. IdU has been used as a radiosensitizing agent in cancer patients and could slow cellular proliferation (Kajstura *et al*., 2010). Higher doses of BrdU have been shown to encourage entrance to the cell cycle in haematopoietic stem cells (Wilson *et al.*, 2008). Another study, examining the pancreas, has found there to be no difference in proliferation in beta-cell proliferation after long term administration of BrdU (Tuttle *et al*., 2010), suggesting that any affects thymidine analogues have on proliferation may be dependent on the tissue involved, the animal model and indeed the actual analogue used. I found no significant differences in cardiomyocyte proliferation when using either 0.25mg/ml or 0.5mg/ml CldU and IdU, suggesting that these doses do not affect proliferation of zebrafish cardiomyocytes (Figure 3.23).

CldU incorporation appears to be higher after IdU incorporation in zebrafish cardiomyocytes (Figure 3.24). This may be due to cumulative stress of the zebrafish being in a relatively small volume of water and unfamiliar environment. This emphasizes the importance of using proper controls in terms of housing zebrafish in the same sized tanks for the same length of time when making comparisons.

The use of ethynyl deoxyuridine (EdU) to label proliferating cardiomyocytes may indeed supersede CldU and IdU (Chehrehasa *et al.*, 2009). Kits using EdU are available which make EdU relatively easier to use than other traditional thymidine

156

analogues. In these kits fluorescent probes are used which are permeable to cells and can work on double stranded DNA means tissue would need less, potentially destructive, processing. However, currently there is only one EdU 'analogue', which means multiplexing analogues for different time points is not possible, and EdU is relatively more expensive than IdU and CldU.

Nevertheless all these problems can be overcome by the use of suitable controls and using the lowest possible dose of analogues to reduce any potential toxic affects without impairing labelling efficiencies, making the use of halogenated thymidine analogues the superior method for my experimental objectives.

As there are thousands of cardiomyocytes per section of zebrafish heart and sections are too large to be captured in one image a specialised method needed to be developed using FIJI to allow a relatively high throughput of zebrafish hearts to be processed semi automatically. The method firstly needed to be able to stitch together multiple images with different fluorescent channels to form a full patchwork heart, without loss of cells. The different channels then needed to be thresholded to remove any background fluorescence whilst still keeping real signals from cells in order to automatically count cells. The methods were verified by counting by hand and various quality control steps were put in place, such as no primary antibody negative controls and periodically verifying counts by hand.

Being able to measure cardiomyocyte apoptosis in a way that is comparable between zebrafish is important when measuring cardiomyocyte proliferation. The levels of apoptosis can help indicate whether proliferation is contributing to cardiac growth (low apoptotic rate) or regenerating lost cardiomyocytes (high apoptotic rate). Although thymidine analogue incorporation and presence of cleaved caspase 3 are not directly comparable; thymidine analogue pulsing is user defined and can therefore be more specific, whereas cleaved caspase 3 levels are transient and are more of a 'snap shot' of what is going on at the time of death, the amount of cleaved caspase 3 cardiomyocytes can give a good indication of the role of proliferation. It has been reported that the length of apoptosis is between 6-24 hours (Saraste, 1999; Saraste and Pulkki, 2000). This means that the rate of apoptosis can be roughly compared to the rate of thymidine incorporation over a defined time period.

Choosing an appropriate marker for cardiomyocytes is also very important when measuring turnover of cardiomyocytes. If cardiomyocytes are not accurately distinguished from other cells in the heart, this could influence the reported turnover rate (Soonpaa *et al.*, 2013). This indeed was one of the criticisms of the Bergman *et al.,*

2009 study. It has been argued that the marker used in this study, cTroponin, may only be expressed by senescent cardiomyocytes (Kajstura *et al.*, 2012b). Initially I planned on using transgenic zebrafish which expressed a nuclear localised fluorescent protein, DSred, under the control of the cardiac specific *cmlc2* promoter (Lepilina *et al.*, 2006). I planned on using an antibody to fluorescently detect the cardiomyocyte specific DSred. However, it appeared that the hydrochloric acid treatment needed in order to detect thymidine analogues with immunofluorescence was not compatible with the antibody for DSred. I next turned my attention to *mef2*. In zebrafish *mef2* expression has been shown to be necessary for cardiomyocyte differentiation (Hinits *et al.*, 2012) and is widely accepted as a suitable marker of cardiomyocytes, particularly in zebrafish (Wills *et al.*, 2008; Chablais *et al.*, 2011). When performing immunofluorescence to detect cleaved caspase 3 it was not possible to use the anti-mef2 antibody with the anti-cleaved caspase 3 antibody, as they were both raised in rabbit. In this instance I used an ant-MF20 antibody instead of an anti-mef2 antibody. MF20 is also a widely used marker of cardiomyocytes in zebrafish (Jopling *et al.*, 2010; Itou *et al.*, 2012b). However, MF20 is not nuclear localised, which made counting cells more difficult. This was solved by counter staining cells with DAPI in order to mark the nucleus of cells, making counting cardiomyocytes possible.

3.5.3. *Cardiomyocyte turnover in zebrafish*

In humans and mammalian models, many of the pathogenic changes that occur in the ageing heart are the result of a net reduction in cardiomyocytes (Shih *et al.*, 2011). There is a reduction in cardiomyocytes due to the increased rate of apoptosis (Kajstura *et al.*, 2010a) and a possible decreased rate of proliferation (Bergmann *et al.*, 2009; Bergmann *et al.*, 2011), or at least a rate of proliferation insufficient at replacing lost cardiomyocytes (Kajstura *et al.*, 2010a; Kajstura *et al.*, 2010b; Elser and Margulies, 2012). I sought to find out if this was also true of zebrafish hearts.

The rate of proliferation was measured in zebrafish cardiomyocytes showing that there is no change in proliferation in the different age groups I examined. This implies that cardiomyocyte proliferation remains static up to 23 months, at least, in zebrafish. 23 months is a significant portion of a zebrafish lifespan (62%). This is in disagreement with some of what is reported in humans. It has been postulated that cardiomyocyte proliferation increased with age in humans (Kajstura *et al.*, 2010a). A different group used different methods to show a decrease in cardiomyocyte proliferation with age in humans (Bergmann *et al.*, 2009). This group further illustrated how there may be flaws

in the data that showed an increase in proliferation due to ageing (Bergmann *et al.*, 2011). Overall there appears to be some controversy over how cardiomyocyte proliferation changes in response to age in humans, but my data implies there is no change in proliferation of zebrafish cardiomyocytes which, with the background of increasing cardiomyocyte apoptosis, would cause a net reduction in cardiomyocytes. If older zebrafish were examined there is still the possibility that cardiomyocyte proliferation may increase.

To compare my zebrafish cardiomyocyte proliferation data with that reported in humans (Elser and Margulies, 2012) I converted the age of my zebrafish and the age of humans into percentage of elapsed life span, assuming the lifespan is 3.5 and 70 years, respectively. This would allow for comparison between different aged humans and zebrafish. The human cardiomyocyte proliferation data is often expressed as annual turnover. Expressing cardiomyocyte turnover in zebrafish as annual turnover would not be appropriate as 1 year is almost a third of their lifespan. Instead I worked out that 1 year is the equivalent of 1.4% of a human lifespan. 1.4% of a zebrafish lifespan is 17.89 days. I extrapolated the percentage of IdU positive cardiomyocytes to 17.89 days for zebrafish and plotted this data on the hybrid cardiomyocyte proliferation graph from Elser and Margulies, 2012 (Figure 3.35). I used IdU incorporation rather than CldU as CldU incorporation was higher may be due to the stress of sequential immersing and IdU incorporation would therefore be a truer reflection of basal cardiomyocyte turnover. My laboratory are planning to confirm this by immersing zebrafish in CldU followed by IdU. The proliferation of zebrafish cardiomyocytes appears to fit in well with the hybrid model of human cardiomyocyte proliferation with age (Elser and Margulies, 2012). This suggests that the frequency of cardiomyocyte proliferation is similar between humans and zebrafish when differences in lifespan are accounted for.

The rate of cardiomyocyte apoptosis was higher in zebrafish aged 23 months than the younger age groups, suggesting that apoptosis in cardiomyocytes increases as zebrafish age, as has been reported in humans (Kajstura *et al.*, 2010a). Adding the rate of cardiomyocyte apoptosis in zebrafish to the human data collected by Kajstura et al., 2010 can be done by converting the percentage of cells undergoing apoptosis to the rate of apoptosis per 10^6 cells and converting the ages into percentage of elapsed lifespan rather that absolute age (Figure 3.36). This shows that the frequency of cardiomyocyte apoptosis in zebrafish is also in agreement to that of human data. In humans and zebrafish apoptosis appears to increase with age and the magnitude of apoptosis appears similar.

159

In order to make a model of absolute cardiomyocyte turnover in the zebrafish heart I needed to work out the rate of cardiomyocyte proliferation and apoptosis over a defined period of time. Cardiomyocyte proliferation was recorded over a defined period of time, 24 hours in this series of experiments, as thymidine analogues were used to measure proliferation. As cleaved caspase 3 was used as a marker of apoptosis this is a bit trickier. The cells positive for cleaved caspase 3 are cells that are in the process of apoptosis at the time the zebrafish was euthanized. It has been reported that apoptosis lasts between 6 to 24 hours (Saraste, 1999; Saraste and Pulkki, 2000). Assuming that the average length of apoptosis is 15 hours the daily apoptotic rate of cardiomyocytes can be calculated and plotted alongside the daily cardiomyocyte proliferation rate for different aged zebrafish (Figure 3.37). The rate of proliferation appears to remain stable, with approximately 0.76% of cardiomyocytes proliferating in a day. Cardiomyocyte apoptosis is lower than the rate of proliferation up until approximately 20 months. This suggests that there is an increasing number of cardiomyocytes in the zebrafish heart, although this does not take into account loss of cardiomyocytes via necrosis. At the point where cardiomyocyte proliferation is equal to apoptosis there would be no net increase in the number of cardiomyocytes. At the point where the rate of cardiomyocyte apoptosis surpasses that of cardiomyocyte proliferation, loss of cardiomyocytes will occur. It may be that this reduction in cardiomyocytes leads to the pathogenic remodelling I have observed. Although there are less cardiomyocytes in the heart from 20 months of age, in this model, it may be that there is a critical threshold whereby if cardiomyocyte number is reduced below this point fibrosis occurs to compensate for the loss of cardiomyocytes. The fact that the model predicts that at approximately 20 months of age is when there is a decrease in the number of cardiomyocytes is interesting in light of the observation of cardiac fibrosis in zebrafish aged 21 months old. This suggests that the fibrosis occurring is reactive fibrosis and is a response to a reduction of cardiomyocytes.

There was no significant difference in the proportion of cells that re-enter the cell cycle between different aged adult zebrafish (Figure 3.25). Approximately 15% of cardiomyocytes that had divided re-entered the cell cycle over the course of the immersing schedule (5 days in total, Figure 3.25). This suggests that there is a population of clonally dominant cardiomyocytes in zebrafish hearts that are more likely to proliferate than other cardiomyocytes. These cardiomyocytes may described as 'stem-like'. This is in agreement with a report elsewhere (Gupta and Poss, 2012). The majority of IdU positive cardiomyocytes were observed either as singular cells (51.69%)

or doublets (46.02%) (Figure 3.27). Only a small proportion were observed as clusters (2.29%). Given more time the frequency of IdU positive cardiomyocyte clusters would be expected to increase as more of the 'stem like' cardiomyocytes re-enter the cell cycle. I may have experienced so few due to the immersing schedule being too short in duration. Given a longer time more cells would be labelled and more would proliferate. Also, sectioning may have separated clusters of cells, so IdU positive cardiomyocyte clusters may have been underestimated.

(A)

Age (months)	Age (life expectancy elapsed, %)	IdU+ve cardiomyocytes (%)	Yearly proliferation (%)	Yearly proliferation ('zebrafish years', %)
7	15	0.67	244.55	11.99
12	31	0.79	288.35	14.13
15	38	0.58	211.70	10.38
23	62	1.01	368.65	18.07
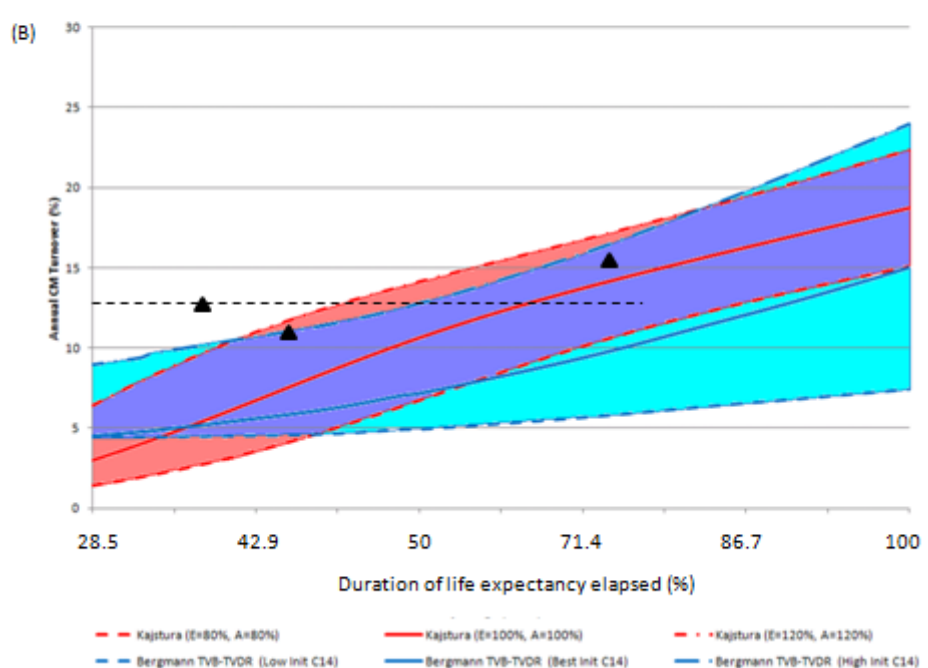
Figure 3.35: Comparing human and zebrafish cardiomyocyte proliferation
To compare the proliferation rate of human cardiomyocytes to that of zebrafish at comparative ages I firstly decided to convert the ages of zebrafish and people to percentage of life expectancy elapsed (A). Next, I worked out that 1 year was equivalent to 1.4% of a human lifespan. 1.4% of a zebrafish lifespan is 17.89 days. To compare annual turnover I extrapolated the rate of daily IdU incorporation to a period of 17.89 days, giving the equivalent annual turnover in 'zebrafish years' (A). I then plotted these points onto the hybrid graph of cardiomyocyte proliferation in humans to see how zebrafish fit in (black triangles and black dashed line) (B). Although I found no increase in cardiomyocyte proliferation, the level of proliferation I observed fits into this model.
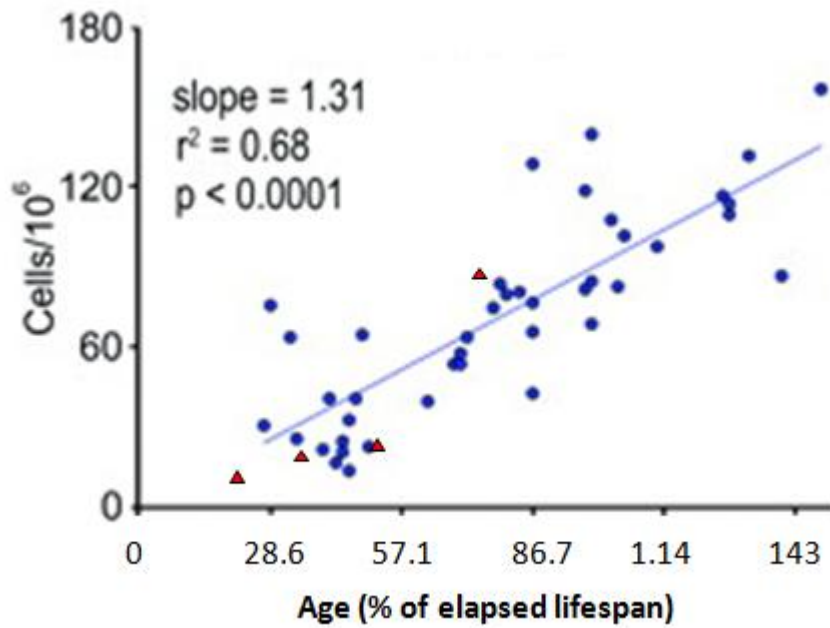
Figure 3.36: Comparing human and zebrafish cardiomyocyte apoptosis
The data from Kajstura et al., 2010 displayed cardiomyocyte apoptosis in terms of apoptotic events per 10^6 cells. I converted my zebrafish apoptotic data from a percentage into apoptotic cells per 10^6 cells and plotted this on the male apoptosis data from Kajstura et al., 2010 (red triangles). These data sets appear quite similar as they are of a similar magnitude and show increasing apoptosis with age.

(A)

(B)

Age (months)	Age (life expectancy elapsed, %)	Caspase 3 positive cardiomyocytes (%)	Daily apoptosis (%)	Daily proliferation	Daily cardiomyocyte net turnover (%)
7	15	0.13	0.20	0.67	0.41
12	31	0.18	0.27	0.79	0.43
15	38	0.25	0.38	0.58	0.08
23	62	0.81	1.22	1.01	-0.61
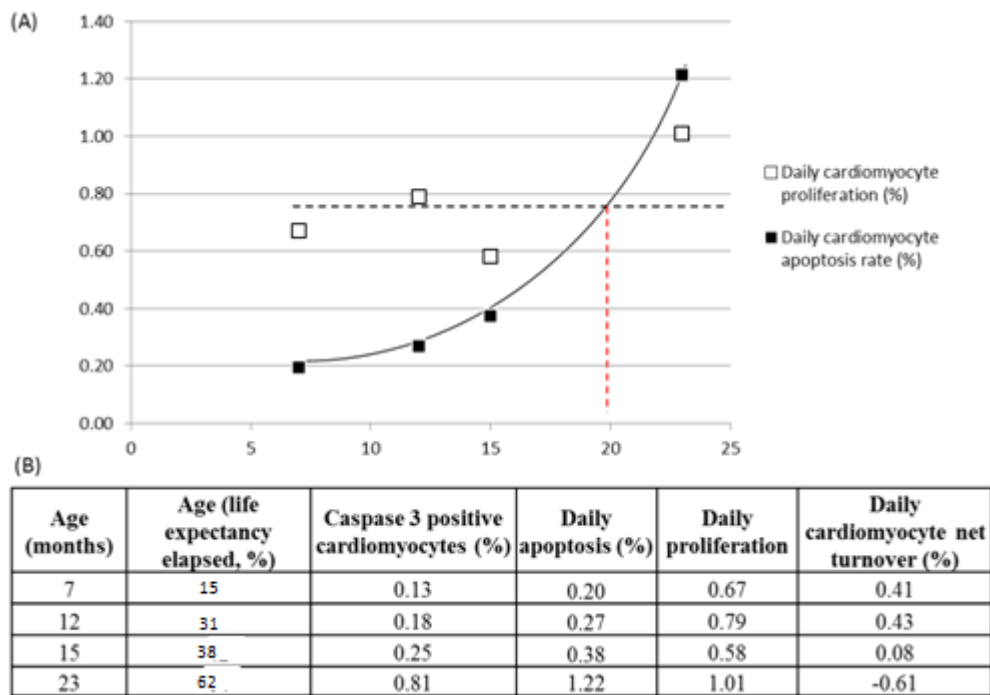
Figure 3.37 Net turnover of cardiomyocytes in zebrafish

I have assumed apoptosis takes on average 15 hours to occur and plotted data for zebrafish (solid black line) alongside the daily IdU incorporation rate for different aged zebrafish (dashed black line). Where the two lines intersect is the age where cardiomyocyte apoptosis exceeds proliferation and the total number of cardiomyocytes begins to decrease (red dashed line). This may be the point where pathogenic cardiac remodelling is initiated.

3.5.4. Changes in the heart due to fibrosis

When the proliferative and apoptotic rates were compared in cardiomyocytes of 23 months old zebrafish with cardiac fibrosis and no cardiac fibrosis it was found that apoptosis was higher in zebrafish with cardiac fibrosis. There was no significant difference in cardiomyocyte proliferation between 23 months old zebrafish with or without cardiac fibrosis. This suggests that fibrosis may be occurring to compensate for a loss of cardiomyocytes. Even though cardiomyocyte proliferation is still occurring, the level of proliferation is not sufficient enough to compensate for the increase in apoptosis. The fibrous tissue may temporarily repair the heart but cause problems in the longer term such as conduction problems and ventricular stiffness.

3.5.5. Limitations

Although all experiment were thoroughly planned and carried out as well as possible, this chapter does have limitiations. Some of the limitations are based on measurements of certain parameters of the zebrafish hearts, such as fibrosis, wall thickness and adipose deposits.

A scoring system for the level of fibrosis, rather than whether it is present or not may have been more appropriate. This could have been done by developing a scoring system for the severity of fibrosis or by quantifying the percentage of myocardium that was fibrotic.

Measuring the thickness of the ventricular walls in zebrafish is a very difficult process due to changes in thickness at different positions. Although all effort was made to use mid line sections, using other methods would have been more appropriate. This could have been done my echocardiography. This was not possible for me to do due to technical difficulties and the cost of very expensive specialist equipment that would be required.

Masons Trichrome staining is not a recogniszed method for identifying adipose tissue. A more suitable way to identify adipose tissue would be to use oil red. To remove user bias that a qualitative scoring system can sometimes incur, it would have been beneficial for other people to use my scoring system to see if we achieved corroborative results.

Another limitation could be the number of zebrafish in some of the experiments was not sufficient to achieve statistical power. Some observations were not statistically significant although the results look like they may have been different. This is particularly true when analysing the cardiomyocyte proliferation data. No statistical

significance was shown when a one-way ANOVA was performed on the data. Although the number of animals used was carefully considered and statistical tests were carried out to ensure power was achieved without using an unnecessarily large number of animals (Festing *et al.,* 1998). It may be that if more animals were examined statistical significance could be achieved.

It is difficult to compare the lifespan of humans to different animals, particularly zebrafish , which have a much shorter lifespan (Gerhard et al., 2002). This therefore limits the interpretation of comparing the cardiomyocyte turnover rates of zebrafish to that of humans. Overall, comparing the turnover rates can give a reasonable assumption to how cardiomyocyte turnover compares.

3.5.6. *Further work*

There are a few experiments that I would have liked to perform which were beyond the scope of this thesis and would add to the data I have garnered in this chapter. I would have liked have developed a way of monitoring cardiac output *in vivo* in adult zebrafish. Although I have reported potentially pathogenic changes which occur in the zebrafish heart due to ageing, I would have liked to have confirmed that they have a significant effect on the output of the heart. This would further validate the used of zebrafish as a model of human cardiac ageing if the decline in cardiac output is similar to that which is reported in humans (Vigorito and Giallauria, 2014). Cardiac output could have been measured using echocardiography. This was not possible for me to do as I did not have access to the specialist equipment required to perform echocardiography in zebrafish (Ho *et al.*, 2002)

I have shown that there is an increase in the rate of apoptosis in cardiomyocytes due to ageing, with no increase in cardiomyocyte proliferation. This would result in a net reduction of cardiomyocytes. However, knowing the absolute number of cardiomyocytes in hearts of zebrafish at different ages would prove conclusively that there is a reduction in the number of cardiomyocytes as zebrafish age. This could have been done using flow cytometry, which has been successfully carried out on whole mouse hearts (Song *et al.*, 2012; Malliaras *et al.*, 2013). Hearts from zebrafish treated with IdU and CldU could have been digested and then separated with flow cytometry to get the absolute number of cardiomyocytes (using a marker for cardiomyocytes, e.g. mef2c), IdU positive cardiomyocytes, CldU positive cardiomyocytes and IdU and CldU double positive cardiomyocytes. Flow cytometry could have also been used to measure apoptosis in cardiomyocytes. Data gathered from these types of experiments could

unequivocally prove whether there is a change in cardiomyocyte number in zebrafish as they age. However, I chose to section zebrafish hearts instead. I decided that sectioning hearts would preserve the structure of the heart so I could also examine morphological and histological changes, as well as changes in turnover. I would have liked to have used flow cytometry to merely confirm a reduction in the absolute number of cardiomyocytes as zebrafish age, however time and lack of reagents did not permit this.

I would have also liked to have analysed some zebrafish hearts older than 23 months old. It would have been interesting to see if all zebrafish hearts develop fibrosis if given enough time. I would have also been able to see if cardiomyocyte apoptosis continued to decrease with age and if there are any changes in cardiomyocyte proliferation due to ageing in the very old. This was not done as my main aim was to examine the genesis of pathological remodelling in zebrafish, which I achieved. It is also expensive and time consuming generating animals that are extremely old.

3.5.7. Summary and conclusion

In the work I have presented in this chapter I have demonstrated for the first time that zebrafish exhibit morphological changes in the heart due to ageing. These include thickening of the ventricle wall, fibrosis, increasing adipose tissue deposition and dilation of the ventricle. These changes have been associated with pathogenesis in human hearts. The changes observed in the zebrafish heart due to ageing may be due to changes in the turnover of cardiomyocytes. Although the rate cardiomyocyte proliferation remains static throughout the adult zebrafish life, cardiomyocyte the frequency of cardiomyocyte apoptosis appears to increase. This likely results in a net decrease of cardiomyocytes within the aged zebrafish heart. This is a likely cause for the potentially pathogenic remodelling I have reported.

Chapter 4 : Exercise and p38MAPK inhibition in the zebrafish heart

4.1. Introduction

In the previous chapter I have demonstrated the changes that occur in zebrafish hearts in response to ageing. These changes have been associated with cardiac disease in humans and animal models. One of the mechanism behind cardiac morphology changes in ageing appears to be the reduction of cardiomyocytes. I demonstrated this in the previous chapter, as there was an increase in cardiomyocyte apoptosis with no increase in proliferation as zebrafish age. Increasing cardiomyocyte proliferation or reducing cardiomyocyte apoptosis may help prevent some of the morphological changes in the heart, such as fibrosis. In this chapter I will discuss p38MAPK inhibition and exercise. These are two interventions I then applied to zebrafish to see if they have a positive effect on cardiomyocyte turnover. This could reduce the amount of fibrosis and remodelling in ageing zebrafish hearts.

4.2. Aims of the chapter

In this chapter I aimed to see if cardiomyocyte proliferation could be increased or cardiomyocyte apoptosis reduced in zebrafish. To do this I used pharmacological intervention in the form of p38MAPK inhibition and physiological intervention in the form of exercise. Then, I examined if these two interventions had any additive affect when used together. The underlying mechanisms of any changes in cardiomyocyte turnover were elucidated via RT-qPCR of candidate genes.

4.3. Results

4.3.1. Choosing the optimum dose of p38MAPK inhibitor

In order to determine the optimum dose of SB203580, a p38MAPK inhibitor (Engel *et al.,* 2005), to increase cardiomyocyte proliferation in zebrafish, 6 months old zebrafish were immersed in IdU for 24 hours, then either aquarium water, 2.5μM, 5μM or 10μM SB203580 for 12 hours and then CldU for 24 hours (Figure 4.1A). The length of immersion time was not changed. 12 hours was choosen as an acute period of p38MAPK inhibition and in order to carry out the experiment it was more practical to use different dilutions rather than varying immersion time.

Dose (μM)	0	2.5	5	10
No. of zebrafish	3	4	4	3
MEF2+ve	2586	3173	3595	4049
Mef2 and IdU+ve	2.67	3.04	2.67	3.11
IdU+ve cardiomyocytes (%)	0.10	0.10	0.07	0.08
SEM	0.06	0.05	0.02	0.00
Mef2 and CldU+ve	2.83	4.29	17.67	36.44
CldU+ve cardiomyocytes (%)	0.11	0.14	0.49	0.90
SEM	0.03	0.02	0.12	0.10
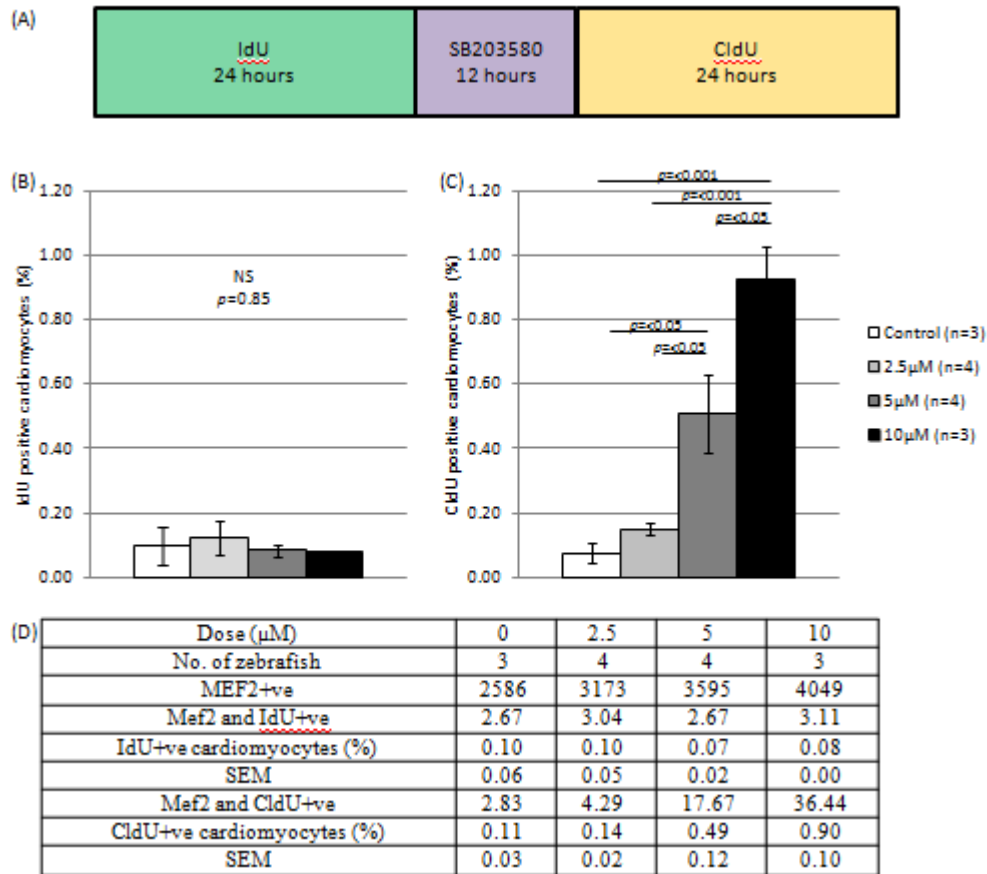
Figure 4.1: Selecting the optimum dose of SB203580 to increase cardiomyocyte proliferation

To determine the optimum dose of SB203580 to increase cardiomyocyte proliferation zebrafish were immersed in IdU for 24 hours, then either aquarium water, 2.5μM, 5μM or 10μM SB203580 for 12 hours and then CldU for 24 hours (A). There was no significant difference in the frequency of IdU positive cardiomyocytes in the hearts of zebrafish immersed in different doses of SB203580 (pre-SB203580 proliferation rate, B). There was a significant increase in the frequency of CldU positive cardiomyocytes in the hearts of zebrafish treated with 5μM and 10μM SB203580 compared to zebrafish treated with aquarium water or 2.5μM SB203580 (post-SB203580 proliferation rate C). Error bars are SEM, statistical analysis was done using a one-way ANOVA. Mean values for each dosage group are shown in table (D).

The range of SB203580 dosing was chosen based on previous work undertaken in the Chaudhry laboratory, examining the effects of SB203580 in tissue culture. A treatment time of 12 hours of SB203580 was selected as I thought this would give acute inhibition of p38MAPK. In a study examining the affects of p38MAPK inhibition after experimental myocardial infarction in rats, rats were injected with SB203580 only once, which had a beneficial effect (Engel *et al.*, 2006a). This would also result in short term p38MAPK inhibition. Prolonged inhibition of p38MAPK may have been deleterious due to the widespread nature of p38MAPK signalling.

The percentage of cardiomyocytes that had incorporated IdU was not significantly different between groups of zebrafish receiving different doses of SB203580, suggesting that basal cardiomyocyte proliferation was the same in all zebrafish prior to p38MAPK inhibition (Figure 4.1B). The percentage of CldU positive cardiomyocytes was significantly higher in the 5μM and 10μM SB203580 groups than in the aquarium water control and 2.5μM SB203580 treated zebrafish (Figure 4.1C). This implied that both 5μM and 10μM SB203580 increases cardiomyocyte proliferation. There was significantly more CldU positive cardiomyocytes in the hearts of zebrafish treated with 10μM SB203580 than those treated with 5μM SB203580.

The amount of cleaved caspase 3 positive cardiomyocytes was next monitored to see if p38MAPK exerted any effect on cardiomyocyte apoptosis. It appeared that there was no significant difference in the percentage of cleaved caspase 3 positive cardiomyocytes with different concentrations of SB203580 (Figure 4.2).

Altogether these results suggest that 5μM and 10μM SB203580 can promote cardiomyocyte proliferation without having any effect on apoptosis. As p38MAPK has many roles in lots of different cellular processes I decided to use a dose of 5μM SB203580 to try and minimise any possible off target effects.

(A)

(B)

Dose (μM)	0	2.5	5	10
No. of zebrafish	4	4	4	4
MF20+VE	2326	2701	3123	3295
MF20 and Cleaved caspase 3 +ve	1.83	1.42	3.50	5.00
Cleaved caspase 3 +ve cardiomyocytes (%)	0.08	0.05	0.11	0.15
SEM	0.03	0.02	0.04	0.05
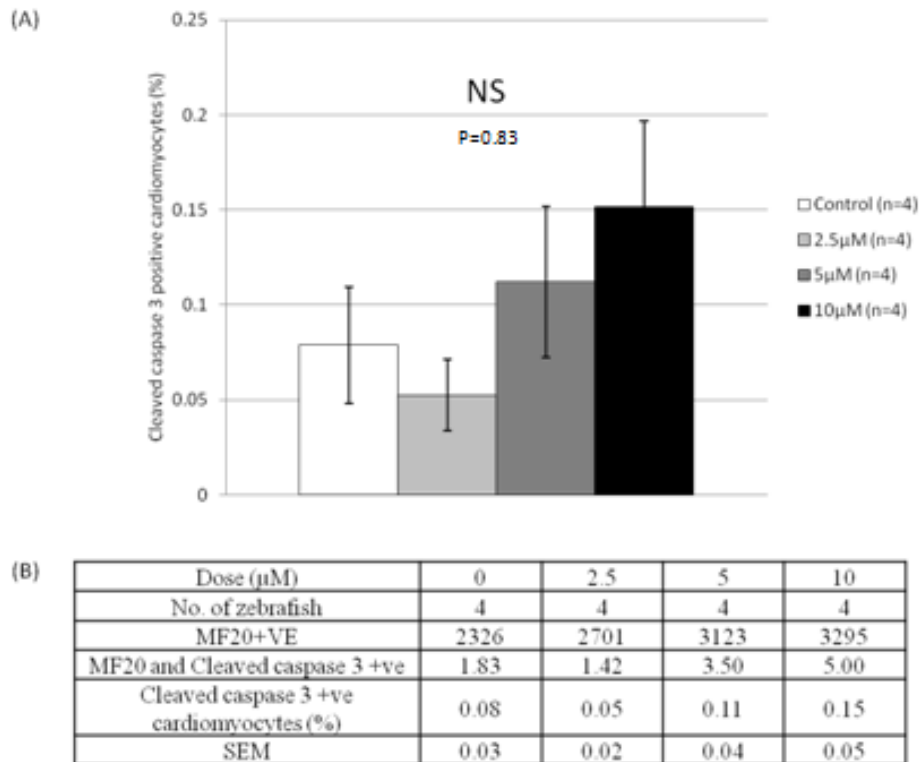
Figure 4.2: SB203580 does not increase cardiomyocyte apoptosis
The different doses of SB203580 tested to increases cardiomyocyte proliferation did not increase cardiomyocyte apoptosis (A). Error bars are SEM, statistical analysis was done using a one-way ANOVA. Mean values for each dosage group are shown in table (B).

4.3.2. Selecting a suitable intensity of enforced exercise in zebrafish

To determine the optimum intensity of exercise for zebrafish to experience in a swim tunnel, I decided to work out the average swimming speed of different aged zebrafish. To do this, data collected from the zebrafish used in the previous results chapter was used.

Swimming speed was monitored in zebrafish of different ages, for three 1 minute intervals, using a camera mounted above a tank housing individual zebrafish. Zebrafish were left for 5 minutes to allow them to settle into their new environment before recording began. Using the software Fiji, the speed of individual zebrafish was determined. The speed of zebrafish from cm per second was converted to BL/s using the length of each individual zebrafish to do so. This takes into account the size of each zebrafish and makes comparing speed more comparable. The 7 months old zebrafish swam at a speed of 3.49BL/s, the 12 months old 3.12BL/s, the 15 months old 3.86BL/s and the 23 months old at 2.10BL/s (Figure 4.3). The 23 months old zebrafish were significantly slower than the 7 months old zebrafish and the 15 months zebrafish. The 23 months old zebrafish were almost twice as slow as the 7 months and 15 months old zebrafish (Figure 4.3).

I therefore decided that an appropriate swimming speed for 21 months old zebrafish would be 3BL/s, 10cm/s in this case. Preliminary experiments demonstrated that 7 months old zebrafish could not survive more than 24 hours in the swim tunnel at speeds over 3BL/s. I therefore chose to keep the speed at 3BL/s, 7cm/s in this case, for the 7 months old zebrafish.
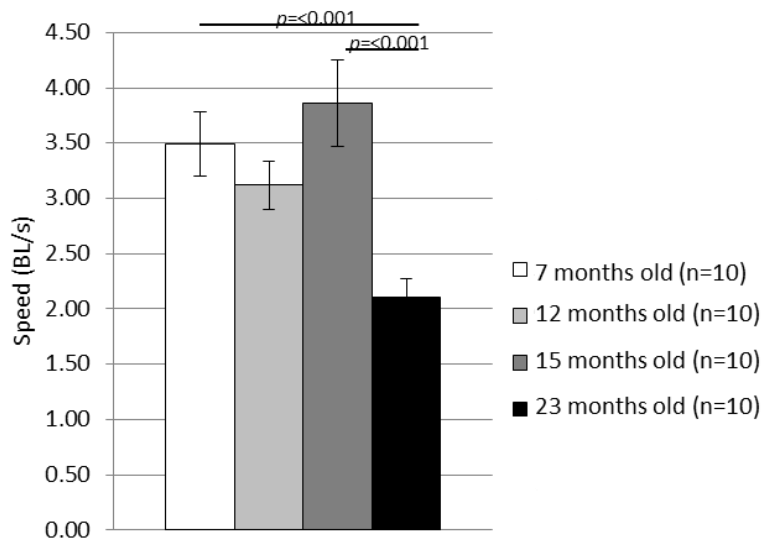
Figure 4.3: Passive swimming speed decreases as zebrafish age
Speed (BL/s) is lower in 23 months old zebrafish compared to 7 and 15 months old zebrafish when zebrafish are house in tanks with no water flow. Error bars are STD, statistical significance was observed using a one-way ANOVA.

4.3.3. *Exercise increases cardiomyocyte proliferation in elderly zebrafish*

Acute enforced exercise was then utilised in an attempt to increase cardiomyocyte proliferation. I decided to carry out this series of experiments on male zebrafish, as in the previous chapter I mapped cardiac turnover in male zebrafish. If I had used female zerbafish in this experiment I may not have been able to fairly compare cardiomyocyte turnover or morphological changes between the sexes. It has been reported that rate of cardiomyocyte proliferation is higher and apoptosis lower in females compared to male humans (Kajstura *et al.,* 2010). I chose to perform these experiments on zebrafish aged 7 months and 21 months. Zebrafish aged 7 months were chosen as I wanted to see if cardiomyocyte turnover could be influenced from an early age in adults. If this was possible it may prevent or delay adverse cardiac remodelling in later life. I decided to choose zebrafish which were 21 months old as this is around the age in zebrafish in which I predicted, in the previous chapter, daily cardiomyocyte apoptosis to exceed that of proliferation. This age may therefore be the genesis of adverse cardiac remodelling and increasing cardiomyocyte proliferation, and/or reducing cardiomyocyte apoptosis may delay the onset of remodelling.

Zebrafish were immersed in IdU for 24 hours, forced to exercise in a swim tunnel at a speed of 3BL/s or rest for 3 days, immersed in aquarium water for 12 hours and then immersed in CldU for 24 hours prior to terminal anaesthesia and dissection (Figure 4.4A). Zebrafish were immersed in aquarium water for 12 hours prior to CldU treatment as I later used this schedule to test if p38MAPK inhibition, via SB203580 treatment, in conjunction with exercise increases cardiomyocyte turnover. As the schedules were similar this allowed me to more accurately compare interventions and allowed me to use the same control zebrafish in multiple experiments.

There was no significant difference in IdU incorporation, used to determine pre-treatment cardiomyocyte proliferation, in cardiomyocytes of zebrafish aged 7 months or zebrafish aged 21 months that were sedentary or exercising. In the 7 months old zebrafish 0.24% and 0.73% of cardiomyocytes were IdU positive in the sedentary and exercised zebrafish, respectively (Figure 4.4B). In the 21 months old zebrafish 0.43% and 0.58% of cardiomyocytes were IdU positive in the sedentary and exercised zebrafish. This implies that the basal cardiomyocyte proliferation rate was the same between sedentary and exercising zebrafish prior to treatment (Figure 4.4B).

In the 7 months old zebrafish there was no significant change in CldU positive cardiomyocytes between sedentary and exercised zebrafish, with 1.14% and 1.24% of cardiomyocytes CldU positive, respectively. This implies that that exercise does not

increase cardiomyocyte proliferation in younger zebrafish. However in 21 months old zebrafish there was a significant increase in CldU positive cardiomyocytes in zebrafish that had been exercised compared to sedentary zebrafish. This infers that exercise increases cardiomyocyte proliferation in older zebrafish. In 21 months old zebrafish the percentage of CldU positive cardiomyocytes increased from approximately 2.31% to 9.46% after exercise, an increase of approximately 4 fold.

Age (months)	7	7	21	21
Intervention	Sedentary	Exercise	Sedentary	Exercise
No. of zebrafish	9	10	8	8
Mef2+ve	7298	7963	6658	6409
Mef2 and IdU+ve	17.22	58.40	28.63	37.00
IdU+ve cardiomyocytes(%)	0.24	0.73	0.43	0.58
SEM	0.02	0.16	0.06	0.06
Mef2 and CldU+ve	83.44	98.90	153.50	606.25
CldU+ve cardiomyocytes	1.14	1.24	2.31	9.46
SEM	0.10	0.17	0.69	4.19
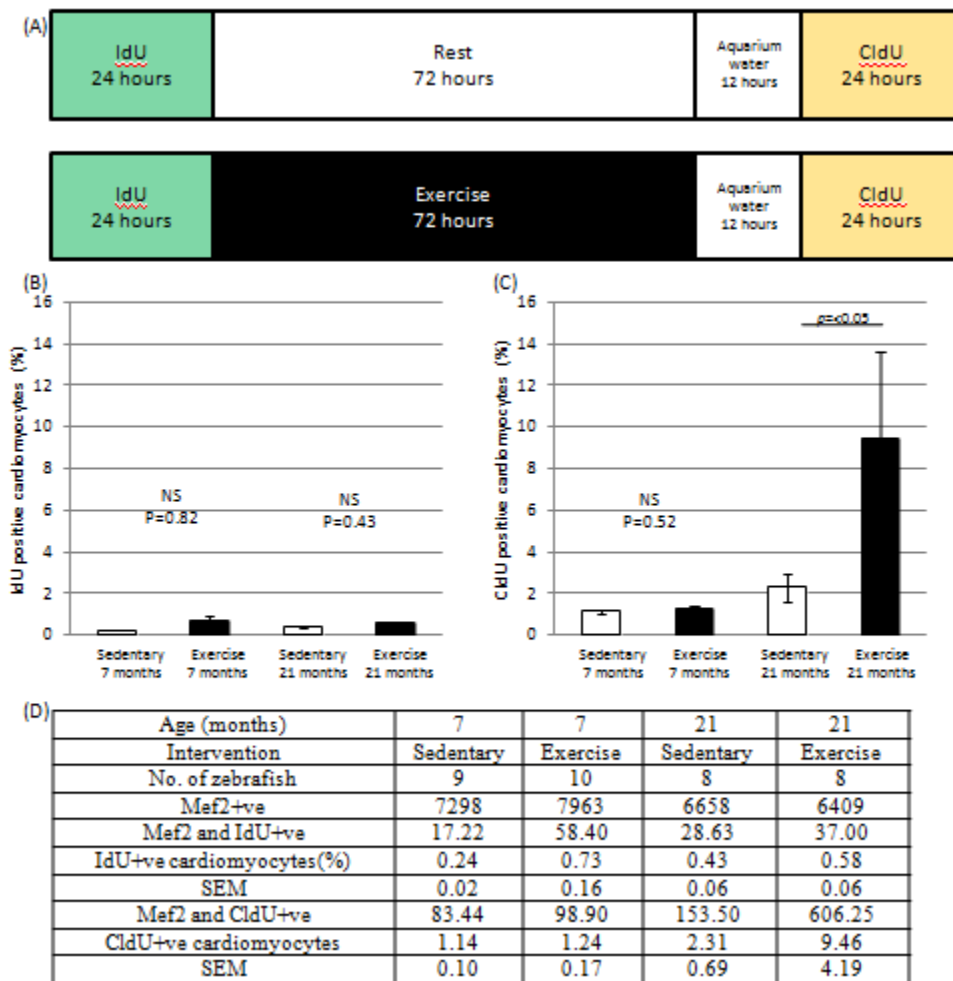
Figure 4.4: Exercise increases cardiomyocyte proliferation in elderly zebrafish
Zebrafish were immersed in IdU for 24 hours then either rested or exercised in a swim tunnel at a speed of 3BL/s ('sedentary' and 'exercise', respectively, A). Zebrafish were then placed in static aquarium water for 12 hours and then CldU for 24 hours. There was no significant difference in the frequency of IdU positive cardiomyocytes between 'sedentary' and 'exercise' zebrafish aged 7 months or 21 months (pre-treatment proliferation rate, B). There was no significant difference in the frequency of CldU positive cardiomyocytes in 7 months old zebrafish between 'sedentary' and 'exercise' zebrafish (post-treatment proliferation rate, C). There was a significant increase in the frequency of CldU positive cardiomyocytes between 21 months old 'sedentary' and 'exercise' zebrafish. Error bars are SEM. Statistical analysis was performed using a Student's T-test. Mean values for each age and intervention are shown in table (D). All data were normally distributed.

4.3.4. *Exercise does not increase cardiomyocyte apoptosis*

Next the rate of apoptosis was analysed to see if exercise could reduce cardiomyocyte apoptosis. This was done using immunofluorescence to detect cleaved caspase 3 in cardiac sections, as described in the previous chapter.

There was no significant difference in the frequency of cardiomyocyte apoptosis between sedentary or exercised zebrafish in either age group. In the 7 months old sedentary zebrafish 0.15% of cardiomyocytes were found to be cleaved caspase 3 positive compared to 0.14% in the exercise group (Figure 4.5). In the 21 months old zebrafish 0.64% and 0.46% of cardiomyocytes were cleaved caspase 3 positive in the sedentary and exercise groups, respectively. This suggests that in zebrafish acute enforced exercise does not reduce cardiomyocyte apoptosis. There appeared to be a difference in cardiomyocyte apoptosis between different aged zebrafish. This is in agreement with what I reported in the previous chapter; cardiomyocyte apoptosis increases as zebrafish age.

(A)

(B)

Age (months)	7	7	21	21
Intervention	Sedentary	Exercise	Sedentary	Exercise
No. of zebrafish	9	9	10	7
MF20+ve	7461	10334	13760	13401
MF20 and cleaved caspase 3+ve	11.56	14.89	87.40	61.43
Cleaved caspase 3 positive cardiomyocytes (%)	0.15	0.14	0.64	0.46
SEM	0.02	0.02	0.13	0.11

Figure 4.5: Exercise does not increase cardiomyocyte apoptosis
There was no significant difference in the frequency of apoptosis between 'sedentary' or 'exercise' zebrafish aged 7 months and 21 months (A). Error bars are SEM. Statistical analysis was performed using a Student's T-test. Mean values for each age and intervention are shown in table (B).

4.3.5. *p38MAPK inhibition does not increase cardiomyocyte proliferation*

As I had previously demonstrated that p38MAPK inhibition could increase cardiomyocyte proliferation in zebrafish I decided to test whether p38MAPK inhibition would have an additive or even synergistic effect on increasing cardiomyocyte proliferation, when administered in conjunction with exercise. In order to do this I firstly decided to test if p38MAPK inhibition has an effect on cardiomyocyte proliferation using a immersing schedule similar to that which was used to exercise zebrafish (Figure 4.6).

Zebrafish aged 7 months and 21 months were immersed in IdU for 24 hours, rested in the rest flume for 3 days, then immersed in aquarium water or 5μM SB203580 and then immersed in CldU for 24 hours prior to euthanizing (Figure 4.6). The sedentary zebrafish from the previous experiment were used as controls for comparison.

There appeared to be no significant difference in IdU positive cardiomyocytes, with or without SB203580, in the 7 months old zebrafish or the 21 months old zebrafish (Figure 4.6). 0.24% of cardiomyocytes were IdU positive in both the sedentary and SB203580 groups of 7 months old zebrafish and 0.43% and 0.35% in the sedentary and SB203580 21 months old zebrafish, respectively. This implies that the basal cardiomyocyte proliferation rate was the same between interventions of zebrafish the same age.

There was no significant difference in the rate of CldU incorporation between interventions in either age group (Figure 4.6). 1.14% and 1.25% of cardiomyocytes were CldU positive in the 7 months old sedentary and SB203580 treated zebrafish, respectively. In the 21 months old zebrafish 2.31% of cardiomyocytes in the sedentary group and 1.25% of cardiomyocytes in the SB203580 group were CldU positive. This implies that p38MAPK inhibition has no effect on cardiomyocyte proliferation in this schedule in either young or old zebrafish. This was surprising as SB203580 treatment had previously increased the frequency of CldU positive cardiomyocytes (Figure 4.1). This suggests that the process of transferring the zebrafish to unfamiliar tanks (the static swim tunnel) is sufficient to induce cardiomyocyte proliferation. This proliferation may be due to the stress of being in an alien environment and appears to mask the increase in cardiomyocyte proliferation expected to be seen from inhibiting p38MAPK when a dose of 5μM SB203580 is used.

(A)

| IdU 24 hours | Rest 72 hours | Aquarium water 12 hours | CldU 24 hours |

| IdU 24 hours | Rest 72 hours | SB203580 12 hours | CldU 24 hours |

(B) IdU positive cardiomyocytes (%)

NS P=0.64   NS P=0.72

Sedentary 7 months | SB203580 7 months | Sedentary 21 months | SB203580 21 months

(C) CldU positive cardiomyocytes (%)

NS P=0.14   NS P=0.14

Sedentary 7 months | SB203580 7 months | Sedentary 21 months | SB203580 21 months

(D)

Age (months)	7	7	21	21
Intervention	Sedentary	SB203580	Sedentary	SB203580
No of zebrafish	9	9	8	9
Mef2+ve	7298	6053	6658	8284
Mef2 and IdU+ve	17.22	14.78	28.63	29.00
IdU positive cardiomyocytes (%)	0.24	0.24	0.43	0.35
SEM	0.02	0.06	0.06	0.05
Mef2 and CldU+ve	83.44	75.78	153.50	103.67
CldU positive cardiomyocytes (%)	1.14	1.25	2.31	1.25
SEM	0.10	0.17	0.69	0.12

Figure 4.6: SB203580 does not increase cardiomyocyte proliferation in zebrafish
Zebrafish were immersed in IdU for 24 hours then rested in the static swim tunnel for 72 hours (A). Zebrafish were then placed in aquarium water or 5μM SB203580 for 12 hours ('sedentary' and 'SB203580', respectively) and then CldU for 24 hours. There was no significant difference in the frequency of IdU positive cardiomyocytes between 'sedentary' and 'SB203580' zebrafish aged 7 months or 21 months (pre-treatment proliferation rate, B). There was no significant difference in the frequency of CldU positive cardiomyocytes between 'sedentary' and 'SB203580' zebrafish (post-treatment proliferation rate, C). Error bars are SEM. Statistical analysis was performed using a Student's T-test. Mean values for each age and intervention are shown in table (D).

4.3.6. p38MAPK inhibition does not increase cardiomyocyte apoptosis

The frequency of cleaved caspase 3 positive cardiomyocytes was then compared in zebrafish aged 7 months and 21 months which had been immersed in either aquarium water or 5μM SB203580. The frequency of cleaved caspase 3 positive cardiomyocytes was 0.15% in 7 months old sedentary zebrafish compared to 0.14% in SB203580 treated zebrafish (Figure 4.7). This was not significantly different. Similarly, the frequency of cleaved caspase 3 positive cardiomyocytes was not significantly different in 21 months old zebrafish that were sedentary or treated with SB203580. 0.64% and 0.55% of cardiomyocytes were cleaved caspase 3 positive in each group, respectively. From this data it can be deduced that p38MAPK inhibition using 5μM SB203580, had no effect on the rate of cardiomyocyte apoptosis in either age group.

(B)

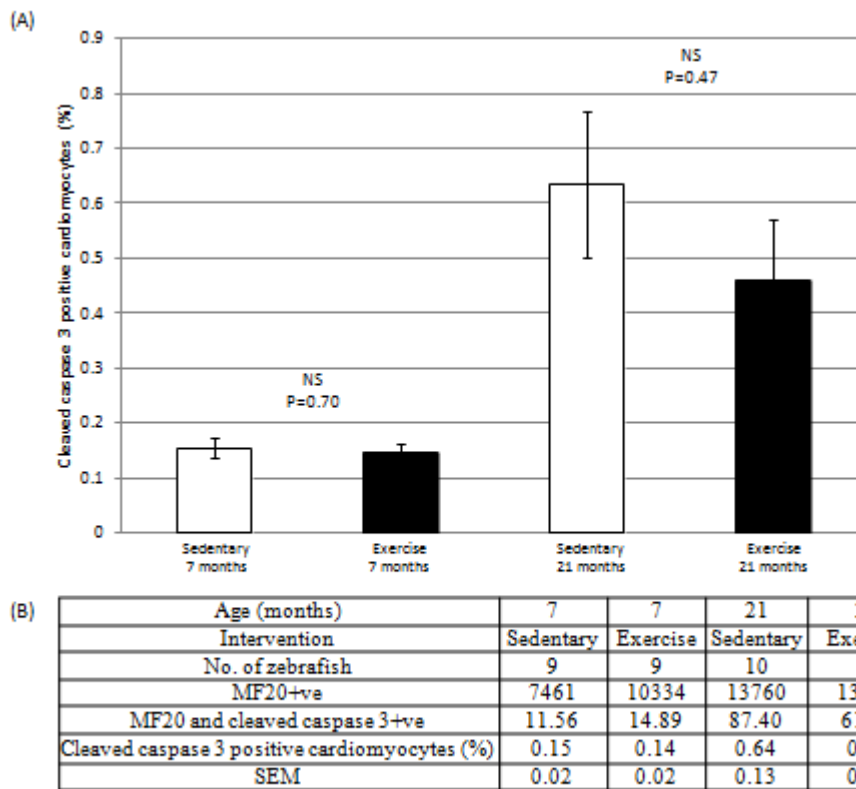| Age (months)	7	7	21	21
Intervention	Sedentary	SB203580	Sedentary	SB203580
No. of zebrafish	9	9	10	9
MF20+ve	7461	8998	13760	12468
MF20 and cleaved caspase 3+ve	11.56	12.22	87.40	69.11
Cleaved caspase 3 positive cardiomyocytes (%)	0.15	0.14	0.64	0.55
SEM	0.02	0.02	0.13	0.06

Figure 4.7: SB203580 does not increase cardiomyocyte apoptosis
There was no significant difference in the frequency of apoptosis between 'sedentary' or 'SB203580' zebrafish aged 7 months and 21 months (A). Error bars are SEM. Statistical analysis was performed using a Student's T-test. Mean values for each age and intervention are shown in table (B).

4.3.7. Exercise and p38MAPK inhibition does not have an additive effect on cardiomyocyte proliferation in zebrafish

As p38MAPK inhibition was shown in cultured cardiomyocytes and in rat hearts to have an additive effect on cardiomyocyte proliferation in conjunction with FGF administration, (Engel *et al.,* 2005; Engel *et al.,* 2006), I next sought to discover if p38MAPK inhibition would have similar additive, or even synergistic, effect on cardiomyocyte proliferation in conjunction with exercise.

7 months old and 21 months old zebrafish were immersed in IdU for 24 hours, exercised at a speed of 3BL/s for three days then immersed in 5μM SB203580 for 12 hours and then immersed in CldU for 24 hours prior to dissection (Figure 4.8). I have labelled this experimental group of zebrafish 'exercise and SB203580'. To see if SB203580 and exercise increases cardiomyocyte proliferation more than exercise alone I compared the 'exercise and SB203580' zebrafish to the 'exercise' zebrafish from previous experiments (Figure 4.4).

In zebrafish aged 7 months there was no significant difference in the incidence of IdU or CldU positive cardiomyocytes when subjected to exercise alone or exercise and 5μM SB203580 (Figure 4.8). 0.73% and 0.30% of cardiomyocytes were IdU positive and 1.14% and 1.25% were CldU positive in the 'exercise' group and 'exercise and SB203580' group respectively. This implies that the cardiomyocyte proliferation rate in these zebrafish was not increased by inhibiting p38MAPK in addition to exercise.

The same was true of zebrafish aged 21 months old; there was no significant difference in thymidine analogue incorporation in cardiomyocytes suggesting no change in proliferation between the two interventions. 0.58% and 0.48% of cardiomyocytes were IdU positive and 9.46% and 7.08% CldU positive in the 'exercise' group and 'exercise and SB203580' group respectively. This demonstrates that inhibiting p38MAPK does not cause an additional increase to cardiomyocyte proliferation when carried out in conjunction with exercise.

(A)

| IdU 24 hours | Exercise 72 hours | Aquarium water 12 hours | CldU 24 hours |

(B)

| IdU 24 hours | Exercise 72 hours | SB203580 12 hours | CldU 24 hours |

(B)

(C)

(D)

Age (months)	7	7	21	21
Intervention	Exercise	Exercise + SB203580	Exercise	Exercise + SB203580
No of zebrafish	10	10	8	8
Mef2+ve	7963	5084	6409	9602
Mef2 and IdU+ve	58.40	15.50	37.00	45.75
IdU positive cardiomyocytes (%)	0.73	0.30	0.58	0.48
SEM	0.16	0.03	0.06	0.06
Mef2 and CldU+ve	98.90	61.30	606.25	680.13
CldU positive cardiomyocytes (%)	1.24	1.21	9.46	7.08
SEM	0.17	0.15	4.19	0.95

Figure 4.8:Exercise and p38MAPK inhibition does not have an additive affect on cardiomyocyte proliferation in zebrafish

Zebrafish were immersed in IdU for 24 hours then exercised in the swim tunnel at a speed of 3BL/s for 72 hours (A). Zebrafish were then placed in aquarium water or 5μM SB203580 for 12 hours ('exercise' and 'exercise and SB203580', respectively) and then CldU for 24 hours. There was no significant difference in the frequency of IdU positive cardiomyocytes between 'exercise' and 'exercise and SB203580' zebrafish aged 7 months or 21 months (pre-treatment proliferation rate, B). There was no significant difference in the frequency of CldU positive cardiomyocytes between 'exercise' and 'exercise and SB203580' zebrafish (post-treatment proliferation rate, C). Error bars are SEM. Statistical analysis was performed using a Student's T-test. Mean values for each age and intervention are shown in table (D).

4.3.8. Exercise and p38MAPK inhibition does not increase cardiomyocyte apoptosis

There also appears to be no difference in the amount of cardiomyocyte apoptosis when comparing exercise alone to exercise and p38MAPK; no significant difference was observed in the percentage of cleaved caspase 3 positive cardiomyocyte between zebrafish that were exercised or exercised and treated with 5µM SB203580 in neither the 7 months old zebrafish, nor the 21 months old zebrafish (Figure 4.9).

(A)

(B)

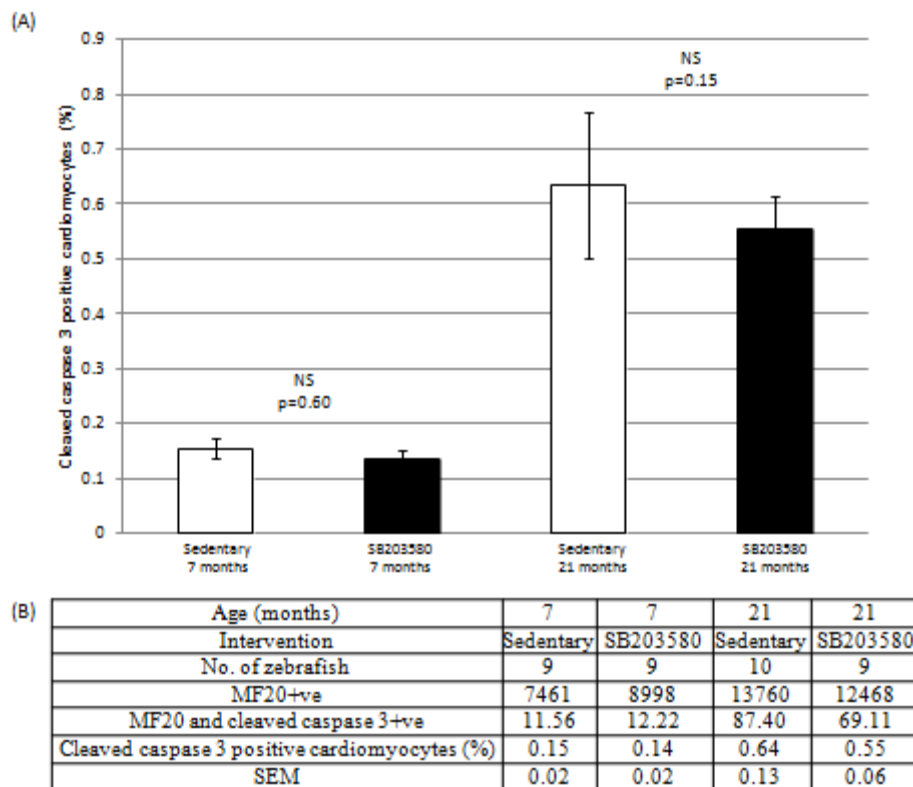| Age (months)	7	7	21	21
Intervention	Exercise	Exercise and SB203580	Exercise	Exercise and SB203580
No. of zebrafish	9	10	7	7
MF20+ve	10334	9194	13401	8829
MF20 and cleaved caspase 3+ve	14.89	16.40	61.43	44.00
Cleaved caspase 3 positive cardiomyocytes (%)	0.14	0.18	0.46	0.50
SEM	0.02	0.01	0.11	0.05
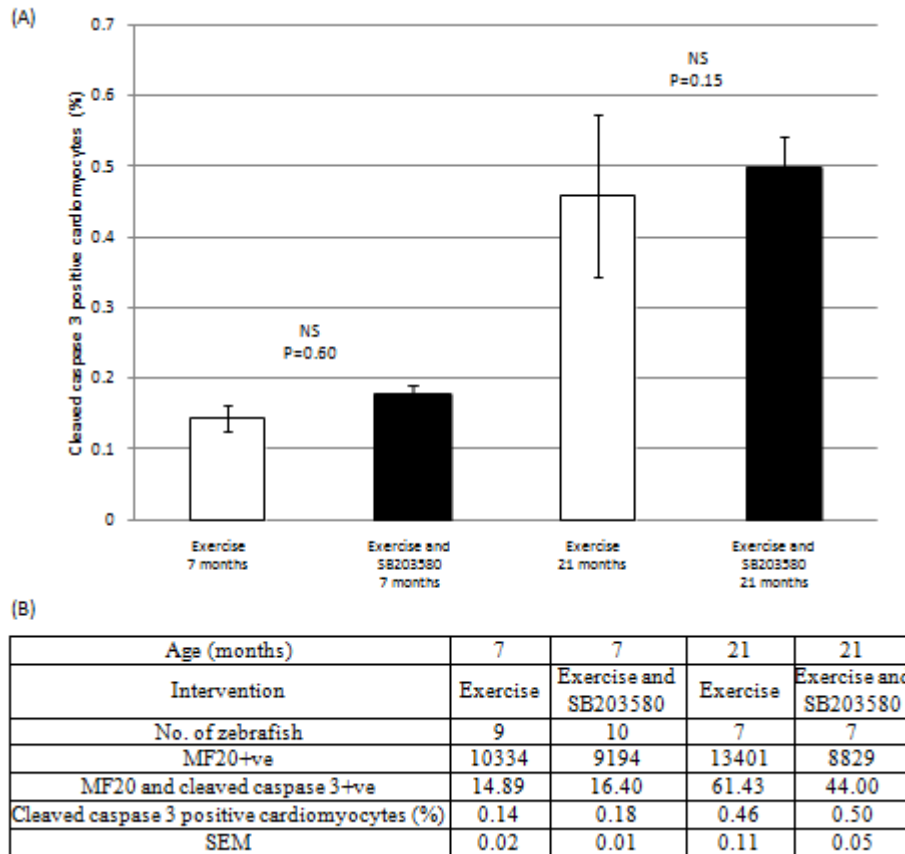
Figure 4.9:Exercise and SB203580 does not increase cardiomyocyte apoptosis
There was no significant difference in the frequency of apoptosis between 'exercise' or 'exercise and SB203580' zebrafish aged 7 months and 21 months (A). Error bars are SEM. Statistical analysis was performed using a Student's T-test. Mean values for each age and intervention are shown in table (B).

4.3.9. Changes in cardiac morphology due to exercise

As acute enforced exercise increased cardiomyocyte proliferation the next aim was to see if this burst of increased proliferation was sufficient to reverse the potentially pathological remodelling I have previously observed in the aged zebrafish heart.

Mason's trichrome staining of hearts from sedentary and exercised zebrafish aged 21 months showed that non-fibrotic (Figure 4.10A) and fibrotic hearts (Figure 4.10B) occurred at a similar frequency; 1 in 9 hearts versus 1 in 10 hearts had cardiac fibrosis in sedentary and exercised zebrafish, respectively (Figure 4.10C). This suggests that acute exercise had no effect at reducing or preventing cardiac fibrosis. This is understandable as I would not expect to observe changes in fibrosis after three days of treatment. The sedentary group of zebrafish used here were included in analysis of fibrosis in different aged zebrafish in the previous chapter.

Hearts were then scored for adipose tissue deposits in 21 months old zebrafish, using the same technique that I developed in the previous chapter. There was significantly less adipose tissue in hearts from zebrafish that had been exercised than the sedentary zebrafish (Figure 4.11A). There appeared to be no difference in ventricular wall thickness between sedentary zebrafish and exercised zebrafish aged 21 months (Figure 4.11B). The lumen area as a percentage of total ventricular volume was also not significantly changed due to exercise in 21 months old zebrafish (Figure 4.11C). However the cross sectional area of cardiomyocytes on sections appeared significantly larger in 23 months old zebrafish that had been exercised than sedentary zebrafish (Figure 4.11D), suggesting that cardiomyocyte hypertrophy may occur in response to exercise.

Overall, there were subtle morphological changes in the aged zebrafish heart in response to acute enforced exercise. It may be that if the duration of exercise was longer then there may have been more long term changes to remodelling. A longer period of exercise would allow more cardiomyocyte proliferation to occur and the new cardiomyocytes may stop reactive fibrosis occurring as there may be a reduction in the net number of cardiomyocytes.
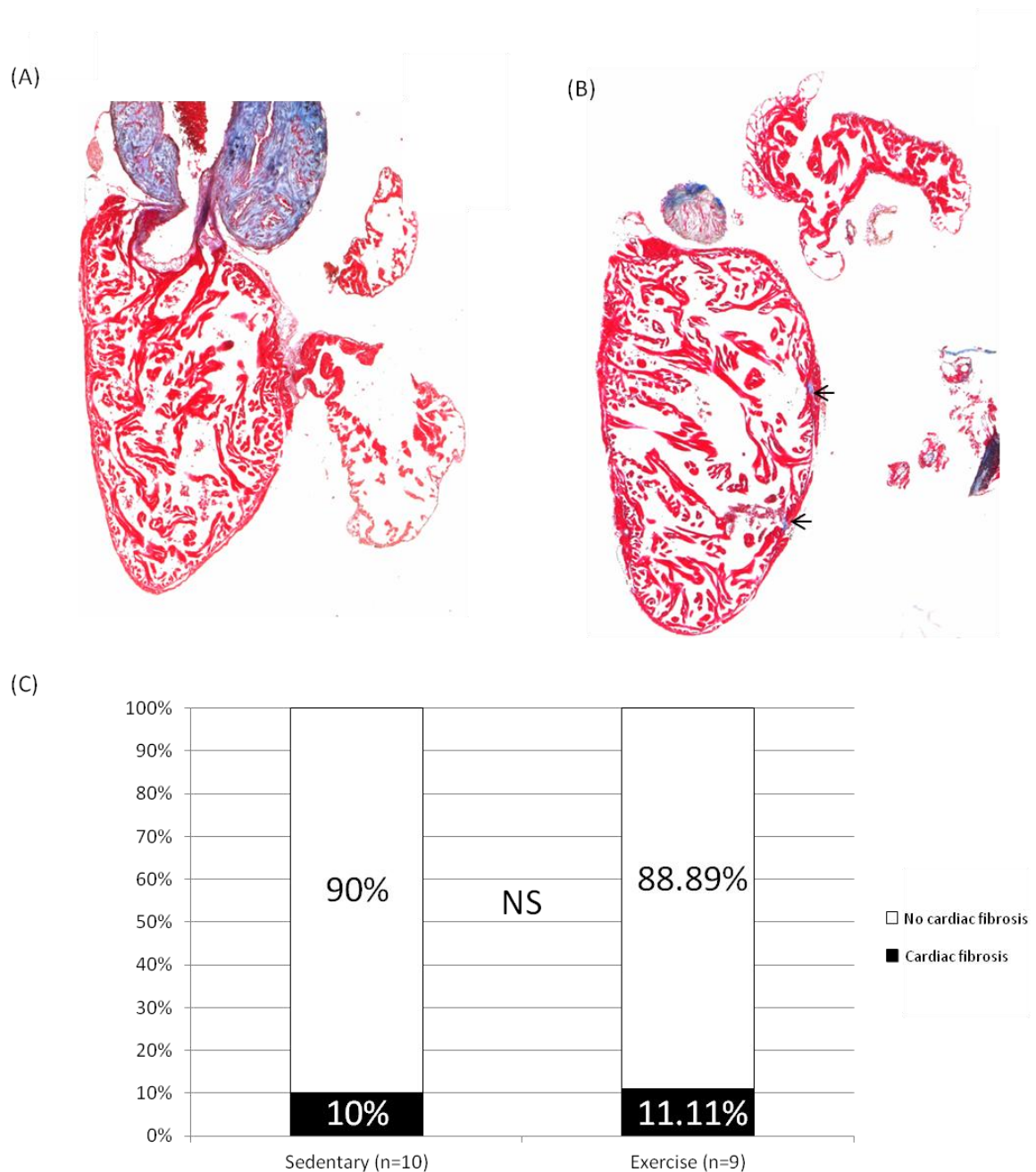
Figure 4.10:Acute exercise does not reduce the incidence of cardiac fibrosis in 21 months old zebrafish hearts

The increased frequency of cardiomyocyte proliferation in 21 months old zebrafish hearts in response to acute exercise did not reduce the incidence of cardiac fibrosis. 9/10 21 months old 'exercise' hearts did not have cardiac fibrosis (A), one heart did (B). There was no significant difference in the frequency of cardiac fibrosis between 'sedentary' and 'exercise' 21 months old zebrafish. Statistical analysis was performed using a Student's T-test.
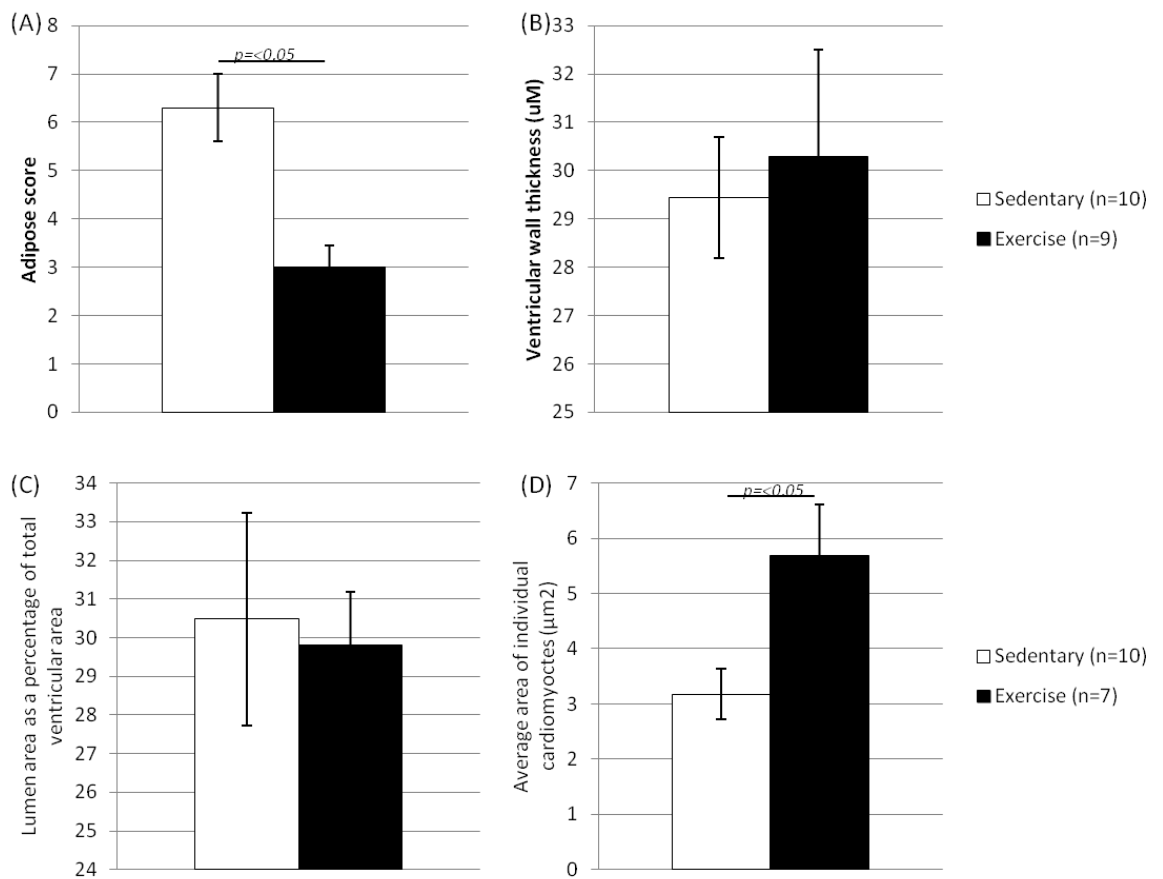
Figure 4.11: Exercise changes some morphological features in the ageing zebrafish heart

Adipose tissue deposits were reduced due to exercise in zebrafish aged 21 months old (A). Ventricular wall thickness did not change due to exercise in zebrafish aged 21 months (B). Lumen area as a percentage of total ventricular area does not change due to exercise in zebrafish aged 21 months (C). The average area of individual cardiomyocytes increases due to exercise in zebrafish aged 21 months (D). Area was calculated as the number of DAPI positive cells in MF20 positive tissue divided by the area of MF20 positive tissue (µm2). Error bars are SEM, significant difference was observed using a student's T-test.

4.3.10. Using RT-qPCR to identify changes in gene expression in zebrafish hearts

Next, I wanted to determine some of the signalling pathways important for the changes I had observed in zebrafish hearts in response to ageing and exercise. The analysis of gene expression is becoming increasingly important within research as the understanding of genes involved in complex signalling pathways and diseases can lead to a greater understanding and identification of potential therapeutic targets. One way of measuring the amount of target genes being transcribed in tissue is real time quantitative PCR (RT-qPCR). RT-qPCR has many advantages over other techniques used to measure transcriptome abundance as small amounts of tissue can be used and the process is fairly automated, allowing for a high throughput, when compared to DNA microarrays and Northern blots, respectively.

In order to successfully perform RT-qPCR various variables need to be accounted for, like the amount of tissue used and variances in the extraction of RNA and production of cDNA. The amount of tissue can be accounted for by using the same number of cells in each RNA extraction, however when using whole organs this can be difficult, particularly when comparing organs from different sized animals. This variable can be controlled by using the same amount of RNA for cDNA production, however, there can be variations in the efficiency of the transcriptase enzyme and the total amount of RNA consists mainly of rRNA meaning the mass may not necessarily be representative of the total mRNA content (Solanas *et al.*, 2001).

The general consensus is that the best way to control variances in cDNA used for RT-qPCR is to analyse the expression of internal control genes, otherwise known as reference genes or housekeeping genes, alongside the analysis of genes of interest. Reference genes should be genes that are constitutively expressed in every cell type in the tissues or cells that are being examined. In many studies reference genes are often used as reference genes due to popularity rather than actually verification in specific tissue types. Various studies have shown that many well accepted 'reference genes' can vary considerably within different tissues or cells or under experimental settings (Ryo *et al.*, 1999; Bustin, 2000; Warrington *et al.*, 2000). It was therefore imperative that a panel of reference genes were tested specifically in the same tissues that were treated under experimental conditions to ensure that there is as little variation as possible.

Deciding which was the most suitable reference gene was a very circular question with no straight forward answer. In order to answer it a panel of genes needed be tested in different samples. Different reference genes are expressed at different levels, largely dependent on the function of the gene. The most practical way to

determine which genes are most stable was to compare the ratios of the reference genes with each other in all of the different samples. To get the most use out of this analysis the samples had to be representative of the breadth of different experimental conditions. A program called geNorm has been developed which can do this automatically from the ΔCt values of the genes in each sample (Vandesompele *et al.*, 2002). geNorm works on the assumption that the variation in the ratio between the most stable reference genes is negligible, despite experimental interventions or cell type. geNorm calculates the control gene stability measure (M) as the pairwise variation between all of the tested genes by comparing the standard deviation of the log expression ratio. Low M values denote a stably expressed gene.

The geometric mean of three genes that are expressed most stably under experimental conditions can be used thereafter as internal controls (Vandesompele *et al.*, 2002). Using only one or two reference genes can lead to erroneous results whereas the benefit of using four is diminished by the disadvantages of an increased work load and requirement of more cDNA, with increasing the amount of reference genes providing little increases in accuracy.

Few studies have been carried out in zebrafish to determine the most suitable reference genes in different tissues and different aged embryos (Tang *et al.*, 2007; McCurley and Callard, 2008; Casadei *et al.*, 2011). Although these studies are very extensive they are limited to development and comparisons between different tissues. Any more specific transcriptional analysis would require the testing of various housekeeping genes under different experimental conditions. I therefore decided it was imperative for me to determine to most stable reference genes in the zebrafish heart under my experimental conditions.

4.3.10.1. *Choosing the most stable reference genes for RT-qPCR*

Nine reference genes were chosen on the basis of their use in literature in zebrafish and other organisms; *β-actin1, β-actin2, β-2-microglobulin, elongation factor 1a, gapdh, rpl13a, s18, sl25a5* and *usp5* (Tang *et al*., 2007; McCurley *et al*., 2008; Casadei *et al*., 2011; Vandesompele *et al*., 2002). I chose to test the housekeeping genes in hearts from three zebrafish in three different experimental conditions; untreated 7 months old wild type, wild type 21 months old that have been exercised and treated with a p38MAPK inhibitor and 6 months old *desma* null zebrafish (other members of the Chaudhry lab were working on desmin). The three experimental situations were chosen as they would be the most varied conditions analysed and using a panel of 9

genes in a total of 9 samples would give the study suitable statistical power (Vandesompele *et al*., 2009). RNA extraction, cDNA synthesis, primer design and qPCR were carried out as described in the methods section.

Primer efficiencies were calculated and melt curves produced using cDNA produced from a pool of wild type hearts via the program LinRegPCR (Figure 4.12). A primer efficiency of above 1.85 was acceptable, all primers were above this efficiency. Melting curves illustrate the primers were specific to desired target as only one clear peak can be observed in each curve (Figure 4.12).

Next, Ct values were converted to comparative Ct values (ΔCt) for all the samples and genes. Using geNorm (Vandesompele *et al*., 2002) the average expression stability (M) was calculated and plotted (Figure 4.13). This shows the most stable genes were *usp5* and *rpl13a*, and the least stable genes, *sl25a5* and *gapdh*. After *usp5* and *rpl13a* the next stable gene was *β-2-microglobulin*. As these three genes were the most stable they were used in subsequent RT-qPCR analysis.

β-actin 1 — Efficiency=1.875 R²=1.000

β-actin 2 — Efficiency=1.879 R²=1.000

s-18 — Efficiency=1.914 R²=1.000

usp5 — Efficiency=1.937 R²=1.000

b2m — Efficiency=1.899 R²=1.000

slc25a5 — Efficiency=1.922 R²=1.000

rpl13a — Efficiency=1.885 R²=1.000

gapdh — Efficiency=1.934 R²=0.999

ef1a — Efficiency=1.924 R²=1.000
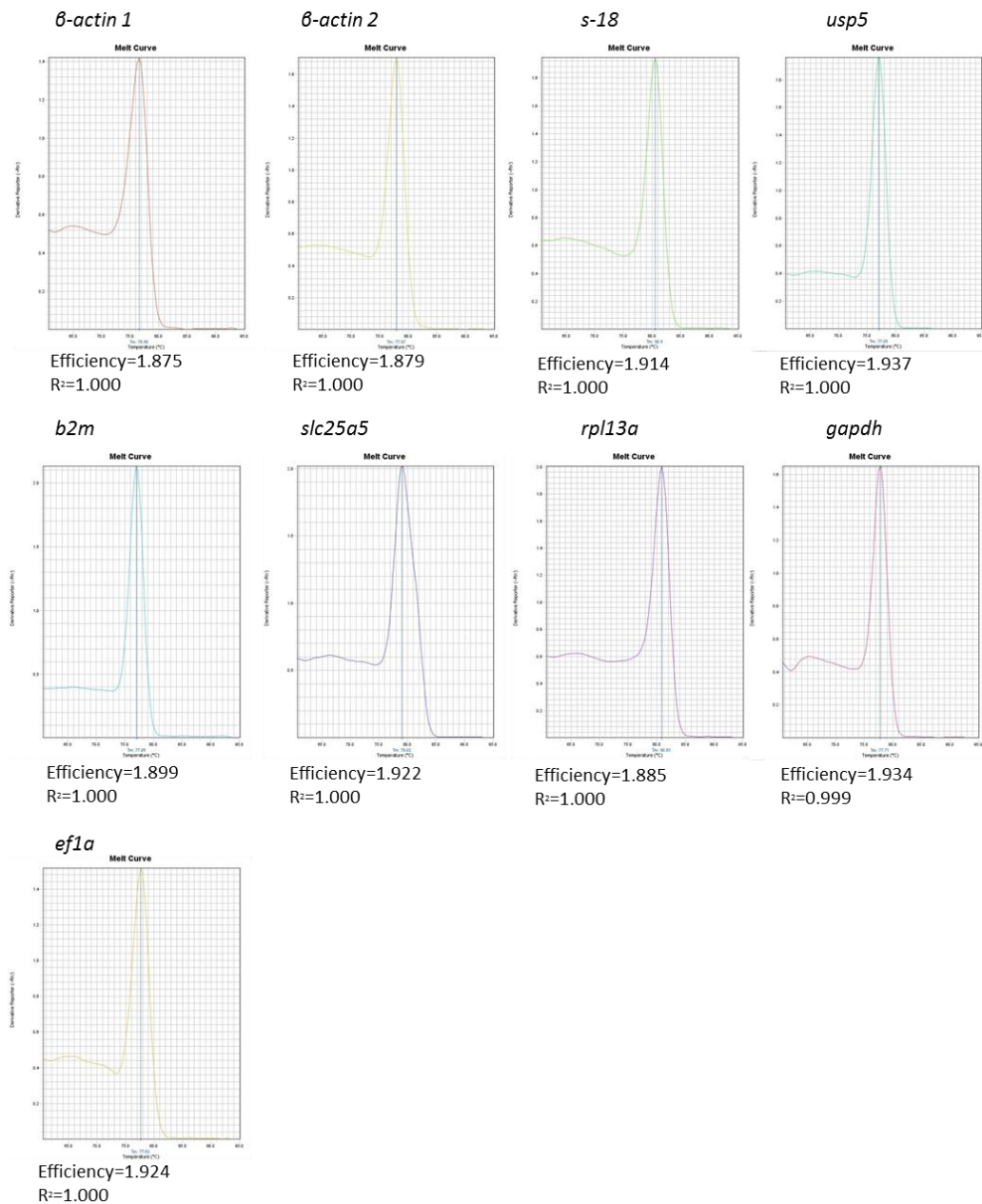
Figure 4.12: Melt curves for potential reference genes
Melt curves, efficiency and R^2 values for all reference genes tested in zebrafish hearts. Melt curves were calculated during the qPCR run using a Taqman 7500 machine and supplied software (SDS), efficiency and R^2 values were calculated *post-hoc* using LinRegPCR.
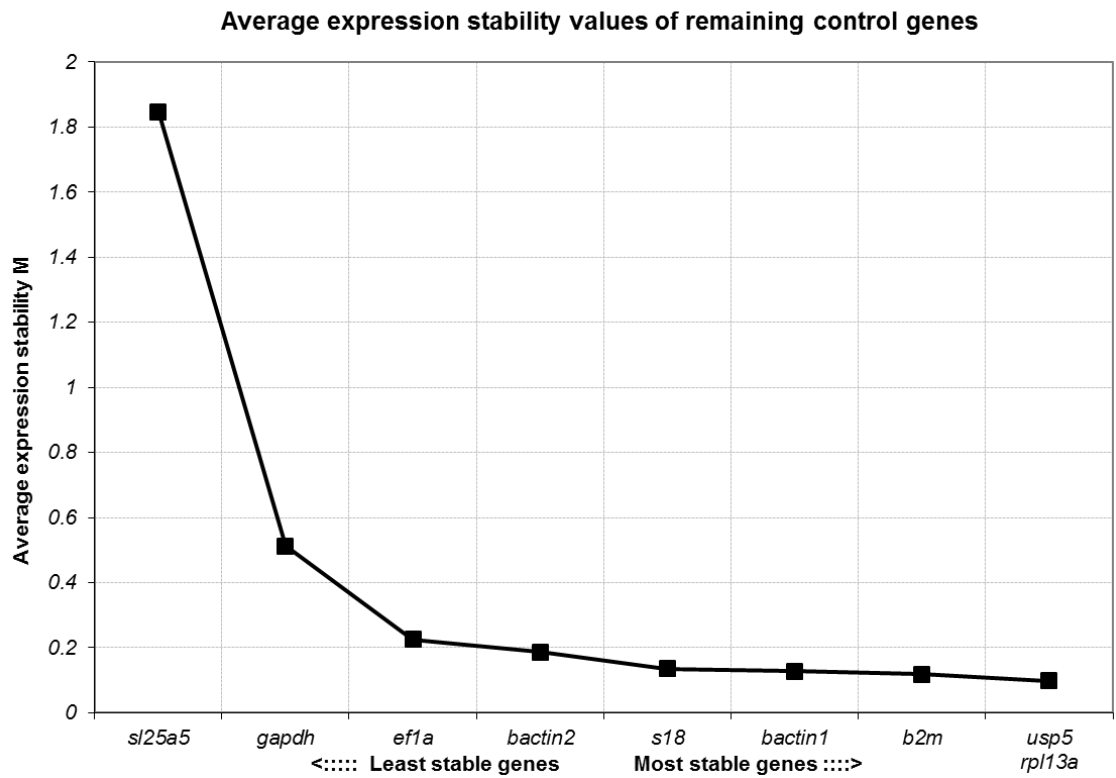
Figure 4.13: Testing the stability of reference genes for RT-qPCR in zebrafish hearts

GeNorm software was used to determine the most stable reference genes in 9 different zebrafish hearts over a variety of different experimental conditions. The most stable genes are then used for subsequent RT-qPCR experiments as reference genes.

4.3.11. Changes in gene expression due to ageing

I first aimed to elucidate potential changes in signalling pathways associated with ageing. To do this I compared four 7 months old zebrafish, and four 21 months old zebrafish. As I next planned to compare how exercise affects these pathways, I treated these zebrafish with the same protocol as the 'sedentary' zebrafish (Figure 4.4). This allowed me to later compare these zebrafish to clutchmates which had experienced the 'exercise' protocol.

4.3.11.1.   *Expression of natriuretic peptides in different aged sedentary zebrafish*

As the fetal gene program is often reactivated during pathological cardiac remodelling fetal gene program I considered these genes prime candidates to study in qPCR analyses of ageing in zebrafish hearts. Atrial natriuretic peptide (ANP, *nppa*) and brain natriuretic peptide (BNP, *nppb*) were analysed first as ANP and BNP have been associated with age and disease related cardiac hypertrophy (Younes *et al.,* 1995) and levels in the plasma and cardiac secretion amplitude is increased with ageing (Charloux *et al.,* 2008).

    The relative expression of *nppa* and *nppb* was compared between zebrafish aged 7 months and 21 months. Levels of *nppa* and *nppb* expression were 2.5 and 3.5 fold significantly higher in 21 months old zebrafish compared to 7 months old zebrafish, respectively (

Figure **4.14**). Similar observations have been made in human and mammalian models of cardiac pathology (Gardner, 2003; Richards, 2007; Wang *et al.*, 2003; Mori *et al.*, 2004).

Figure 4.14: Expression of natriuretic peptides was higher in older zebrafish hearts

The expression of *nppa* (A) and *nppb* (B) in the heart was significantly increased in 21 months old zebrafish when compared to 7 months old zebrafish. Error bars are SEM. Statistical analysis was performed using a student's t-test.

4.3.11.2. *Expression of cardiac specific transcription factors in different aged sedentary zebrafish*

Next, I wanted to see if the cardiac specific transcription factors, a subset of the fetal gene program, were affected by cardiac ageing. The expression of cardiac specific transcription factors such as *gata4* and *mef2c* are essential for the differentiation of newly proliferated cardiomyocytes and are part of the fetal gene program which are a set of genes highly expressed in the fetal heart and then redeployed in the ageing adult heart (Oka *et al.*, 2007b; Barry *et al.*, 2008).

There appeared to be no significant changes in the expression of *gata4*, *mef2c*, *nkx2.5*, *tbx5a* or *tbx5b* between 7 and 21 months old zebrafish (Figure 4.15). This may be because in the context of ageing and pathology transcription factors are mainly involved in hypertrophy (Oka *et al.*, 2007a; Barry *et al.*, 2008). I demonstrated in the previous chapter that cardiomyocyte hypertrophy was not a feature of ageing in zebrafish cardiomyocytes.
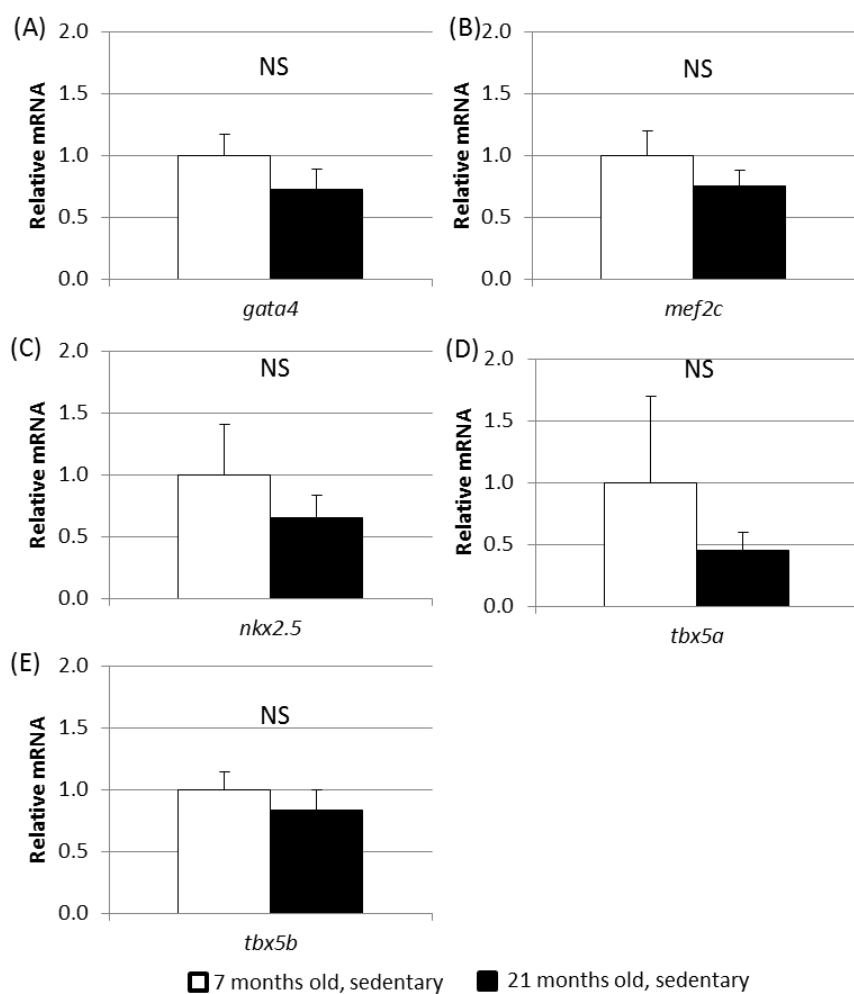
Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	p-value
7	Sedentary	4	gata4	1.00	0.34	0.17	0.34
21	Sedentary	4		0.72	0.33	0.16	
7	Sedentary	4	mef2c	1.00	0.40	0.20	0.29
21	Sedentary	4		0.75	0.27	0.14	
7	Sedentary	3	nkx2.5	1.00	0.70	0.40	0.19
21	Sedentary	4		0.65	0.35	0.18	
7	Sedentary	3	tbx5a	1.00	1.39	0.70	0.06
21	Sedentary	4		0.45	0.30	0.15	
7	Sedentary	4	tbx5b	1.00	0.29	0.15	0.48
21	Sedentary	4		0.83	0.34	0.17	

Figure 4.15: Expression of cardiac specific transcription factors was the same in hearts from different aged zebrafish
The expression of *gata4* (A), *mef2c* (B), *nkx2.5* (C), *tbx5a* (D) and *tbx5b* (E) was not significantly different in hearts of zebrafish aged 7 months old compared to 21 months old. Error bars are SEM. Statistical analysis was performed using a student's t-test.

4.3.11.3. *Expression of genes associated with epicardial activation in different aged sedentary zebrafish*

When zebrafish are moved from densely stocked aquariums to less densely stocked aquariums they experience an increase in cardiomyocyte proliferation and an increase in cardiac growth (Wills *et al.*, 2008). Epicardial activation was demonstrated to be an important mediator of this growth. In response to physical trauma to the ventricle, the zebrafish epicardium also becomes activated (Lepilina *et al.*, 2006; Kikuchi *et al.*, 2011b; Schnabel *et al.*, 2011). I therefore sought to determine if epicardial activation plays a central role in cardiac ageing in zebrafish. To do this I compared the expression levels of *aldh2* (the zebrafish orthologue of *RALDH2)* and *gata5,* two genes associated with epicardial activation (Lepilina *et al.*, 2006; Pérez-Pomares and de la Pompa, 2011), between 7 months old and 21 months old zebrafish. There appeared to be no significant difference in the expression of either *raldh2* or *gata5* between 7 months and 21 months old zebrafish (Figure 4.16). This suggests that epicardial activation does not play a pivotal role in ageing. The pathological signalling pathways associated with ageing are therefore distinct to that of physical trauma in the zebrafish heart.

(C)	Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	*p*-value
	7	Sedentary	4	*aldh2*	1	0.34	0.17	0.18
	21	Sedentary	3		1.51	0.81	0.47	
	7	Sedentary	3	*gata5*	1	0.68	0.39	0.90
	21	Sedentary	4		1.06	0.41	0.21	
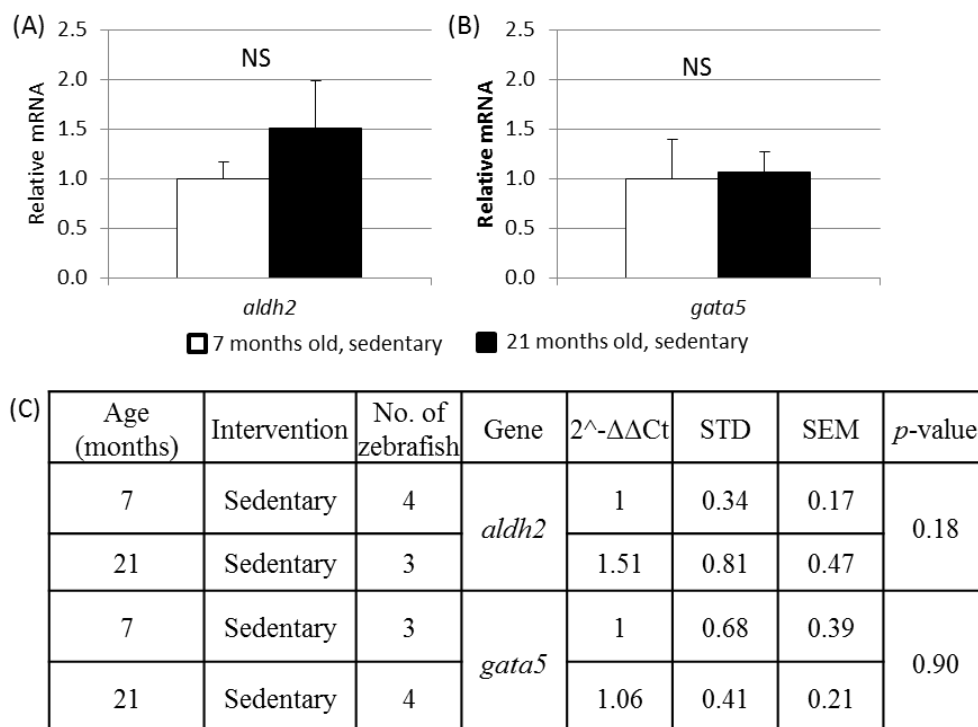
Figure 4.16: Expression of genes associated with epicardial activation was the same in hearts from different aged zebrafish
The expression of *aldh2* (A) and *gata5* (B) was not significantly different in hearts of zebrafish aged 7 months old compared to 21 months old. Error bars are SEM. Statistical analysis was performed using a student's t-test.

4.3.12. Changes in gene expression due to exercise

As I have previously shown in this chapter that exercise increases cardiomyocyte proliferation in 21 months old zebrafish I wanted to use RT-qPCR to identify a potential mechanism. To do this I wanted to determine if the previously used panel of genes were affected by exercise. I treated 4 zebrafish that were the same age (7 months and 21 months old) and clutchmates to those used in the previous set of RT-qPCR to the exercise protocol I have previously used (Figure 4.4).

4.3.12.1. Expression of natriuretic peptides after exercise

As expression of natriuretic peptides was increased in 21 months old zebrafish compared to 7 months old zebrafish, I firstly wanted to determine if exercise could alter the expression of natriuretic peptides. Exercise has been shown to reduce the expression of natriuretic peptides in humans and other models and may be a mechanism of exercise exerting beneficial effects in the heart.

There appeared to be no significant difference in the expression of *nppa* or *nppb* between the 7 months old sedentary and exercise zebrafish (Figure 4.18). In 21 months old zebrafish the exercise group of zebrafish had a statistically significant 0.01 fold reduction in *nppa* expression when compared to sedentary zebrafish of the same age (Figure 4.17). The expression of *nppb* was not significantly different between the exercise and sedentary zebrafish (Figure 4.17). This suggests that a reduction in *nppa* may be important in increasing cardiomyocyte proliferation or may be a secondary effect of increased proliferation. It may be that a reduction in *nppa* expression in response to exercise is an age specific response. This could be because *nppa* expression is not elevated in untreated younger zebrafish hearts, whereas expression is elevated in older untreated hearts.

(C)

Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	p-value
21	Sedentary	4	anp	1.00	0.87	0.44	0.00
21	Exercise	4	anp	0.01	0.00	0.00	0.00
21	Sedentary	4	bnp	1.00	0.44	0.22	0.07
21	Exercise	4	bnp	2.19	1.14	0.66	0.07
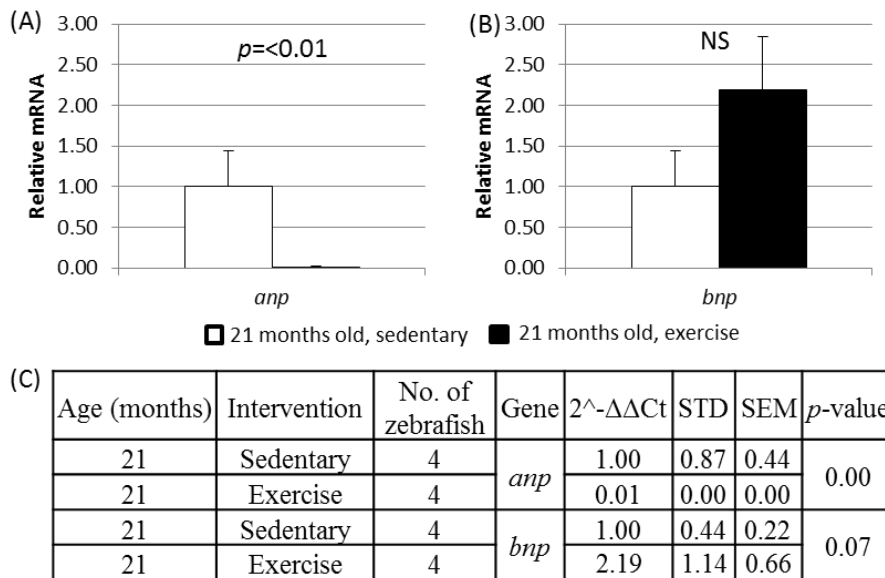
Figure 4.17: Expression of *nppa*, but not *nppb*, was reduced due to exercise in 21 months old zebrafish hearts

The expression of *nppa* (A) but not *nppb* (B) was significantly different in hearts of sedentary zebrafish aged 21 months old compared to hearts of exercised 21 months old zebrafish. Error bars are SEM. Statistical analysis was performed using a student's t-test.

(B)

Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	p-value
7	Sedentary	4	anp	1.00	0.59	0.29	0.59
7	Exercise	4	anp	0.83	0.35	0.18	0.59
7	Sedentary	4	bnp	1	0.65	0.32	0.09
7	Exercise	4	bnp	0.86	0.33	0.16	0.09

Figure 4.18: Expression of natriuretic peptides did not change due to exercise in 7 months old zebrafish hearts

The expression of *nppa* (A) and *nppb* (B) was not significantly different in hearts of sedentary zebrafish aged 7 months old compared to hearts of exercised 7 months old zebrafish. Error bars are SEM. Statistical analysis was performed using a student's t-test.

4.3.12.2. *Expression of cardiac specific transcription factors after exercise*

When comparing expression levels of these cardiac specific transcription factors in sedentary 7 months old zebrafish to exercised 7 months old zebrafish there was no significant difference (Figure 4.21).

Next I wanted to examine if expression of cardiac specific transcription factors is linked with the observed increase in cardiomyocyte proliferation when 21 months old zebrafish are exercised. In 21 months old zebrafish that had been exercised there was a statistically significant 39, 618, 16, 6 and 6 fold increase in the expression of *gata4*, *mef2c*, *nkx2.5*, *tbx5a* and *tbx5b* respectively, compared to sedentary 21 months old zebrafish (Figure 4.22).

(F)	Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	p-value
	7	Sedentary	4	gata4	1.00	0.34	0.17	0.30
	7	Exercise	4		0.42	0.42	0.21	
	7	Sedentary	4	mef2c	1.00	0.40	0.20	0.59
	7	Exercise	4		0.53	0.53	0.26	
	7	Sedentary	4	nkx2.5	1.00	0.70	0.40	0.16
	7	Exercise	4		0.16	0.16	0.08	
	7	Sedentary	4	tbx5a	1.00	1.39	0.70	0.70
	7	Exercise	4		1.04	1.04	0.52	
	7	Sedentary	4	tbx5b	1.00	0.29	0.15	0.83
	7	Exercise	4		1.17	1.17	0.58	

Figure 4.19: Expression of cardiac specific transcription factors did not change due to exercise in 7 months old zebrafish hearts

The expression of *gata4* (A), *mef2c* (B), *nkx2.5* (C), *tbx5a* (D) and *tbx5b* (E) was not significantly different in hearts of sedentary zebrafish aged 7 months old compared to hearts of exercised 7 months old zebrafish. Error bars are SEM. Statistical analysis was performed using a student's t-test.
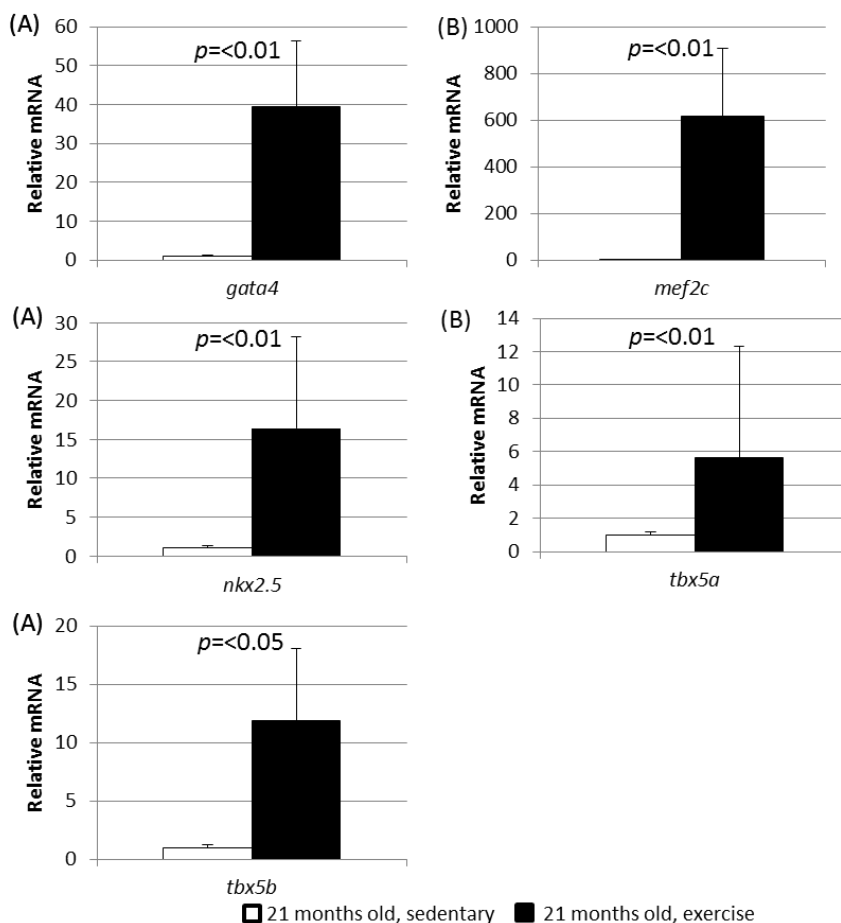
(B) Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	*p*-value
21	Sedentary	4	gata4	1.00	0.45	0.23	0.00
21	Exercise	4		39.34	34.05	17.03	
21	Sedentary	4	mef2c	1.00	0.36	0.18	0.00
21	Exercise	4		617.73	579.20	289.60	
21	Sedentary	4	nkx2.5	1.00	0.54	0.27	0.01
21	Exercise	4		16.44	23.44	11.72	
21	Sedentary	4	tbx5a	1.00	0.66	0.33	0.01
21	Exercise	4		5.62	13.45	6.73	
21	Sedentary	4	tbx5b	1.00	0.41	0.20	0.04
21	Exercise	4		11.86	12.30	6.15	

Figure 4.20: Expression of cardiac specific transcription factors was increased due to exercise in 21 months old zebrafish hearts

The expression of *gata4* (A), *mef2c* (B), *nkx2.5* (C), *tbx5a* (D) and *tbx5b* (E) was significantly increased in hearts of exercised zebrafish aged 21 months old compared to hearts of sedentary 21 months old zebrafish. Error bars are SEM. Statistical analysis was performed using a student's t-test.
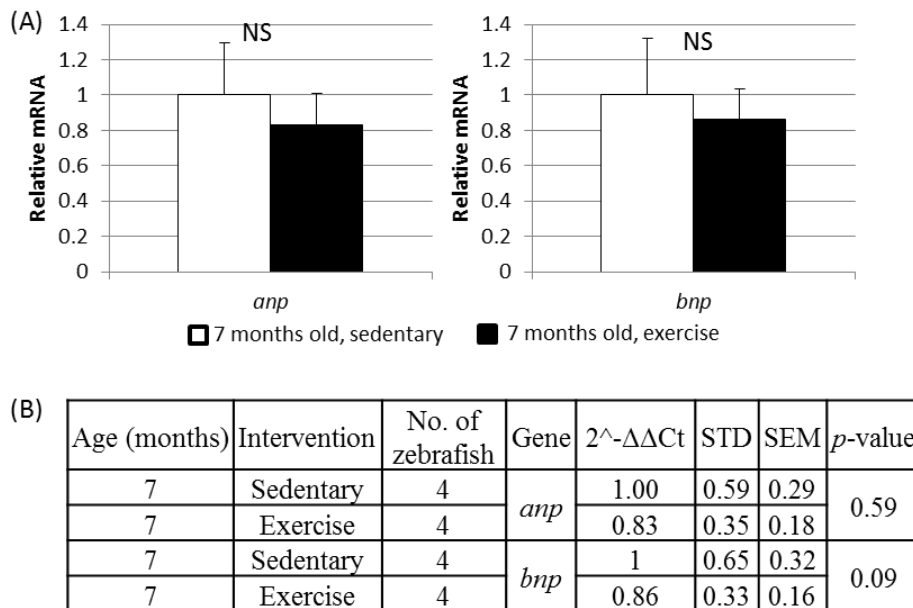
4.3.12.3. Expression of genes associated with epicardial activation after exercise

I then wanted to see if epicardial activation instigated the increased proliferation of cardiomyocytes in response to exercise. There was no significant difference in the expression of genes associated with epicardial activation in 7 months old zebrafish that were sedentary or exercised (Figure 4.21). Although there was no significant difference there looks like there may actually be an increase in epicardial signalling. There was also no significant difference in the expression of *aldh2* and *gata5* after exercise in 21 months old zebrafish when compared to 21 months old sedentary zebrafish (Figure 4.22). These results suggest that the increase in cardiomyocyte proliferation in elderly zebrafish occurs independently of epicardial activation.

(C)	Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	*p*-value
	7	Sedentary	4	*aldh2*	1	0.34	0.17	0.06
	7	Exercise	4		3.2	3.84	1.92	
	7	Sedentary	3	*gata5*	1	0.68	0.39	0.30
	7	Exercise	4		5.53	5.53	2.76	
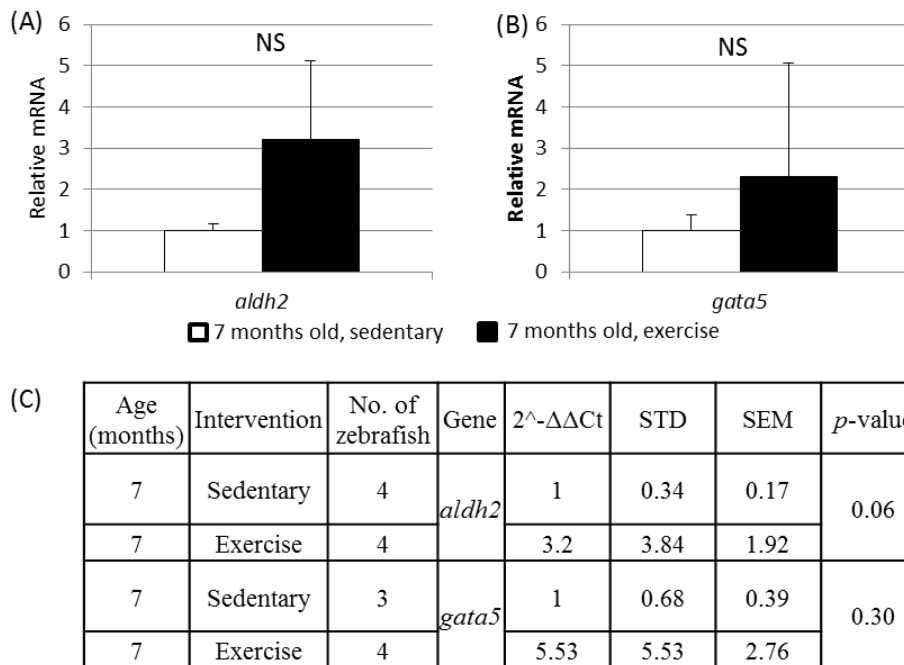
Figure 4.21: Expression of genes associated with epicardial activation did not change due to exercise in 7 months old zebrafish hearts
The expression of *aldh2* (A) and *gata5* (B) was not significantly different in hearts of sedentary zebrafish aged 7 months old compared to hearts of exercised 7 months old zebrafish. Error bars are SEM. Statistical analysis was performed using a student's t-test.
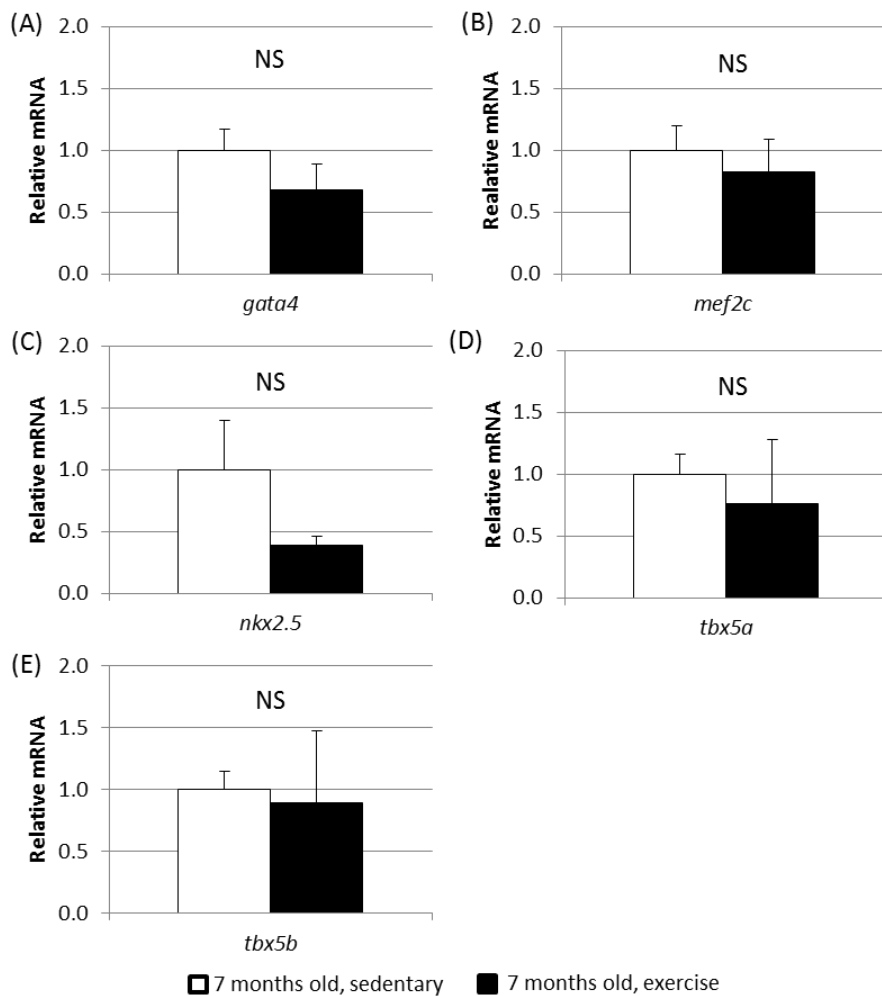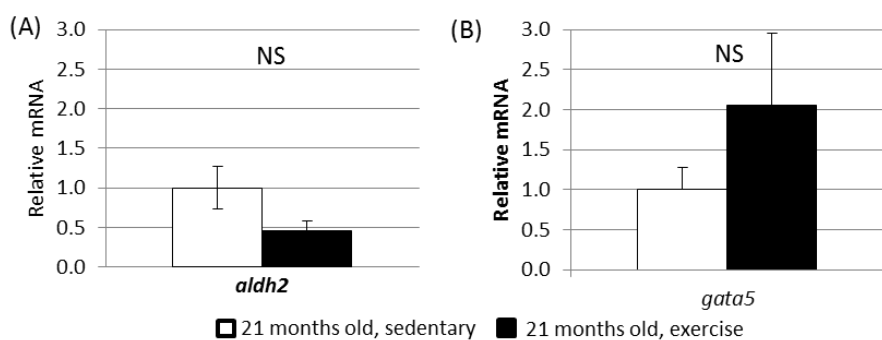

Age (months)	Intervention	No. of zebrafish	Gene	2^-ΔΔCt	STD	SEM	p-value
21	Sedentary	4	aldh2	1.00	0.53	0.27	0.67
21	Exercise	4		0.46	0.25	0.12	
21	Sedentary	4	gata5	1.00	0.39	0.19	0.19
21	Exercise	4		2.05	1.80	0.90	

Figure 4.22: Expression of genes associated with epicardial activation did not change due to exercise in 21 months old zebrafish hearts
The expression of *aldh2* (A) and *gata5* (B) was not significantly different in hearts of sedentary zebrafish aged 21 months old compared to hearts of exercised 21 months old zebrafish. Error bars are SEM. Statistical analysis was performed using a student's t-test.

4.4. Discussion

4.4.1. *Exercise in the zebrafish heart*

In mammals it has been demonstrated that exercise can have many positive effects on the heart (Ellison *et al.*, 2012), including pro-proliferative and anti-apoptotic effects (Kwak *et al.*, 2006; Boström *et al.*, 2010). I decided to test the hypothesis that exercise may increase cardiomyocyte proliferation and/or reduce apoptosis, increasing the amount of cardiomyocytes in the zebrafish heart, reversing some of the pathological changes I discovered to be present in ageing zebrafish hearts. In elderly zebrafish hearts it did indeed appear true that exercise increased cardiomyocyte proliferation but did not reduce apoptosis. This would be expected to result in a slower rate of decline in the population of cardiomyocytes due to ageing. Many cardiac pathologies that occur due to ageing are the result of a net loss of cardiomyocytes. My findings therefore suggests that exercise may be a way of preventing heart disease in the elderly by slowing down the age associated decrease in cardiomyocytes within the heart.

In the previous chapter I demonstrated that cardiomyocyte proliferation remains at a similar level from the age of 7 months to 23 months in zebrafish. I also demonstrated that there is a drastic increase in cardiomyocyte apoptosis between the ages of 15 months and 23 months. This would cause a net decrease in the population of

cardiomyocytes in the zebrafish heart. Using data from the previous chapter, regarding cardiomyocyte proliferation and apoptosis, I have produced a speculative model of how exercise may positively affect the turnover of cardiomyocytes in the zebrafish heart (Figure 4.23). In this model a reduction in the number of cardiomyocytes does not occur until around 35 months when exercise is maintained. This is the point I have predicted where cardiomyocyte apoptosis outstrips proliferation. Of course there are many caveats of this model. Firstly, it assumes cardiomyocyte proliferation remains high. This may not happen as the rate of proliferation may decrease as the heart adapts to be more comfortable with the increased workload. This increase in proliferation would increase the number of cardiomyocytes in the heart. This would mean more cardiomyocytes would need to be lost for signs of cardiac pathology to be present. This assumes there is a critical number of cardiomyocytes, below which remodelling occurs. The model also assumes cardiomyocyte apoptosis will continue to increase at the same rate. Again this may not be true. Cardiomyocyte apoptosis may increase more rapidly in extreme old age or may even slow. Overall this model is intended to give a flavour of what may be occurring in terms of cardiomyocyte turnover in response to ageing and exercise.

In a study using a swim training protocol in mice as exercise stimuli, the amount of PHH3 positive cardiomyocytes was approximately 8 fold higher in exercised mice compared to sedentary controls (Boström *et al.*, 2010). In the 21 months old zebrafish cardiomyocyte proliferation was approximately 4 fold higher in exercised zebrafish when compared to sedentary controls. Although different exercise protocols and markers of proliferation were utilised between my experiments and this data, this demonstrates that the increase in cardiomyocyte proliferation in zebrafish is not too dissimilar to that of mice. In my experiments I have used three days of constant swimming using thymidine analogues to determine proliferation, compared to a daily swim protocol using PHH3 as a marker of proliferation. This further validates the use of zebrafish as a model of cardiac disease.

The average area of cardiomyocytes on sections increased in 21 months old zebrafish in response to exercise (Figure 4.11), this is suggestive of cardiomyocyte hypertrophy. Although this technique has been reported in the literature (Jean *et al.*, 2012) a better way to determine if cardiomyocyte hypertrophy has occurred may have been to use a haemocytometer. This would accurately give the volume of cardiomyocytes. This was not done as there were not enough zebrafish hearts to process in this way in the time that I had. Another way to accurately determine if

cardiomyocyte hypertrophy has occurred would have been to stain the cardiac sections for a cardiomyocyte specific marker, such as mef2 or MF20, and also a membrane specific marker, such as laminin or wheat germ agglutinin. The area of cardiomyocytes could then be measured. This technique would also rule out the possibility of binucleation occurring, although this has never been reported in zebrafish hearts. Cardiomyocyte hypertrophy appeared to occur in response to exercise in elderly zebrafish, which is in agreement with the physiological hypertrophy which has been observed in human and murine hearts in response to exercise (Boström *et al.*, 2010; Ellison *et al.*, 2012).

Other changes in cardiac morphology were not observed such as a reduction in cardiac fibrosis, changes in lumen area, or a reversal of ventricular wall thickening. However, I would not expect these morphological changes to occur in response to acute exercise. In these experiments zebrafish were exercised for three days and culled shortly afterwards.

I did find that there was less adipose tissue deposits in the hearts of 21 months old zebrafish which that had been exercised compared to sedentary zebrafish. This may be because long term endurance exercise uses lipids as an energy source (Jeppesen *et al.*, 2012) and endurance training in humans has been reported to change body fat composition (Schwartz *et al.*, 1991). It may be that adipose tissue has been used as an energy source in the exercised zebrafish. Although the changes in body fat composition in humans were reported to occur over 6 months (Schwartz *et al.*, 1991), the duration of exercise in zebrafish, three days, appears considerably shorter. Although 6 months would be the equivalent of 0.5% of a human lifespan which would be 5 days in zebrafish. This is not too different the length of the exercise treatment protocol when the rest period and CldU immersing is taken into consideration (3 days exercise + 0.5 days rest + 1 day in CldU = 4.5 days). A reduction in epicardial fat deposits due to exercise would be beneficial to humans. An increase in epicardial fat is associated with ageing and is a risk factor in cardiac disease (Wong *et al.*, 2011; Bertaso *et al.*, 2013).

A period of acute exercise was deliberately chosen to look at potential mechanisms underpinning changes rather than long term outcomes, such as a reduction in fibrosis or an increased lifespan. Looking at short term outcomes allowed me to look at short term changes which may alleviate the burden of disease. Although longer term studies measuring changes in life expectancy would be a better outcome, studies using lifespan as an output are technically difficult, take a long time and are very expensive to conduct. It was therefore not feasible to look at longer term outcomes in this thesis.

A reduction of cardiac fibrosis may be expected if the exercise was for a longer duration as an increase in cardiomyocyte proliferation may lessen the need for fibrosis in order to maintain cardiac structure and the zebrafish heart is capable of replacing pathogenic fibrotic tissue from the heart (Wills *et al.*, 2008; Schnabel *et al.*, 2011). In rodent models swim training and treadmill running exercise has been shown to reduce the amount of infarcted tissue and reduce the amount of ventricular remodelling after myocardial infarction (McElroy et al., 1978; Orenstein et al., 1995; Zhang et al., 2000; Wisloff et al., 2002). The duration of the exercise programs in these studies varied between 5-8 weeks. Although these studies were conducted in the context of myocardial infarction, they do suggest that if given for a long enough duration then exercise can have profound effects on collagen depositions and ventricular remodelling. As the duration of the exercise in these studies varied from 5-8 weeks, maybe if the zebrafish were allowed to exercise for this length of time there would be a decrease in fibrosis. A clear link between cardiomyocyte proliferation or apoptosis and cardiac fibrosis needs to be established. This could be determined by inhibiting cardiomyocyte apoptosis or proliferation via pharmacological intervention and examining if fibrosis occurs.

It could be argued that no increase in proliferation was experienced in 7 months old zebrafish in response to exercise as the exercise was not intense enough. This could be argued due my findings that 7 months old zebrafish swim naturally at a speed of 3.5BL/s (Figure 4.3). Although zebrafish were allowed 5 minutes to 'settle' in the tanks before video sequences were captured for determining speed, it may have been that the 7 months old zebrafish were more stressed and swimming faster than their basal speed. This would mean that the basal swimming speed of zebrafish aged 7 months was over estimated. It is very likely that younger zebrafish would find this scenario a more stressful situation than older zebrafish, as older zebrafish would have become more accustomed to being netted and placed in different tanks, as this occurs on an almost weekly basis. A more suitable way to determine the optimum speed of the swim tunnel may have been to work out the critical swimming speed of different aged zebrafish. This would involve steadily increasing the speed of the flume over a short time period until the zebrafish could no longer tolerate the speed.
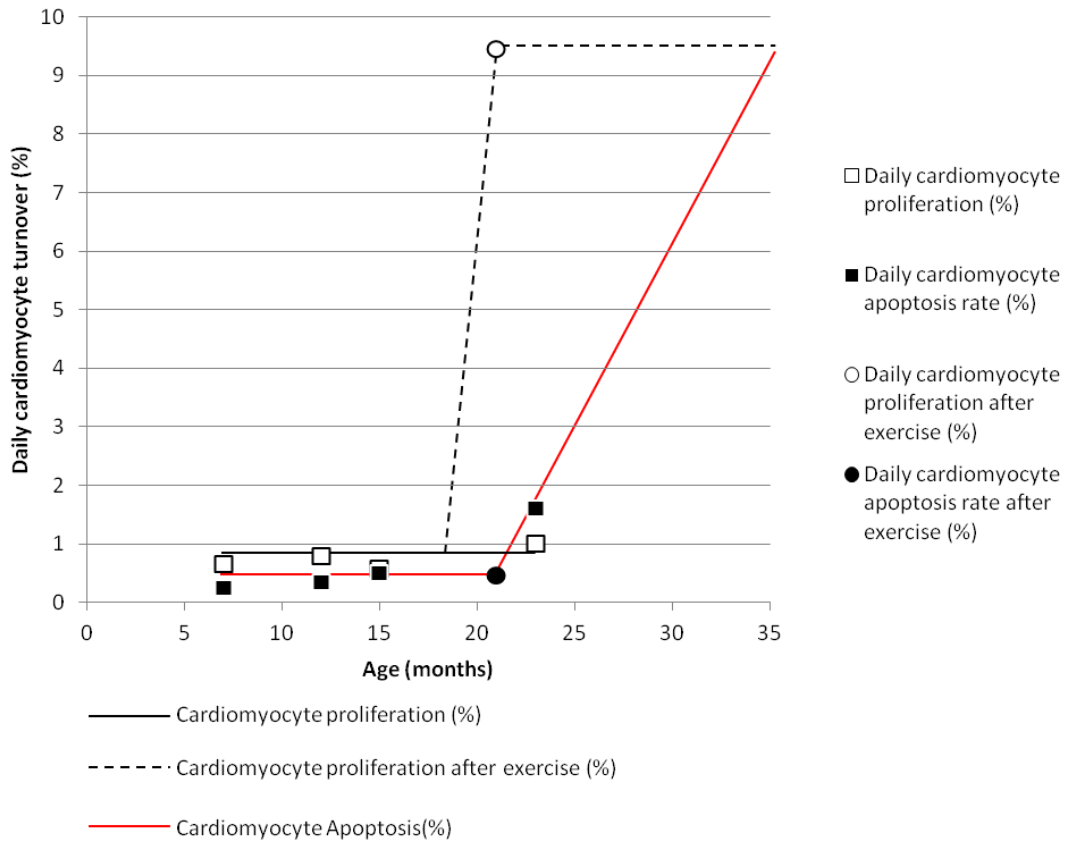
Figure 4.23: Modelling cardiomyocyte turnover in zebrafish hearts after exercise
In the previous chapter I mapped cardiomyocyte proliferation (White square, black line) and apoptosis (Black square, red line). I have plotted this data with the data regarding cardiomyocyte proliferation after exercise in 21 months old zebrafish (white circle, black dashed line). I have assumed that cardiomyocyte proliferation remains raised if exercise is continued (black dashed line) and extrapolated the increasing frequency of cardiomyocyte apoptosis with age (red line). This model predicts that the increasing rate of cardiomyocyte apoptosis will not equal cardiomyocyte proliferation until around 35 months of age. At this age the total cardiomyocyte population in the heart would decline. This would be the point where it cardiac remodelling would occur.

4.4.2. p38MAPK inhibition in the zebrafish heart

Initially when testing the dose responsiveness of different concentrations of the p38MAPK inhibitor SB203580 there was a significant increase in cardiomyocyte proliferation with a dose of 5μM for 12 hours. However when 5μM SB203580 was administered in a similar regimen to that of enforced exercise, with three days in the control flume so as to be able to later test the combination of p38MAPK and exercise, no significant increase was observed in cardiomyocyte proliferation when compared to controls in both 7 months old and 21 months old zebrafish. This may have been because cardiomyocyte proliferation is increased when zebrafish are in the control flume compared to normal aquarium conditions, thus masking any effect of p38MAPK inhibition. Reasons for an increase in proliferation in the static swim tunnel, used as a control to exercise treatment, may be because of stress induced from being in an alien environment, the control flume is very narrow and long compared to the tank zebrafish are housed in. This underlines the importance of using an appropriate 'control swim tunnel' with no current which has exactly the same dimensions and volume of water as the actual swim tunnel.

Being undeterred from the lack of a proliferative response after SB203580 treatment as in tissue culture and animals FGF administration was needed in conjunction with SB203580 to increase proliferation in cardiomyocytes (Engel *et al.*, 2005; Engel *et al.*, 2006a), I decided to see if p38MAPK inhibition would enhance the ability of exercise to increase cardiomyocyte proliferation. Administering 5μM SB203580 in conjunction with exercise had no significant increase in proliferation in cardiomyocytes of zebrafish aged 7 months or zebrafish aged 21 months. This implies that p38MAPK inhibition, or at least the dosage of SB203580 applied, had no effect on cardiomyocyte proliferation. It may be that a higher dosage of SB203580 was required, although this may run the risk of causing off target effects as p38MAPK signalling is ubiquitously used in cells.

It could be argued that p38MAPK is not being inhibited by 5μM SB203580. I believe that 5μM SB203580 is sufficient as increases in cardiomyocyte proliferation were experienced in preliminary experiments assessing the dosage of SB203580 (Figure 4.1). One way to confirm that p38MAPK is being inhibited in zebrafish hearts would be to perform a western blot on hearts treated with 5μM SB203580 and untreated hearts using an antibody for phosphorylated (active) p38MAPK. If 5μM SB203580 is sufficient to inhibit p38MAPK it would be expected that there would be less

phosphorylated p38MAPK in hearts from zebrafish treated with 5μM SB203580 than compared to untreated controls.

4.4.3. *Selecting the optimum reference genes for RT-qPCR in zebrafish hearts*

Determining the most stable housekeeping genes in particular experimental conditions is an often overlooked aspect of transcriptome analysis. Various studies have highlighted the importance of determining the most suitable genes used in specific experimental scenarios and the perils of plumping for an often used 'popular' housekeeping gene without subsequent tests (Tang *et al*., 2007; McCurley *et al*., 2008; Casadei *et al*., 2011; Vandesompele *et al*., 2002). Here I have determined the most stable housekeeping genes in my experimental scenarios, which whilst not in complete disagreement with the literature, does highlight some potential pitfalls of housekeeping gene selection, such as the suggestion of sl25a5 expression being one of the most stable across different tissue types by Tang *et al*., 2007, when in the zebrafish heart under my experimental conditions it was the least stable. For further RT-qPCR analysis I used *usp5, rpl13a* and *b2m* as these were determined to be the most stable.

The roles of *usp5, rpl13a* and *b2m* are not interlinked; *usp5*is involved in ubiquitination (Nakajima *et al.*, 2014), *rpl13a*is a ribosomal protein (Poddar *et al.*, 2013) and *b2m* is part of the major histocompatibility complex (Kumar *et al.*, 2014). It is important to select reference genes that have roles that are not connected because if something does happen which increases the expression of one reference gene, there is less chance of it also affecting the other genes if they have unconnected roles. For example, if all three reference genes played a role in proliferation and expression levels were increased due to cellular proliferation, the expression of genes of interest will not be correctly measured in cases where increased cellular proliferation occurs. This could result in a masking of actual changes in gene expression of genes of interest or perceived increases or decreases in gene expression when none occurs.

4.4.4. *Changes in gene expression due to ageing*

Reactivation of the fetal gene program has widely been reported in ageing and failing hearts (Oka *et al.*, 2007b; Barry *et al.*, 2008). Components of the fetal gene program include cardiac transcription factors and natriuretic peptides. An increased expression of the natriuretic peptides *NPPA* and *NPPB* has been widely reported in ageing hearts (Younes *et al.*, 1995; Charloux *et al.*, 2008). Indeed in the zebrafish heart there was a significant increase in *nppa* and *nppb* expression in 21 months old zebrafish compared

to 7 months old zebrafish. This suggests that a similar signalling network is at play that causes aging associated pathology in humans. This may be the driving factor or a secondary effect of the morphological and cellular changes I described in the aged zebrafish heart in the previous chapter. Such morphological and cellular changes include an increased incidence of cardiac fibrosis, thickening of the ventricle walls and an increase in cardiomyocyte apoptosis. Although there was no evidence of cardiomyocyte hypertrophy which is commonly associated with reactivation of the fetal gene program.

Reactivation of cardiac transcription factors, such as GATA4 and MEF2C, is often associated with cardiac hypertrophy (Oka *et al.*, 2007b; Barry *et al.*, 2008). The expression of these transcription factors were not significantly higher in zebrafish aged 21 months old when compared to zebrafish age 7 months old. This suggests that expression of cardiac transcription factors is not reactivated due to ageing. This may be because unlike the mammalian heart, the zebrafish heart does not respond to ageing associated stresses by becoming hypertrophied and thus, cardiac transcription factors are not re-expressed.

When zebrafish hearts undergo injury epicardial activation occurs in which *raldh2* is globally expressed in the epicardium and then later localized to the sight of injury (Kikuchi *et al.*, 2011a; Schnabel *et al.*, 2011; van Wijk *et al.*, 2012). *aldh2* expression was compared in 7 months old zebrafish to 21 months old zebrafish to see if the ageing activates a similar pathway to physical trauma. There was no significant difference in *aldh2* expression suggesting that the pathological changes that occur due to ageing does not activate the epicardium via *aldh2*, unlike a physical injury. Physical trauma in the zebrafish results in increased cardiomyocte proliferation in order to replace lost cardiomyocytes and repair any damage (Poss *et al.*, 2002; Jopling *et al.*, 2010; Schnabel *et al.*, 2011), unlike ageing which did not affect cardiomyocyte proliferation in zebrafish.

There appears to be changes in gene expression due to ageing in zebrafish hearts. These changes may be the driving force behind the observed morphological and cellular changes I have observed in the zebrafish heart in response to aging. One way to determine if the changes in gene expression I have observed in this chapter are the cause or a secondary effect of morphological and cellular changes in the heart would be to histologically examine zebrafish hearts from the ages of 15 months to 21 months to determine the point at which remodelling occurs and apoptosis increases. When this age has been determined RT-qPCR analysis could be conducted on hearts from

zebrafish at the age prior to pathological changes. If there is an increased expression of natriuretic peptides at this age, it is likely that an increased expression of natriuretic peptides is the driving factor behind morphological changes in zebrafish hearts observed in response to ageing. If there is no increase in natriuretic peptides, it would imply that increases in natriuretic peptides are a secondary affect of the morphological and cellular changes.

In mice, when hypertrophy does occur in *Nppa* deficient hearts the expression of extracellular matrix proteins is increased (Wang *et al.*, 2003). Hypertension, hypertrophy, fibrosis and heart failure occurs in *Npra* null mice (Oliver *et al.*, 1997; Kishimoto *et al.*, 2001; Knowles *et al.*, 2001). *Nppb* deletion in mice has been demonstrated to cause interstitial ventricular fibrosis (Tamura *et al.*, 2000b). This body of data implies that natriuretic peptide signalling is important for the prevention of pathological cardiac remodelling. This may suggest that natriuretic peptides could be upregulated in the aging zebrafish hearts in an attempt to decrease the extent of pathological remodelling. This would imply that increased natriuretic peptide signalling is a secondary response to pathological changes. The natriuretic signalling pathway may be activated in order to reduce the severity of damage in the heart.

Changes in natriuretic peptide expression suggest that some of the mechanisms of pathogenesis are similar to that of humans and other mammals. However there are some genes that would be expected to be reactivated in response to ageing, such as cardiac transcription factors, however the main role of re-expression of cardiac transcription factors is to promote cardiomyocyte hypertrophy which does not occur in zebrafish hearts, so it is maybe no surprise that these genes are not reactivated.

4.4.5. *Changes in gene expression due to exercise in the zebrafish heart*

It appeared that exercise significantly reduced the expression of *nppa*, a natriuretic peptide associated with pathogenic changes in the heart. In the human heart ANP serum levels have been reported to be raised during exercise and then reduced afterwards (Follenius and Brandenberger, 1988; Scharhag *et al.*, 2013). *Nppa* expression has also been reported to be reduced in rat hearts with exercise induced hypertrophy (Diffee *et al.*, 2003). It may be that during exercise in old zebrafish *nppa* expression is initially raised to promote cardiomyocyte hypertrophy, after exercise when the stimulus is not present ANP levels may be reduced as an adaption to training. As the levels of natriuretic peptides were not increased in aged zebrafish after exercise the hypertrophy observed can be described as healthy physiological hypertrophy, which is often

observed in athletes (Scharhag *et al.*, 2013), rather than pathological hypertrophy, often observed in cardiac diseases (Oka *et al.*, 2007a; Barry *et al.*, 2008). Levels of natriuretic peptide can be raised due to extreme exercise in humans, in which myocardial damage occurs (Ohba *et al.*, 2001), suggesting the exercise regimen chosen for the zebrafish was not too extreme.

Levels of cardiac transcription factors were also increased after exercise in elderly zebrafish. Cardiac transcription factors, such as *GATA4*, *MEF2C* and *NKX2.5* are part of the fetal gene program which is reactivated in the heart due to pathological changes associated with ageing. Because the level of the natriuretic peptide *nppa* was reduced, which is normally raised in pathological hypertrophy (Diffee *et al.*, 2003; Scharhag *et al.*, 2013), the increased expression of cardiac transcription factors may be mediating physiological hypertrophy in the aged zebrafish heart post exercise. Physiological hypertrophy in response to exercise is not associated with increased natriuretic peptide expression (Ellison *et al.*, 2012). Levels of cardiac transcription factors may also be raised due to the increased cardiomyocyte proliferation observed, which would result in differentiation of the newly generated cardiomyocytes both processes would require expression of cardiac transcription factors (Pikkarainen *et al.*, 2004; Kikuchi *et al.*, 2010; Schlesinger *et al.*, 2011).

In zebrafish hearts it has been demonstrated that during heart regeneration after physical trauma epicardial activation occurs. This involves the expression of *aldh2*, the rate limiting enzyme of retinoic acid, in the epicardium (Lepilina *et al.*, 2006; Kikuchi *et al.*, 2011a; Schnabel *et al.*, 2011). Inhibition of raldh2 in zebrafish hearts prevents regeneration by stopping proliferation of cardiomyocytes (Kikuchi *et al.*, 2011b). It has also been demonstrated that *aldh2* expression is upregulated in the epicardium when 8-10 week old zebrafish are stimulated to grow by being placed in less densely stocked aquaria (Wills *et al.*, 2008).

Although the increase in *aldh2* and *gata5* expression in 7 months old zebrafish after exercise was not statistically significant, it is indeed interesting. It may be that if more zebrafish were used to increase the power of the experiment, the increase in *aldh2* and *gata5*expression would be significant. This would suggest that the way younger zebrafish respond to exercise is distinct to that of older zebrafish. Younger zebrafish respond by epicardial activation, whereas older zebrafish respond by a reduction of natriuretic peptides and activation of cardiac specific transcrioption factors. Indeed, it has been demonstrated that the epicardial activation in young zebrafish can occur in response to environmental stimuli (Wills *et al.*, 2008). Expression of *aldh2* was

unchanged between exercised and sedentary 21 months old zebrafish hearts although proliferation was increased after exercise in elderly hearts, suggesting that the mechanism of increasing cardiomyocyte proliferation occurs independently of *aldh2* and *gata5* and may therefore be independent of epicardial activation. I would have also liked to examine the expression of other genes associated with epicardial activation, such as *tbx18, dhand,* and *wt1*. The expression of these genes may be increased in 7 months old zebrafish after exercise if epicardial activation did occur. This may suggest that exercise stimulates pathways involved in the fetal gene program in aged zebrafish, whereas in younger zebrafish exercise may stimulate epicardial activation. Understanding this difference may help to underpin why aged hearts show signs of pathology.

4.4.6. Limitations

Finding the appropriate level of exercise for zebrafish for different sized and aged zebrafish was a difficult challenge. Mapping out the speed of different aged zebrafish in terms of body length per second and then generating a relative flow rate appeared to be a good idea in theory. However in practise this did not work and the level of exercise for the 7 months old zebrafish initially appeared too high for them to keep up. This may have been because of younger zebrafish being less accustomed to the stress of being netted and responded by swimming faster than they would under normal circumstances. Whereas the older zebrafish would have been well accustomed to being netted and would likely have swam nearer their basal speed when being tracked. I therefore decided to use a lower flow rate for the younger zebrafish. It was difficult to tell if the flow rate chosen was having an effect on the younger zebrafish.

An alternative approach which may have been more successful at determining the appropriate level of exercise for different aged and different sized zebrafish may have been to carry out an exercise tolerance test. This could have been done by increasing the flow rate when a group of same aged zebrafish are in it at defined time periods. When a flow rate is achieved that exhausts them the test could be stopped and would give an indication of the maximum swimming speed for that group of zebrafish.

4.4.7. Summary

I have demonstrated there is an increase in natriuretic peptide expression in aged zebrafish compared to younger zebrafish. This is likely a response to changes in cardiac morphology due to ageing that I reported in the previous chapter. My work in this

chapter adds to the literature that suggests that exercise in advanced age is particularly of benefit to cardiovascular health (Giallauria *et al.*, 2005; Hollmann *et al.*, 2007; Fujimoto *et al.*, 2010). Increased cardiomyocyte proliferation in response to exercise has not been demonstrated in humans but has been reported in mice (Boström *et al.*, 2010). The fetal gene program appears to play an important role in the increase in cardiomyocyte proliferation in response to exercise in elderly zebrafish as the expression of natriuretic peptides appeared to decrease in response to exercise, whereas the expression of cardiac specific transcription factors increased.

Chapter 5 : Discussion

5.1. Introduction

A net reduction of cardiomyocytes is the underlying pathogenic mechanism in many ailments of the heart and is particularly true in aged hearts (Kajstura *et al.*, 2010a). Finding ways to reduce the declining population of cardiomyocytes would be of significant clinical value. This could be achieved by reducing the incidence of cardiomyocyte apoptosis, or increasing the rate of cardiomyocyte proliferation. One way of elucidating factors and mechanisms used to increase cardiomyocyte number is via the use of animal models.

Zebrafish have become an increasingly popular model for the study of ageing and heart development and disease, however no one has examined how the zebrafish heart responds to ageing. My main objectives in this thesis were to characterise morphological changes which may occur in the zebrafish heart in response to ageing, determine if there are changes in cardiomyocyte turnover in zebrafish hearts due to ageing and then to find interventions to promote cardiomyocyte proliferation in zebrafish hearts. Fulfilling these objectives would demonstrate that zebrafish are a novel model of cardiac ageing which may have benefits over other existing models. Interventions found to increase cardiomyocyte proliferation could also be considered therapeutic agents for ageing humans trying to offset cardiac ageing.

5.2. Summary of findings

I have shown that as the zebrafish heart ages some develop abnormal collagen deposits, thinning of ventricular walls, increasing adipose tissue deposits and a reduction in trabeculae, similar characteristics have also been observed in hearts in humans and animal models (Vandenbossche *et al.*, 1988; Mukherjee and Sen, 1993; Hees *et al.*, 2002). At a cellular level, these changes may be due to a decrease in cardiomyocyte turnover; I observed an increase in cardiomyocyte apoptosis, due to ageing, with cardiomyocyte proliferation remaining constant throughout the ages examined. Unlike in mammals pathological cardiomyocyte hypertrophy was not observed. These changes in zebrafish hearts appear to share a same molecular pathway to pathology in mammalian hearts as *nppb* expression was significantly increased, a genetic marker of many cardiac pathologies in humans and animal models.

As the pathology I described in the zebrafish hearts with advancing age appeared to be due to a reduction in cardiomyocyte turnover, I next sought interventions which

may increase the number of cardiomyocytes by either reducing cardiomyocyte apoptosis or increasing cardiomyocyte proliferation. The interventions I decided to try were exercise and p38MAPK inhibition. Exercise has been shown to increase cardiomyocyte proliferation and reduce apoptosis in animal models (Boström *et al.*, 2010). Inhibition of p38MAPK has been demonstrated to increase cardiomyocyte proliferation in animal models and tissue culture (Engel *et al.*, 2005; Engel *et al.*, 2006a; Boström *et al.*, 2010). Acute enforced exercise significantly increased cardiomyocyte proliferation and hypertrophy in elderly zebrafish with no affect on apoptosis but had no affect on cardiomyocyte turnover in younger zebrafish, this may be due to the exercise not being intense enough as the basal swimming rate in younger zebrafish was later discovered to be faster than that of the exercise flume. Gene expression analysis in the older zebrafish hearts illustrated that the positive affect of exercise on cardiomyocyte proliferation may be mediated by a reduction in *anp* expression, an increase in expression of the cardiac specific transcription factors *mef2c, gata4* and *nkx2.5* and also *tert.* These changes in gene expression have been reported in other animal models and humans in response to exercise in order to mediate physiological hypertrophy (Calderone *et al.*, 2001; Boström *et al.*, 2010). However, these hearts did not have a decrease in cardiac fibrosis, most likely because the duration of the exercise regime was not sufficient to have positive remodelling affects at an organ level.

Although inhibition of the p38MAPK inhibitor initially appeared to increase cardiomyocyte proliferation during experiments aiming to deduce the optimal dosage to use, when used in a similar schedule in tanks used for acute enforced exercise, no difference was observed. This may be because the increase in cardiomyocyte proliferation due to p38MAPK inhibition was masked by the increase in proliferation also observed by being in the 'unusually' shaped tank of the control flume. This underlines the necessity of using tanks of the same dimensions when comparing treatment groups, which was done throughout this project.

This body of results suggest that zebrafish have many of the fundamental pathologies associated with cardiac ageing. Acute exercise appeared to have a beneficial effect on cardiomyocyte proliferation, sharing many of the molecular pathways associated with exercise induced hypertrophy in other models.

5.3. Further work

There are many unanswered questions that were beyond the scope of these studies, which owing to time limitations could not be answered. Given enough time I would have liked to study how a longer term exercise regime may affect the ageing heart. Maybe if the 21 months old zebrafish were given enough time there may be reduced incidences of cardiac fibrosis in exercised hearts at 23 months. I would have also liked to have developed ways of measuring the performance of the heart, such as ejection fraction, in adult zebrafish to see if this would be affected by ageing and exercise. Another albeit longer study I would have relished conducting would be to see when is the best time of life to exercise to get the maximum benefit to the heart; is it better to exercise all throughout life or could similar results be obtained from exercising exclusively in old age. Studies like this would be difficult to conduct in animals and humans due to the length of time involved and, in humans, other lifestyle factors.

It could also be possible to develop a pharmacological screening process, in which various drugs or combination of drugs could be tested to see if they have an effect on cardiomyocyte proliferation in zebrafish and if they produce additive or even synergistic affects when applied in conjunction with exercise.

Using exercise as a cardioprotective intervention could also be tested in other scenarios in zebrafish, other than ageing, such as hereditary cardiomyopathies. Desmin related cardiomyopathy is a cardiac disease caused by a heterozygous dominant mutation in the intermediate filament protein desmin (Itou *et al.*, 2012b). Preliminary experiments I conducted showed that *desa* null zebrafish develop fibrosis prematurely, likely due to an increase in cardiomyocyte apoptosis (Figure 5.1). Reducing apoptosis in a desmin null mouse model of desminopathy by overexpressing *Bcl-2* did indeed reduce cardiac fibrosis and improved cardiac function (Vigorito and Giallauria, 2014). I would have like to see if exercise could help improve cardiomyocyte turnover in this model of cardiomyopathy and slow down collagen deposition in the heart.

I would have liked to have delineated more of the mechanisms behind the ageing I observed in the zebrafish heart and also the mechanisms behind an increase in cardiomyocyte proliferation in 21 months old zebrafish hearts after exercise. One process that I would like to look at would be autophagy. Autophagy a key part of ageing in the heart as the accumulation of damaged organelles, particularly mitochondria, is often reported in aged cardiomyocytes (Terman *et al.*, 2003; Sheydina *et al.*, 2011). It may be that in aged zebrafish hearts the accumulation of damaged organelles is too excessive which may be the cause of cardiomyocyte apoptosis.

Exercise has been shown to reduce the age related decline of autophagy (Wohlgemuth *et al.*, 2010). It may be that this is also true of the 21 months old zebrafish hearts after exercise which may convey some of the beneficial effects in cardiomyocyte turnover that I experienced. It may therefore also be of benefit to cardiomyocyte turnover to try and increase autophagy in aged zebrafish hearts. This could be done by calorie restriction (Han and Ren, 2010) or administration of rapamycin, a known promotor of autophagy (Sciarretta *et al.*, 2014). Carried out in conjunction with exercise, calorie restriction and/or administration of rapamycin may cause an additive effect to cardiomyocyte proliferation in elderly zebrafish. Carried out over a longer period, these treatments may also slow down or even reverse cardiac fibrosis.

One way to find out if autphagy is reduced in aged zebrafish hearts and increased in exercised zebrafish hearts would be to use transmission electron microscopy (TEM) (Barth *et al.*, 2010). TEM could be used to detect autophagic vesicles in sections of zebrafish heart, making it possible to see if there are fewer vesicles in aged hearts or more in aged hearts after exercise. Another method would be to measure autophagic flux, the actual turnover of autophagic vesicles (Barth *et al.*, 2010). This can be done using a luciferase assay (Ju *et al.*, 2009) or there are many commercially available kits for measuring autophagic flux.

Cellular senescence is the loss of the cells ability to proliferate and has been strongly associated with the shortening of telomeres due to ageing (Fossel, 2000; Campisi, 2003; Itahana *et al.*, 2004; Rodier *et al.*, 2005). In zebrafish, telomeres are a similar length to those of humans (Anchelin *et al.*, 2013) and expression of telomerase and length of telomeres have been demonstrated to decline due to ageing (Anchelin *et al.*, 2011). I would have liked to see if the telomeres in aged zebrafish hearts were shortened and if there was an increase in cellular scenescence due to ageing. There has been many links suggesting that telomeres in active individuals are longer than those in sedentary people (Kim *et al.*, 2012a; Denham *et al.*, 2013; Valenzuela, 2013). I would like to see if this is also true in zebrafish and if exercise throughout the lifespan of a zebrafish results in longer telomeres and reduced cardiac remodelling in later life. Telomere length can be measured by qPCR techniques (O'Callaghan and Fenech, 2011) or using commercially available kits.
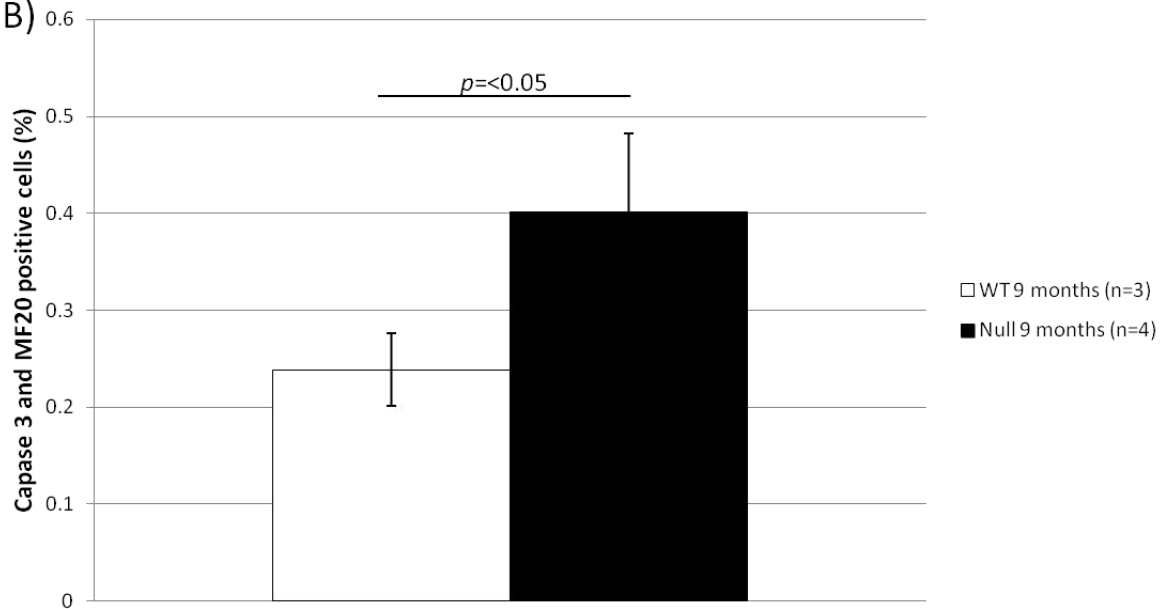
Figure 5.1: Desminopathy in zebrafish hearts
Masson's trichrome staining of a section of 11 months old desmin zebrafish heart illustrating extensive fibrosis in the ventricle (blue, A). Apoptosis in *desma* null 9 month old zebrafish was higher than that of wild type clutchmates. Significant differences were observed using a t-test, error bars are SEM.

5.4. Future perspectives

This thesis demonstrates that zebrafish can be used as a model to aid understanding of cardiac ageing. I have described how the zebrafish heart appears as it ages and delineated some of the cellular and molecular changes. Many questions remain unanswered which may facilitate our understanding of cardiac ageing in more mammalian hearts, which are undoubtedly more complex.

I have also demonstrated that exercise promotes cardiomyocyte proliferation in elderly zebrafish. This adds further knowledge to the benefit of exercise in the heart, particularly in the elderly. This further reinforces the idea that exercise and increasing activity levels should be promoted more, particularly to the elderly. This is important as physical inactivity in humans is increasing and has been associated with reduced lifespans, becoming a global problem (Craig *et al.*, 2012; Lee *et al.*, 2012).

5.5. Conclusions

Overall, there are many key findings in this body of work which could be extrapolated and provide the foundations to studies in mammalian models with the endpoint aim of using to improve cardiac health in elderly people. I have demonstrated that zebrafish are a simple, and previously unexplored model for cardiac ageing. There are many similar markers of pathology in zebrafish hearts when compared to human hearts. There are also many differences, however these differences can be beneficial and exploited to aid understanding of basic processes, such as cardiomyocyte turnover. Exercise in elderly zebrafish appears to increase cardiomyocyte proliferation. This demonstrates one of the ways in which exercise is beneficial to cardiovascular health in the elderly.

Chapter 6 : References

Aanhaanen, W.T.J., Moorman, A.F.M. and Christoffels, V.M. (2011) 'Origin and development of the atrioventricular myocardial lineage: Insight into the development of accessory pathways', *Birth Defects Research Part A: Clinical and Molecular Teratology*, 91(6), pp. 565-577.

Abramoff, M.D., Magalhaes, P.J., Ram, S.J. (2004) 'Image Processing with ImageJ', *Biophotonics International*, 11(7), pp. 36-42.

Abrass, C.K., Adcox, M.J. and Raugi, G.J. (1995) 'Aging-associated changes in renal extracellular matrix', *The American journal of pathology*, 146(3), p. 742.

Adams, R.H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R. and Nebreda, A.R. (2000) 'Essential Role of p38α MAP Kinase in Placental but Not Embryonic Cardiovascular Development', *Molecular Cell*, 6(1), pp. 109-116.

Ahuja, P., Perriard, E., Perriard, J.-C. and Ehler, E. (2004) 'Sequential myofibrillar breakdown accompanies mitotic division of mammalian cardiomyocytes', *Journal of cell science*, 117(15), pp. 3295-3306.

Akazawa, H. and Komuro, I. (2003) 'Roles of Cardiac Transcription Factors in Cardiac Hypertrophy', *Circulation Research*, 92(10), pp. 1079-1088.

Alberts, J., Lewis, Raff, Roberts, and Walter (ed.) (2007) *Molecular Biology of the Cell*.

Alvers, A.L., Fishwick, L.K., Wood, M.S., Hu, D., Chung, H.S., Dunn Jr, W.A. and Aris, J.P. (2009a) 'Autophagy and amino acid homeostasis are required for chronological longevity in Saccharomyces cerevisiae', *Aging cell*, 8(4), pp. 353-369.

Alvers, A.L., Wood, M.S., Hu, D., Kaywell, A.C., Dunn Jr, W.A. and Aris, J.P. (2009b) 'Autophagy is required for extension of yeast chronological life span by rapamycin', *Autophagy*, 5(6), pp. 847-849.

Anchelin, M., Alcaraz-Pérez, F., Martínez, C.M., Bernabé-García, M., Mulero, V. and Cayuela, M.L. (2013) 'Premature aging in telomerase-deficient zebrafish', *Disease Models & Mechanisms*, 6(5), pp. 1101-1112.

Anchelin, M., Murcia, L., Alcaraz-Pérez, F., García-Navarro, E.M. and Cayuela, M.L. (2011) 'Behaviour of telomere and telomerase during aging and regeneration in zebrafish', *PloS one*, 6(2), p. e16955.

Anderson, E.J., Rodriguez, E., Anderson, C.A., Thayne, K., Chitwood, W.R. and Kypson, A.P. (2011) 'Increased propensity for cell death in diabetic human heart is mediated by mitochondrial-dependent pathways', *American Journal of Physiology - Heart and Circulatory Physiology*, 300(1), pp. H118-H124.

Andersson, O., Adams, B.A., Yoo, D., Ellis, G.C., Gut, P., Anderson, R.M., German, M.S. and Stainier, D.Y. (2012) 'Adenosine signaling promotes regeneration of pancreatic β cells in vivo', *Cell metabolism*, 15(6), pp. 885-894.

Annes, J.P., Munger, J.S. and Rifkin, D.B. (2003) 'Making sense of latent TGFβ activation', *Journal of cell science*, 116(2), pp. 217-224.

Anversa, P., Hiler, B., Ricci, R., Guideri, G. and Olivetti, G. (1986) 'Myocyte cell loss and myocyte hypertrophy in the aging rat heart', *Journal of the American College of Cardiology*, 8(6), pp. 1441-1448.

Anversa, P., Palackal, T., Sonnenblick, E.H., Olivetti, G. and Capasso, J.M. (1990a) 'Hypertensive cardiomyopathy. Myocyte nuclei hyperplasia in the mammalian rat heart', *Journal of Clinical Investigation*, 85(4), p. 994.

Anversa, P., Palackal, T., Sonnenblick, E.H., Olivetti, G., Meggs, L.G. and Capasso, J.M. (1990b) 'Myocyte cell loss and myocyte cellular hyperplasia in the hypertrophied aging rat heart', *Circulation Research*, 67(4), pp. 871-885.

Arany, Z., Novikov, M., Chin, S., Ma, Y., Rosenzweig, A. and Spiegelman, B.M. (2006) 'Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-γ coactivator 1α', *Proceedings of the National Academy of Sciences*, 103(26), pp. 10086-10091.

Arbustini, E., Brega, A. and Narula, J. (2008) 'Ultrastructural definition of apoptosis in heart failure', *Heart Failure Reviews*, 13(2), pp. 121-135.

Arnold, M.A., Kim, Y., Czubryt, M.P., Phan, D., McAnally, J., Qi, X., Shelton, J.M., Richardson, J.A., Bassel-Duby, R. and Olson, E.N. (2007) 'MEF2C Transcription Factor Controls Chondrocyte Hypertrophy and Bone Development', *Developmental Cell*, 12(3), pp. 377-389.

Aronsen, J.M., Swift, F. and Sejersted, O.M. (2013) 'Cardiac sodium transport and excitation–contraction coupling', *Journal of Molecular and Cellular Cardiology*, 61(0), pp. 11-19.

Azevedo, A.S., Grotek, B., Jacinto, A., Weidinger, G. and Saúde, L. (2011) 'The Regenerative Capacity of the Zebrafish Caudal Fin Is Not Affected by Repeated Amputations', *PloS one*, 6(7), p. e22820.

Baker, A.B. and Tang, Y.Q. (2010) 'Aging performance for masters records in athletics, swimming, rowing, cycling, triathlon, and weightlifting', *Experimental aging research*, 36(4), pp. 453-477.

Bakkers, J. (2011) 'Zebrafish as a model to study cardiac development and human cardiac disease', *Cardiovascular research*.

Ball, A.J. and Levine, F. (2005) 'Telomere‐independent cellular senescence in human fetal cardiomyocytes', *Aging cell*, 4(1), pp. 21-30.

Banerjee, I., Fuseler, J.W., Price, R.L., Borg, T.K. and Baudino, T.A. (2007) 'Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse', *American Journal of Physiology - Heart and Circulatory Physiology*, 293(3), pp. H1883-H1891.

Banerjee, I., Yekkala, K., Borg, T.K. and Baudino, T.A. 1080 (2006) 'Dynamic interactions between myocytes, fibroblasts, and extracellular matrix'. pp. 76-84. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-33845654352&partnerID=40&md5=ac4d1752fdbc36b2d8e777c0db850470.

Barrionuevo, W.R. and Burggren, W.W. (1999) 'O2 consumption and heart rate in developing zebrafish (Danio rerio): influence of temperature and ambient O2', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 276(2), pp. R505-R513.

Barry, S.P., Davidson, S.M. and Townsend, P.A. (2008) 'Molecular regulation of cardiac hypertrophy', *The international journal of biochemistry & cell biology*, 40(10), pp. 2023-2039.

Barth, S., Glick, D. and Macleod, K.F. (2010) 'Autophagy: assays and artifacts', *The Journal of pathology*, 221(2), pp. 117-124.

Basson, C.T., Bachinsky, D.R., Lin, R.C., Levi, T., Elkins, J.A., Soults, J., Grayzel, D., Kroumpouzou, E., Traill, T.A. and Leblanc-Straceski, J. (1997) 'Mutations in human cause limb and cardiac malformation in Holt-Oram syndrome', *Nature Genetics*, 15(1), pp. 30-35.

Basson, C.T., Cowley, G.S., Solomon, S.D., Weissman, B., Poznanski, A.K., Traill, T.A., Seidman, J.G. and Seidman, C.E. (1994) 'The clinical and genetic spectrum of the Holt-Oram syndrome (heart-hand syndrome)', *New England Journal of Medicine*, 330(13), pp. 885-891.

Basson, C.T., Huang, T., Lin, R.C., Bachinsky, D.R., Weremowicz, S., Vaglio, A., Bruzzone, R., Quadrelli, R., Lerone, M. and Romeo, G. (1999) 'Different TBX5 interactions in heart and limb defined by Holt–Oram syndrome mutations', *Proceedings of the National Academy of Sciences*, 96(6), pp. 2919-2924.

Baudino, T.A., Carver, W., Giles, W. and Borg, T.K. (2006) 'Cardiac fibroblasts: friend or foe?', *American Journal of Physiology-Heart and Circulatory Physiology*, 291(3), pp. H1015-H1026.

Beardmore, V.A., Hinton, H.J., Eftychi, C., Apostolaki, M., Armaka, M., Darragh, J., McIlrath, J., Carr, J.M., Armit, L.J., Clacher, C., Malone, L., Kollias, G. and Arthur, J.S.C. (2005) 'Generation and Characterization of p38β (MAPK11) Gene-Targeted Mice', *Molecular and Cellular Biology*, 25(23), pp. 10454-10464.

Beere, P.A., Russell, S.D., Morey, M.C., Kitzman, D.W. and Higginbotham, M.B. (1999) 'Aerobic Exercise Training Can Reverse Age-Related Peripheral Circulatory Changes in Healthy Older Men', *Circulation*, 100(10), pp. 1085-1094.

Beltrami, A.P., Urbanek, K., Kajstura, J., Yan, S.-M., Finato, N., Bussani, R., Nadal-Ginard, B., Silvestri, F., Leri, A. and Beltrami, C.A. (2001) 'Evidence that human

cardiac myocytes divide after myocardial infarction', *New England Journal of Medicine*, 344(23), pp. 1750-1757.

Bergmann, O., Bhardwaj, R.D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B.A. and Druid, H. (2009) 'Evidence for cardiomyocyte renewal in humans', *Science*, 324(5923), pp. 98-102.

Bergmann, O., Zdunek, S., Alkass, K., Druid, H., Bernard, S. and Frisén, J. (2011) 'Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover', *Experimental cell research*, 317(2), pp. 188-194.

Bernardo, B.C., Weeks, K.L., Pretorius, L. and McMullen, J.R. (2010) 'Molecular distinction between physiological and pathological cardiac hypertrophy: Experimental findings and therapeutic strategies', *Pharmacology & Therapeutics*, 128(1), pp. 191-227.

Bers, D.M., Eisner, D.A. and Valdivia, H.H. (2003) 'Sarcoplasmic reticulum Ca2+ and heart failure roles of diastolic leak and Ca2+ transport', *Circulation Research*, 93(6), pp. 487-490.

Bersell, K., Arab, S., Haring, B. and Kühn, B. (2009) 'Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury', *Cell*, 138(2), pp. 257-270.

Bertaso, A.G., Bertol, D., Duncan, B.B. and Foppa, M. (2013) 'Epicardial fat: definition, measurements and systematic review of main outcomes', *Arquivos brasileiros de cardiologia*, 101(1), pp. e18-e28.

Biben, C. and Harvey, R.P. (1997) 'Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHAND during murine heart development', *Genes and Development*, 11(11), pp. 1357-1369.

Biben, C., Weber, R., Kesteven, S., Stanley, E., McDonald, L., Elliott, D.A., Barnett, L., Köentgen, F., Robb, L., Feneley, M. and Harvey, R.P. (2000) 'Cardiac septal and valvular dysmorphogenesis in mice heterozygous for mutations in the homeobox gene Nkx2-5', *Circulation Research*, 87(10), pp. 888-895.

Biehl, J.K., Yamanaka, S., Desai, T.A., Boheler, K.R. and Russell, B. (2009) 'Proliferation of mouse embryonic stem cell progeny and the spontaneous contractile

activity of cardiomyocytes are affected by microtopography', *Developmental Dynamics*, 238(8), pp. 1964-1973.

Biernacka, A. and Frangogiannis, N.G. (2011) 'Aging and cardiac fibrosis', *Aging and disease*, 2(2), p. 158.

Biga, P.R. and Goetz, F.W. (2006) 'Zebrafish and giant danio as models for muscle growth: determinate vs. indeterminate growth as determined by morphometric analysis', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 291(5), pp. R1327-R1337.

Black, B.L. and Olson, E.N. 14 (1998) 'Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins'. pp. 167-196. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0032437107&partnerID=40&md5=7d1310448f2b918634f1acdd1205150a.

Black, M.A., Cable, N.T., Thijssen, D.H.J. and Green, D.J. (2009) 'Impact of age, sex, and exercise on brachial artery flow-mediated dilatation', *American Journal of Physiology - Heart and Circulatory Physiology*, 297(3), pp. H1109-H1116.

Blackburn, E.H. (1991) 'Structure and function of telomeres', *Nature*, 350(6319), pp. 569-573.

Blasco, M.A. (2007) 'Telomere length, stem cells and aging', *Nature Chemical Biology*, 3(10), pp. 640-649.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S. and Wright, W.E. (1998) 'Extension of life-span by introduction of telomerase into normal human cells', *Science*, 279(5349), pp. 349-352.

Bodyak, N., Kang, P.M., Hiromura, M., Sulijoadikusumo, I., Horikoshi, N., Khrapko, K. and Usheva, A. (2002) 'Gene expression profiling of the aging mouse cardiac myocytes', *Nucleic acids research*, 30(17), pp. 3788-3794.

Boström, P., Mann, N., Wu, J., Quintero, P.A., Plovie, E.R., Panáková, D., Gupta, R.K., Xiao, C., MacRae, C.A., Rosenzweig, A. and Spiegelman, B.M. (2010) 'C/EBPβ Controls Exercise-Induced Cardiac Growth and Protects against Pathological Cardiac Remodeling', *Cell*, 143(7), pp. 1072-1083.

Bratic, A. and Larsson, N.-G. (2013) 'The role of mitochondria in aging', *J Clin Invest*, 123(3), pp. 951-957.

Braz, J.C., Bueno, O.F., Liang, Q., Wilkins, B.J., Dai, Y.-S., Parsons, S., Braunwart, J., Glascock, B.J., Klevitsky, R. and Kimball, T.F. (2003) 'Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling', *Journal of Clinical Investigation*, 111(10), pp. 1475-1486.

Brittsan, A.G., Carr, A.N., Schmidt, A.G. and Kranias, E.G. (2000) 'Maximal inhibition of SERCA2 Ca2+ affinity by phospholamban in transgenic hearts overexpressing a non-phosphorylatable form of phospholamban', *Journal of Biological Chemistry*, 275(16), pp. 12129-12135.

Brocheriou, V., Hagège, A.A., Oubenaïssa, A., Lambert, M., Mallet, V.O., Duriez, M., Wassef, M., Kahn, A., Menasché, P. and Gilgenkrantz, H. (2000) 'Cardiac functional improvement by a human Bcl‑2 transgene in a mouse model of ischemia/reperfusion injury', *The journal of gene medicine*, 2(5), pp. 326-333.

Broderick, T.L., Parrott, C.R., Wang, D., Jankowski, M. and Gutkowska, J. (2012) 'Expression of cardiac GATA4 and downstream genes after exercise training in the db/db mouse', *Pathophysiology*, 19(3), pp. 193-203.

Broecker, W.S., Schulert, A. and Olson, E.A. (1959) 'Bomb carbon-14 in human beings', *Science See Saiensu*, 130.

Brooks, W.W. and Conrad, C.H. (2000) 'Myocardial Fibrosis in Transforming Growth Factor β 1 Heterozygous Mice', *Journal of molecular and cellular cardiology*, 32(2), pp. 187-195.

Brown, M.D. (2003) 'Exercise and coronary vascular remodelling in the healthy heart', *Experimental Physiology*, 88(5), pp. 645-658.

Brown, R.D., Ambler, S.K., Mitchell, M.D. and Long, C.S. 45 (2005) 'The cardiac fibroblast: Therapeutic target in myocardial remodeling and failure'. pp. 657-687. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-13844312743&partnerID=40&md5=85b8eaf67cbb457e84be87020298ce90.

Bruneau, B.G. (2011) 'Atrial natriuretic factor in the developing heart: a signpost for cardiac morphogenesis', *Canadian Journal of Physiology and Pharmacology*, 89(8), pp. 533-537.

Bruneau, B.G., Bao, Z.Z., Tanaka, M., Schott, J.J., Izumo, S., Cepko, C.L., Seidman, J.G. and Seidman, C.E. (2000) 'Cardiac expression of the ventricle-specific homeobox gene Irx4 is modulated by Nkx2-5 and dHand', *Developmental biology*, 217(2), pp. 266-277.

Buckingham, M., Meilhac, S. and Zaffran, S. (2005) 'Building the mammalian heart from two sources of myocardial cells', *Nat Rev Genet*, 6(11), pp. 826-837.

Burgess, M.L., Buggy, J., Price, R.L., Abel, F.L., Terracio, L., Samarel, A.M. and Borg, T.K. (1996) 'Exercise-and hypertension-induced collagen changes are related to left ventricular function in rat hearts', *American Journal of Physiology-Heart and Circulatory Physiology*, 270(1), pp. H151-H159.

Burkauskiene, A., Mackiewicz, Z., Virtanen, I. and Konttinen, Y.T. (2006) 'Age-related changes in myocardial nerve and collagen networks of the auricle of the right atrium', *Acta cardiologica*, 61(5), pp. 513-518.

Burlew, B.S. (2004) 'Diastolic dysfunction in the elderly—the interstitial issue', *The American journal of geriatric cardiology*, 13(1), pp. 29-38.

Burrell, J.H., Boyn, A.M., Kumarasamy, V., Hsieh, A., Head, S.I. and Lumbers, E.R. (2003) 'Growth and maturation of cardiac myocytes in fetal sheep in the second half of gestation', *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology*, 274(2), pp. 952-961.

Bustin, S.A. (2000) 'Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays', *Journal of Molecular Endocrinology*, 25(2), pp. 169-193.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) 'The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments', *Clinical Chemistry*, 55(4), pp. 611-622.

Cai, C.-L., Liang, X., Shi, Y., Chu, P.-H., Pfaff, S.L., Chen, J. and Evans, S. (2003) 'Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart', *Developmental Cell*, 5(6), pp. 877-889.

Cai, C.-L., Martin, J.C., Sun, Y., Cui, L., Wang, L., Ouyang, K., Yang, L., Bu, L., Liang, X. and Zhang, X. (2008) 'A myocardial lineage derives from Tbx18 epicardial cells', *Nature*, 454(7200), pp. 104-108.

Calderone, A., Murphy, R.J.L., Lavoie, J., Colombo, F. and Béliveau, L. (2001) 'TGF-β1 and prepro-ANP mRNAs are differentially regulated in exercise-induced cardiac hypertrophy', *Journal of Applied Physiology*, 91(2), pp. 771-776.

Calderone, A., Thaik, C.M., Takahashi, N., Chang, D.L.F. and Colucci, W.S. (1998) 'Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts', *Journal of Clinical Investigation*, 101(4), pp. 812-818.

Camelliti, P., Borg, T.K. and Kohl, P. (2005) 'Structural and functional characterisation of cardiac fibroblasts', *Cardiovascular research*, 65(1), pp. 40-51.

Camelliti, P., Green, C.R. and Kohl, P. 42 (2006) 'Structural and functional coupling of cardiac myocytes and fibroblasts'. pp. 132-149. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-33744457045&partnerID=40&md5=f2f1a36c5743ea24a88be46aa042c735.

Campisi, J. (2003) 'Cellular senescence and apoptosis: how cellular responses might influence aging phenotypes', *Experimental gerontology*, 38(1), pp. 5-11.

Cao, D., Gillette, T. and Hill, J. (2009) 'Cardiomyocyte autophagy: Remodeling, repairing, and reconstructing the heart', *Current Hypertension Reports*, 11(6), pp. 406-411.

Carter, C.S., Sonntag, W.E., Onder, G. and Pahor, M. (2002) 'Physical Performance and Longevity in Aged Rats', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 57(5), pp. B193-B197.

Casadei, R., Pelleri, M.C., Vitale, L., Facchin, F., Lenzi, L., Canaider, S., Strippoli, P. and Frabetti, F. (2011) 'Identification of housekeeping genes suitable for gene expression analysis in the zebrafish', *Gene Expression Patterns*, 11(3), pp. 271-276.

Castro, V., Grisdale-Helland, B., Helland, S.J., Torgersen, J., Kristensen, T., Claireaux, G., Farrell, A.P. and Takle, H. (2013) 'Cardiac Molecular-Acclimation Mechanisms in Response to Swimming-Induced Exercise in Atlantic Salmon', *PLoS ONE*, 8(1), p. e55056.

Centonze, V.E. and White, J.G. 'Multiphoton Excitation Provides Optical Sections from Deeper within Scattering Specimens than Confocal Imaging', *Biophysical Journal*, 75(4), pp. 2015-2024.

Cesare, A.J., Hayashi, M.T., Crabbe, L. and Karlseder, J. (2013) 'The telomere deprotection response is functionally distinct from the genomic DNA damage response', *Molecular Cell*, 51(2), pp. 141-155.

Chablais, F., Veit, J., Rainer, G. and Jaźwińska, A. (2011) 'The zebrafish heart regenerates after cryoinjury-induced myocardial infarction', *BMC developmental biology*, 11(1), p. 21.

Chai, J. and Tarnawski, A.S. (2002) 'Serum response factor: Discovery, biochemistry, biological roles and implications for tissue injury healing', *Journal of Physiology and Pharmacology*, 53(2), pp. 147-157.

Chang, K.-C., Peng, Y.-I., Dai, S.-H. and Tseng, Y.-Z. (2000) 'Age-Related Changes in Pumping Mechanical Behavior of Rat Ventricle in Terms of Systolic Elastance and Resistance', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 55(9), pp. B440-B447.

Chang, L. and Karin, M. (2001) 'Mammalian MAP kinase signalling cascades', *Nature*, 410(6824), pp. 37-40.

Charloux, A., Brandenberger, G., Piquard, F. and Geny, B. (2008) 'Dysregulation of pulsatility in aging: IV. Pulsatile signaling and cardiovascular aging: Functions and regulation of natriuretic peptide signaling', *Ageing research reviews*, 7(3), pp. 151-163.

Charriaut-Marlangue, C. and Ben-Ari, Y. (1995) 'A cautionary note on the use of the TUNEL stain to determine apoptosis', *Neuroreport*, 7(1), pp. 61-64.

Charron, F., Paradis, P., Bronchain, O., Nemer, G. and Nemer, M. (1999) 'Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression', *Molecular and Cellular Biology*, 19(6), pp. 4355-4365.

Chehrehasa, F., Meedeniya, A.C.B., Dwyer, P., Abrahamsen, G. and Mackay-Sim, A. (2009) 'EdU, a new thymidine analogue for labelling proliferating cells in the nervous system', *Journal of neuroscience methods*, 177(1), pp. 122-130.

Chen, T.H.P., Chang, T.-C., Kang, J.-O., Choudhary, B., Makita, T., Tran, C.M., Burch, J.B.E., Eid, H. and Sucov, H.M. (2002) 'Epicardial Induction of Fetal Cardiomyocyte Proliferation via a Retinoic Acid-Inducible Trophic Factor', *Developmental biology*, 250(1), pp. 198-207.

Childs, A.C., Phaneuf, S.L., Dirks, A.J., Phillips, T. and Leeuwenburgh, C. (2002) 'Doxorubicin Treatment in Vivo Causes Cytochrome c Release and Cardiomyocyte Apoptosis, As Well As Increased Mitochondrial Efficiency, Superoxide Dismutase Activity, and Bcl-2:Bax Ratio', *Cancer research*, 62(16), pp. 4592-4598.

Chimenti, C., Kajstura, J., Torella, D., Urbanek, K., Heleniak, H., Colussi, C., Di Meglio, F., Nadal-Ginard, B., Frustaci, A. and Leri, A. (2003) 'Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure', *Circulation Research*, 93(7), pp. 604-613.

Cho, Y., Park, S., Shin, H. and Chan, F.-M. (2010) 'Physiological consequences of programmed necrosis, an alternative form of cell demise', *Molecules and Cells*, 29(4), pp. 327-332.

Chow, S.-C., Wang, H. and Shao, J. (2007) *Sample size calculations in clinical research*. CRC press.

Chung, C.-C., Kao, Y.-H., Chen, Y.-J. and Chen, Y.-J. (2013) 'Androgen modulates cardiac fibrosis contributing to gender differences on heart failure', *The Aging Male*, 16(1), pp. 22-27.

Cohen, S.B., Graham, M.E., Lovrecz, G.O., Bache, N., Robinson, P.J. and Reddel, R.R. (2007) 'Protein Composition of Catalytically Active Human Telomerase from Immortal Cells', *Science*, 315(5820), pp. 1850-1853.

Condorelli, G., Morisco, C., Stassi, G., Notte, A., Farina, F., Sgaramella, G., de Rienzo, A., Roncarati, R., Trimarco, B. and Lembo, G. (1999) 'Increased Cardiomyocyte Apoptosis and Changes in Proapoptotic and Antiapoptotic Genes bax

and bcl-2 During Left Ventricular Adaptations to Chronic Pressure Overload in the Rat', *Circulation*, 99(23), pp. 3071-3078.

Conn, P.M. (2011) *Handbook of models for human aging*. Academic Press.

Cook, S.A., Sugden, P.H. and Clerk, A. (1999) 'Activation of c-Jun N-terminal kinases and p38-mitogen-activated protein kinases in human heart failure secondary to ischaemic heart disease', *Journal of molecular and cellular cardiology*, 31(8), pp. 1429-1434.

Counter, C.M., Meyerson, M., Eaton, E.N., Ellisen, L.W., Caddle, S.D., Haber, D.A. and Weinberg, R.A. (1998) 'Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase', *Oncogene*, 16(9), pp. 1217-1222.

Craig, C.L., Lambert, E.V., Inoue, S., Alkandari, J.R., Leetongin, G. and Kahlmeier, S. (2012) 'The pandemic of physical inactivity: global action for public health', *The Lancet*, 380(9838), pp. 294-305.

Cuenda, A., Cohen, P., Buée-Scherrer, V. and Goedert, M. (1997) 'Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); Comparison of the specificities of SAPK3 and SAPK2 (RK/p38)', *EMBO Journal*, 16(2), pp. 295-305.

Cuenda, A. and Rousseau, S. (2007) 'p38 MAP-kinases pathway regulation, function and role in human diseases', *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1773(8), pp. 1358-1375.

Das, D., Maulik, N., Sato, M. and Ray, P. (1999) 'Reactive oxygen species function as second messenger during ischemic preconditioning of heart', in Das, D. (ed.) *Stress Adaptation, Prophylaxis and Treatment*. Springer US, pp. 59-67.

Davie, P.S. and Sparksman, R. (1986) 'Burnt tuna: an ultrastructural study of postmortem changes in muscle of yellowfin tuna (Thunnus albacares) caught on rod and reel and southern bluefin tuna (Thunnus maccoyii) caught on handline or longline', *Journal of Food Science*, 51(5), pp. 1122-1128.

Dawson, J.R. and Gibson, D.G. (1989) 'Left ventricular filling and early diastolic function at rest and during angina in patients with coronary artery disease', *British Heart Journal*, 61(3), pp. 248-257.

De Denus, S., Pharand, C. and Williamson, D.R. (2004) 'Brain Natriuretic Peptide in the Management of Heart Failure: The Versatile Neurohormone', *Chest*, 125(2), pp. 652-668.

De Lange, T. (2005) 'Shelterin: The protein complex that shapes and safeguards human telomeres', *Genes and Development*, 19(18), pp. 2100-2110.

De Lange, T. (2009) 'How telomeres solve the end-protection problem', *Science*, 326(5955), pp. 948-952.

De Lange, T. 75 (2010) 'How shelterin solves the telomere end-protection problem'. pp. 167-177. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-80052398381&partnerID=40&md5=983c9e49fee6b57f2c00323c7cb0bcf6.

De Robertis, E.M. (2010) 'Wnt signaling in axial patterning and regeneration: lessons from planaria', *Science signaling*, 3(127), p. pe21.

Denham, J., Nelson, C.P., O'Brien, B.J., Nankervis, S.A., Denniff, M., Harvey, J.T., Marques, F.Z., Codd, V., Zukowska-Szczechowska, E. and Samani, N.J. (2013) 'Longer leukocyte telomeres are associated with ultra-endurance exercise independent of cardiovascular risk factors', *PloS one*, 8(7), p. e69377.

Derveaux, S., Vandesompele, J. and Hellemans, J. (2010) 'How to do successful gene expression analysis using real-time PCR', *Methods*, 50(4), pp. 227-230.

Devlin, R.H., Yesaki, T.Y., Biagi, C.A., Donaldson, E.M., Swanson, P. and Chan, W.-K. (1994) 'Extraordinary salmon growth', *Nature*, 371(6494), pp. 209-210.

Diedrichs, H., Chi, M., Boelck, B., Mehlhorm, U. and Schwinger, R.H.G. (2004) 'Increased regulatory activity of the calcineurin/NFAT pathway in human heart failure', *European journal of heart failure*, 6(1), pp. 3-9.

Diermeier, S., Schmidt‑Bruecken, E., Kubbies, M., Kunz‑Schughart, L.A. and Brockhoff, G. (2004) 'Exposure to continuous bromodeoxyuridine (BrdU) differentially

affects cell cycle progression of human breast and bladder cancer cell lines', *Cell proliferation*, 37(2), pp. 195-206.

Diffee, G.M., Seversen, E.A., Stein, T.D. and Johnson, J.A. (2003) 'Microarray expression analysis of effects of exercise training: increase in atrial MLC-1 in rat ventricles', *American Journal of Physiology - Heart and Circulatory Physiology*, 284(3), pp. H830-H837.

Dinenno, F.A., Seals, D.R., DeSouza, C.A. and Tanaka, H. (2001) 'Age-related decreases in basal limb blood flow in humans: time course, determinants and habitual exercise effects', *The Journal of Physiology*, 531(2), pp. 573-579.

Dirks, A. and Leeuwenburgh, C. (2005) 'The Role of Apoptosis in Age-Related Skeletal Muscle Atrophy', *Sports Medicine*, 35(6), pp. 473-483.

Dorn, G.W. (2007) 'The Fuzzy Logic of Physiological Cardiac Hypertrophy', *Hypertension*, 49(5), pp. 962-970.

Duncker, D.J. and Bache, R.J. (2008) 'Regulation of Coronary Blood Flow During Exercise', *Physiological Reviews*, 88(3), pp. 1009-1086.

Durocher, D., Charron, F., Warren, R., Schwartz, R.J. and Nemer, M. (1997) 'The cardiac transcription factors nkx2-5 and GATA-4 are mutual cofactors', *EMBO Journal*, 16(18), pp. 5687-5696.

Edmondson, D.G., Lyons, G.E., Martin, J.F. and Olson, E.N. (1994) 'Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis', *Development*, 120(5), pp. 1251-1263.

Ellison, G.M., Waring, C.D., Vicinanza, C. and Torella, D. (2012) 'Physiological cardiac remodelling in response to endurance exercise training: cellular and molecular mechanisms', *Heart*, 98(1), pp. 5-10.

Elser, J.A. and Margulies, K.B. (2012) 'Hybrid Mathematical Model of Cardiomyocyte Turnover in the Adult Human Heart', *PloS one*, 7(12), p. e51683.

Engel, F.B., Hsieh, P.C.H., Lee, R.T. and Keating, M.T. (2006a) 'FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues

function after myocardial infarction', *Proceedings of the National Academy of Sciences*, 103(42), pp. 15546-15551.

Engel, F.B., Schebesta, M., Duong, M.T., Lu, G., Ren, S., Madwed, J.B., Jiang, H., Wang, Y. and Keating, M.T. (2005) 'p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes', *Genes & Development*, 19(10), pp. 1175-1187.

Engel, F.B., Schebesta, M. and Keating, M.T. (2006b) 'Anillin localization defect in cardiomyocyte binucleation', *Journal of molecular and cellular cardiology*, 41(4), pp. 601-612.

Eyers, P.A., Craxton, M., Morricel, N., Cohen, P. and Goedert, M. (1998) 'Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution', *Chemistry & Biology*, 5(6), pp. 321-328.

Fan, C., Liu, M. and Wang, Q. (2003) 'Functional analysis of TBX5 missense mutations associated with Holt-Oram syndrome', *Journal of Biological Chemistry*, 278(10), pp. 8780-8785.

Farrell, A.P. and Jones, D.R. (1992) '1 The Heart', *Fish physiology*, 12, pp. 1-88.

Femminella, G.D., Rengo, G., Pagano, G., de Lucia, C., Komici, K., Parisi, V., Cannavo, A., Liccardo, D., Vigorito, C., Filardi, P.P., Ferrara, N. and Leosco, D. (2013) 'β-Adrenergic Receptors and G Protein-Coupled Receptor Kinase-2 in Alzheimer's Disease: A New Paradigm for Prognosis and Therapy?', *Journal of Alzheimer's Disease*, 34(2), pp. 341-347.

Feng, J., Funk, W.D., Wang, S.S., Weinrich, S.L., Avilion, A.A., Chiu, C.P., Adams, R.R., Chang, E., Allsopp, R.C., Yu, J. and al, e. (1995) 'The RNA component of human telomerase', *Science*, 269(5228), pp. 1236-1241.

Feng, J.A., Perry, G., Mori, T., Hayashi, T., Oparil, S. and Chen, Y.F. (2003) 'Pressure-independent enhancement of cardiac hypertrophy in atrial natriuretic peptide-deficient mice', *Clinical and Experimental Pharmacology and Physiology*, 30(5-6), pp. 343-349.

Feng, Q., Li, P., Salamanca, C., Huntsman, D., Leung, P.C.K. and Auersperg, N. (2005) 'Caspase-1α is down-regulated in human ovarian cancer cells and the

overexpression of caspase-1α induces apoptosis', *Cancer research*, 65(19), pp. 8591-8596.

Fiechter, M., Fuchs, T.A., Gebhard, C., Stehli, J., Klaeser, B., Stähli, B.E., Manka, R., Manes, C., Tanner, F.C. and Gaemperli, O. (2013) 'Age-related normal structural and functional ventricular values in cardiac function assessed by magnetic resonance', *BMC medical imaging*, 13(1), p. 6.

Finck, B.N. and Kelly, D.P. (2007) 'Peroxisome Proliferator–Activated Receptor γ Coactivator-1 (PGC-1) Regulatory Cascade in Cardiac Physiology and Disease', *Circulation*, 115(19), pp. 2540-2548.

Fleg, J.L., O'Connor, F., Gerstenblith, G., Becker, L.C., Clulow, J., Schulman, S.P. and Lakatta, E.G. (1995) 'Impact of age on the cardiovascular response to dynamic upright exercise in healthy men and women', *Journal of Applied Physiology*, 78(3), pp. 890-900.

Flicek, P., Amode, M.R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fitzgerald, S., Gil, L., Girón, C.G., Gordon, L., Hourlier, T., Hunt, S., Johnson, N., Juettemann, T., Kähäri, A.K., Keenan, S., Kulesha, E., Martin, F.J., Maurel, T., McLaren, W.M., Murphy, D.N., Nag, R., Overduin, B., Pignatelli, M., Pritchard, B., Pritchard, E., Riat, H.S., Ruffier, M., Sheppard, D., Taylor, K., Thormann, A., Trevanion, S.J., Vullo, A., Wilder, S.P., Wilson, M., Zadissa, A., Aken, B.L., Birney, E., Cunningham, F., Harrow, J., Herrero, J., Hubbard, T.J.P., Kinsella, R., Muffato, M., Parker, A., Spudich, G., Yates, A., Zerbino, D.R. and Searle, S.M.J. (2014) 'Ensembl 2014', *Nucleic Acids Research*, 42(D1), pp. D749-D755.

Follenius, M. and Brandenberger, G. (1988) 'Increase in atrial natriuretic peptide in response to physical exercise', *European Journal of Applied Physiology and Occupational Physiology*, 57(2), pp. 159-162.

Forones, N.M., Carvalho, A.P.S., Giannotti-Filho, O., Lourenço, L.G. and Oshima, C.T.F. (2005) 'Cell proliferation and apoptosis in gastric cancer and intestinal metaplasia', *Arquivos de Gastroenterologia*, 42, pp. 30-34.

Forouhar, A.S., Hove, J.R., Calvert, C., Flores, J., Jadvar, H. and Gharib, M. (2004). Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-11144276067&partnerID=40&md5=56bfc14b5e33762379e4943f68694946.

Fossel, M. (2000) 'Role of cell senescence in human aging', *Journal of Anti-Aging Medicine*, 3(1), pp. 91-98.

Fujimoto, N., Prasad, A., Hastings, J.L., Arbab-Zadeh, A., Bhella, P.S., Shibata, S., Palmer, D. and Levine, B.D. (2010) 'Cardiovascular Effects of 1 Year of Progressive and Vigorous Exercise Training in Previously Sedentary Individuals Older Than 65 Years of Age', *Circulation*, 122(18), pp. 1797-1805.

Gagliano, N., Arosio, B., Grizzi, F., Masson, S., Tagliabue, J., Dioguardi, N., Vergani, C. and Annoni, G. (2002) 'Reduced collagenolytic activity of matrix metalloproteinases and development of liver fibrosis in the aging rat', *Mechanisms of ageing and development*, 123(4), pp. 413-425.

Gagliano, N., Arosio, B., Santambrogio, D., Balestrieri, M.R., Padoani, G., Tagliabue, J., Masson, S., Vergani, C. and Annoni, G. (2000) 'Age-dependent expression of fibrosis-related genes and collagen deposition in rat kidney cortex', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 55(8), pp. B365-B372.

Gagliano, N., Grizzi, F. and Annoni, G. (2007) 'Mechanisms of aging and liver functions', *Digestive diseases*, 25(2), pp. 118-123.

Gallaugher, P., Thorarensen, H., Kiessling, A. and Farrell, A. (2001) 'Effects of high intensity exercise training on cardiovascular function, oxygen uptake, internal oxygen transport and osmotic balance in chinook salmon (Oncorhynchus tshawytscha) during critical speed swimming', *The Journal of experimental biology*, 204(16), pp. 2861-2872.

Gamerdinger, M., Hajieva, P., Kaya, A.M., Wolfrum, U., Hartl, F.U. and Behl, C. (2009) 'Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3', *The EMBO Journal*, 28(7), pp. 889-901.

Gamperl, A.K. and Farrell, A. (2004) 'Cardiac plasticity in fishes: environmental influences and intraspecific differences', *Journal of Experimental Biology*, 207(15), pp. 2539-2550.

Gardner, D.G. (2003) 'Natriuretic peptides: Markers or modulators of cardiac hypertrophy?', *Trends in Endocrinology and Metabolism*, 14(9), pp. 411-416.

Gardner, D.G., Chen, S., Glenn, D.J. and Grigsby, C.L. (2007) 'Molecular biology of the natriuretic peptide system: Implications for physiology and hypertension', *Hypertension*, 49(3), pp. 419-426.

Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K. and Joo, K. (2003a) 'GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5', *Nature*, 424(6947), pp. 443-447.

Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J.C. and Srivastava, D. (2003b) 'GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5', *Nature*, 424(6947), pp. 443-447.

Garrido, C., Gurbuxani, S., Ravagnan, L. and Kroemer, G. (2001) 'Heat shock proteins: endogenous modulators of apoptotic cell death', *Biochemical and biophysical research communications*, 286(3), pp. 433-442.

Gavin, T.P. and Wagner, P.D. (2001) 'Effect of short-term exercise training on angiogenic growth factor gene responses in rats', *Journal of Applied Physiology*, 90(4), pp. 1219-1226.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S.A. (1992) 'Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation', *The Journal of cell biology*, 119(3), pp. 493-501.

Gazoti Debessa, C.R., Mesiano Maifrino, L.B. and Rodrigues de Souza, R. (2001) 'Age related changes of the collagen network of the human heart', *Mechanisms of ageing and development*, 122(10), pp. 1049-1058.

Gerhard, G.S., Kauffman, E.J., Wang, X., Stewart, R., Moore, J.L., Kasales, C.J., Demidenko, E. and Cheng, K.C. (2002) 'Life spans and senescent phenotypes in two strains of Zebrafish *Danio rerio*', *Experimental gerontology*, 37(8), pp. 1055-1068.

Gerstenblith, G., Frederiksen, J., Yin, F.C., Fortuin, N.J., Lakatta, E.G. and Weisfeldt, M.L. (1977) 'Echocardiographic assessment of a normal adult aging population', *Circulation*, 56(2), pp. 273-8.

Giallauria, F., Acampa, W., Ricci, F., Vitelli, A., Torella, G., Lucci, R., Prete, G., Zampella, E., Assante, R., Rengo, G., Leosco, D., Cuocolo, A. and Vigorito, C. (2013) 'Exercise training early after acute myocardial infarction reduces stress-induced hypoperfusion and improves left ventricular function', *European Journal of Nuclear Medicine and Molecular Imaging*, 40(3), pp. 315-324.

Giallauria, F., Cirillo, P., Lucci, R., Pacileo, M., De Lorenzo, A., D'Agostino, M., Moschella, S., Psaroudaki, M., Del Forno, D. and Orio, F. (2008) 'Left ventricular remodelling in patients with moderate systolic dysfunction after myocardial infarction: favourable effects of exercise training and predictive role of N-terminal pro-brain natriuretic peptide', *European Journal of Cardiovascular Prevention & Rehabilitation*, 15(1), pp. 113-118.

Giallauria, F., Del Forno, D., Pilerci, F., De Lorenzo, A., Manakos, A., Lucci, R. and Vigorito, C. (2005) 'IMPROVEMENT OF HEART RATE RECOVERY AFTER EXERCISE TRAINING IN OLDER PEOPLE', *Journal of the American Geriatrics Society*, 53(11), pp. 2037-2038.

Giannuzzi, P., Temporelli, P.L., Corrà, U., Tavazzi, L. and Group, f.t.E.-C.S. (2003) 'Antiremodeling Effect of Long-Term Exercise Training in Patients With Stable Chronic Heart Failure: Results of the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure (ELVD-CHF) Trial', *Circulation*, 108(5), pp. 554-559.

Gilbert, M.J.H., Zerulla, T.C. and Tierney, K.B. (2014) 'Zebrafish (Danio rerio) as a model for the study of aging and exercise: Physical ability and trainability decrease with age', *Experimental Gerontology*, 50(0), pp. 106-113.

Goedert, M., Cuenda, A., Craxton, M., Jakes, R. and Cohen, P. (1997) 'Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); Comparison of its substrate specificity with that of other SAP kinases', *EMBO Journal*, 16(12), pp. 3563-3571.

Goldshmit, Y., Sztal, T.E., Jusuf, P.R., Hall, T.E., Nguyen-Chi, M. and Currie, P.D. (2012) 'Fgf-dependent glial cell bridges facilitate spinal cord regeneration in zebrafish', *The Journal of Neuroscience*, 32(22), pp. 7477-7492.

Goldsmith, E.C., Bradshaw, A.D., Zile, M.R. and Spinale, F.G. (2014) 'Myocardial Fibroblast-Matrix Interactions and Potential Therapeutic Targets', *Journal of molecular and cellular cardiology*.

Gonzalez-Suarez, E., Geserick, C., Flores, J.M. and Blasco, M.A. (2005) 'Antagonistic effects of telomerase on cancer and aging in K5-mTert transgenic mice', 24(13), pp. 2256-2270.

Gosselin, L.E., Adams, C., Cotter, T.A., McCormick, R.J. and Thomas, D.P. (1998) 'Effect of exercise training on passive stiffness in locomotor skeletal muscle: role of extracellular matrix', *Journal of Applied Physiology*, 85(3), pp. 1011-1016.

Gossett, L.A., Kelvin, D.J., Sternberg, E.A. and Olson, E.N. (1989) 'A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes', *Molecular and Cellular Biology*, 9(11), pp. 5022-5033.

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I. and Brand, M. (2006) 'Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate', *Developmental biology*, 295(1), pp. 263-277.

Gratzner, H.G. (1982) 'Monoclonal antibody to 5-bromo-and 5-iododeoxyuridine: a new reagent for detection of DNA replication', *Science*, 218(4571), pp. 474-475.

Gratzner, H.G., Leif, R.C., Ingram, D.J. and Castro, A. (1975) 'The use of antibody specific for bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells and chromosomes', *Experimental cell research*, 95(1), pp. 88-94.

Green, D.R., Galluzzi, L. and Kroemer, G. (2011) 'Mitochondria and the autophagy–inflammation–cell death axis in organismal aging', *Science*, 333(6046), pp. 1109-1112.

Green, E.L. (1966) 'Biology of the laboratory mouse', *Biology of the laboratory mouse*, (2nd ed).

Greider, C.W. and Blackburn, E.H. (1987) 'The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity', *Cell*, 51(6), pp. 887-898.

Grumati, P., Coletto, L., Schiavinato, A., Castagnaro, S., Bertaggia, E., Sandri, M. and Bonaldo, P. (2011) 'Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles', *Autophagy*, 7(12), pp. 1415-1423.

Guerra, S., Leri, A., Wang, X.W., Finato, N., Di Loreto, C., Beltrami, C.A., Kajstura, J. and Anversa, P. (1999) 'Myocyte death in the failing human heart is gender dependent', *Circulation Research*, 85(9), pp. 856-866.

Gum, R.J., McLaughlin, M.M., Kumar, S., Wang, Z., Bower, M.J., Lee, J.C., Adams, J.L., Livi, G.P., Goldsmith, E.J. and Young, P.R. (1998) 'Acquisition of Sensitivity of Stress-activated Protein Kinases to the p38 Inhibitor, SB 203580, by Alteration of One or More Amino Acids within the ATP Binding Pocket', *Journal of Biological Chemistry*, 273(25), pp. 15605-15610.

Gupta, V. and Poss, K.D. (2012) 'Clonally dominant cardiomyocytes direct heart morphogenesis', *Nature*, 484(7395), pp. 479-484.

Gutierrez, J.A. and Perr, H.A. (1999) 'Mechanical stretch modulates TGF-β1 and α1 (I) collagen expression in fetal human intestinal smooth muscle cells', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 277(5), pp. G1074-G1080.

Hambrecht, R., Wolf, A., Gielen, S., Linke, A., Hofer, J., Erbs, S., Schoene, N. and Schuler, G. (2000) 'Effect of Exercise on Coronary Endothelial Function in Patients with Coronary Artery Disease', *New England Journal of Medicine*, 342(7), pp. 454-460.

Han, X. and Ren, J. (2010) 'Caloric restriction and heart function: is there a sensible link?', *Acta Pharmacologica Sinica*, 31(9), pp. 1111-1117.

Hans, F. and Dimitrov, S. (2001) 'Histone H3 phosphorylation and cell division', *Oncogene*, 20(24), pp. 3021-3027.

Hansen, M., Chandra, A., Mitic, L.L., Onken, B., Driscoll, M. and Kenyon, C. (2008) 'A Role for Autophagy in the Extension of Lifespan by Dietary Restriction in <named-content xmlns:xlink="http://www.w3.org/1999/xlink" content-type="genus-species" xlink:type="simple">C. elegans</named-content>', *PLoS Genet*, 4(2), p. e24.

Harley, C.B., Futcher, A.B. and Greider, C.W. (1990) 'Telomeres shorten during ageing of human fibroblasts'.

Hars, E.S., Qi, H., Jin, S.V., Cai, L., Hu, C. and Liu, L.F. (2007) 'Autophagy regulates ageing in C. elegans', *Autophagy*, 3(2), pp. 93-95.

Harvey, P.A. and Leinwand, L.A. (2011) 'Cellular mechanisms of cardiomyopathy', *The Journal of Cell Biology*, 194(3), pp. 355-365.

Hasegawa, K., Lee, S.J., Jobe, S.M., Markham, B.E. and Kitsis, R.N. (1997) 'cis-Acting sequences that mediate induction of β-myosin heavy chain gene expression during left ventricular hypertrophy due to aortic constriction', *Circulation*, 96(11), pp. 3943-3953.

Hasty, P. and Christy, B.A. (2013) 'p53 as an intervention target for cancer and aging', *Pathobiology of aging & age related diseases*, 3.

Hatcher, C.J., Kim, M.-S., Mah, C.S., Goldstein, M.M., Wong, B., Mikawa, T. and Basson, C.T. (2001) 'TBX5 transcription factor regulates cell proliferation during cardiogenesis', *Developmental biology*, 230(2), pp. 177-188.

Hautala, N., Tokola, H., Luodonpää, M., Puhakka, J., Romppanen, H., Vuolteenaho, O. and Ruskoaho, H. (2001) 'Pressure overload increases GATA4 binding activity via endothelin-1', *Circulation*, 103(5), pp. 730-735.

Haykowsky, M., Scott, J., Esch, B., Schopflocher, D., Myers, J., Paterson, I., Warburton, D., Jones, L. and Clark, A. (2011) 'A Meta-analysis of the effects of Exercise Training on Left Ventricular Remodeling Following Myocardial Infarction: Start early and go longer for greatest exercise benefits on remodeling', *Trials*, 12(1), p. 92.

Haykowsky, M.J., Liang, Y., Pechter, D., Jones, L.W., McAlister, F.A. and Clark, A.M. (2007) 'A Meta-Analysis of the Effect of Exercise Training on Left Ventricular Remodeling in Heart Failure Patients: The Benefit Depends on the Type of Training Performed', *Journal of the American College of Cardiology*, 49(24), pp. 2329-2336.

He, C., Bassik, M.C., Moresi, V., Sun, K., Wei, Y., Zou, Z., An, Z., Loh, J., Fisher, J., Sun, Q., Korsmeyer, S., Packer, M., May, H.I., Hill, J.A., Virgin, H.W., Gilpin, C., Xiao, G., Bassel-Duby, R., Scherer, P.E. and Levine, B. (2012) 'Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis', *Nature*, 481(7382), pp. 511-515.

Hees, P.S., Fleg, J.L., Lakatta, E.G. and Shapiro, E.P. (2002) 'Left ventricular remodeling with age in normal men versus women: Novel insights using three-dimensional magnetic resonance imaging', *The American Journal of Cardiology*, 90(11), pp. 1231-1236.

Heineke, J. and Molkentin, J.D. (2006) 'Regulation of cardiac hypertrophy by intracellular signalling pathways', *Nature Reviews Molecular Cell Biology*, 7(8), pp. 589-600.

Heinemeier, K., Langberg, H. and Kjaer, M. (2003) 'Exercise-induced changes in circulating levels of transforming growth factor-β-1 in humans: methodological considerations', *European Journal of Applied Physiology*, 90(1-2), pp. 171-177.

Heiss, N.S., Knight, S.W., Vulliamy, T.J., Klauck, S.M., Wiemann, S., Mason, P.J., Poustka, A. and Dokal, I. (1998) 'X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions', *Nat Genet*, 19(1), pp. 32-38.

Hendzel, M.J., Wei, Y., Mancini, M.A., Van Hooser, A., Ranalli, T., Brinkley, B.R., Bazett-Jones, D.P. and Allis, C.D. (1997) 'Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation', *Chromosoma*, 106(6), pp. 348-360.

Heng, Y.-W. and Koh, C.-G. (2010) 'Actin cytoskeleton dynamics and the cell division cycle', *The international journal of biochemistry & cell biology*, 42(10), pp. 1622-1633.

Henriques, C.M., Carneiro, M.C., Tenente, I.M., Jacinto, A. and Ferreira, M.G. (2013) 'Telomerase Is Required for Zebrafish Lifespan', *PLoS Genet*, 9(1), p. e1003214.

Her, G.M., Hsu, C.-C., Hong, J.-R., Lai, C.-Y., Hsu, M.-C., Pang, H.-W., Chan, S.-K. and Pai, W.-Y. (2011) 'Overexpression of gankyrin induces liver steatosis in zebrafish (< i> Danio rerio</i>)', *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1811(9), pp. 536-548.

Herzig, T.C., Jobe, S.M., Aoki, H., Molkentin, J.D., Cowley A.W, Jr., Izumo, S. and Markham, B.E. (1997) 'Angiotensin II type(1a) receptor gene expression in the

heart: AP-1 and GATA-4 participate in the response to pressure overload', *Proceedings of the National Academy of Sciences of the United States of America*, 94(14), pp. 7543-7548.

Higami, Y. and Shimokawa, I. (2000) 'Apoptosis in the aging process', *Cell and Tissue Research*, 301(1), pp. 125-132.

Higashi, Y., Sukhanov, S., Anwar, A., Shai, S.-Y. and Delafontaine, P. (2012) 'Aging, Atherosclerosis, and IGF-1', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 67A(6), pp. 626-639.

Hilgemann, D.W. (2004) 'New insights into the molecular and cellular workings of the cardiac Na+/Ca2+ exchanger', *American Journal of Physiology-Cell Physiology*, 287(5), pp. C1167-C1172.

Hill, J.A. and Olson, E.N. (2008) 'Cardiac plasticity', *New England Journal of Medicine*, 358(13), pp. 1370-1380.

Hinck, A.P. (2012) 'Structural studies of the TGF-βs and their receptors–insights into evolution of the TGF-β superfamily', *FEBS letters*, 586(14), pp. 1860-1870.

Hinits, Y., Pan, L., Walker, C., Dowd, J., Moens, C.B. and Hughes, S.M. (2012) 'Zebrafish Mef2ca and Mef2cb are essential for both first and second heart field cardiomyocyte differentiation', *Developmental biology*, 369(2), pp. 199-210.

Hinrichs, S., Heger, J., Schreckenberg, R., Wenzel, S., Euler, G., Arens, C., Bader, M., Rosenkranz, S., Caglayan, E. and Schlüter, K.-D. (2011) 'Controlling cardiomyocyte length: the role of renin and PPAR-γ', *Cardiovascular research*, 89(2), pp. 344-352.

Hinton, D.E. and Williams, W.L. (1968) 'Hepatic fibrosis associated with aging in four stocks of mice', *Journal of gerontology*, 23(2), pp. 205-211.

Ho, Y.-L., Shau, Y.-W., Tsai, H.-J., Lin, L.-C., Huang, P.-J. and Hsieh, F.-J. (2002) 'Assessment of zebrafish cardiac performance using Doppler echocardiography and power angiography', *Ultrasound in medicine & biology*, 28(9), pp. 1137-1143.

Hollmann, W., Strüder, H.K., Tagarakis, C.V.M. and King, G. (2007) 'Physical activity and the elderly', *European Journal of Cardiovascular Prevention & Rehabilitation*, 14(6), pp. 730-739.

Holmes, J.W., Borg, T.K. and Covell, J.W. 7 (2005) 'Structure and mechanics of healing myocardial infarcts'. pp. 223-253. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-23844523196&partnerID=40&md5=9a6b40944260a5bc6863037753573057.

Hoover, L.L., Burton, E.G., Brooks, B.A. and Kubalak, S.W. (2008) 'The expanding role for retinoid signaling in heart development', *The Scientific World Journal*, 8, pp. 194-211.

Hsieh, P.C.H., Segers, V.F.M., Davis, M.E., MacGillivray, C., Gannon, J., Molkentin, J.D., Robbins, J. and Lee, R.T. (2007) 'Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury', *Nature medicine*, 13(8), pp. 970-974.

Hu, N., Yost, H.J. and Clark, E.B. (2001) 'Cardiac morphology and blood pressure in the adult zebrafish', *The Anatomical Record*, 264(1), pp. 1-12.

Hua, H. and Kearsey, S.E. (2011) 'Monitoring DNA replication in fission yeast by incorporation of 5-ethynyl-2′ -deoxyuridine', *Nucleic acids research*, 39(9), pp. e60-e60.

Hum, D., Besnard, S., Sanchez, R., Devost, D., Gossard, F., Hamet, P. and Tremblay, J. (2004) 'Characterization of a cGMP-response element in the guanylyl cyclase/natriuretic peptide receptor A gene promoter', *Hypertension*, 43(6), pp. 1270-1278.

Ieda, M., Fu, J.-D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G. and Srivastava, D. (2010) 'Direct Reprogramming of Fibroblasts into Functional Cardiomyocytes by Defined Factors', *Cell*, 142(3), pp. 375-386.

Ing, D.J., Zang, J., Dzau, V.J., Webster, K.A. and Bishopric, N.H. (1999) 'Modulation of Cytokine-Induced Cardiac Myocyte Apoptosis by Nitric Oxide, Bak, and Bcl-x', *Circulation Research*, 84(1), pp. 21-33.

Itahana, K., Campisi, J. and Dimri, G.P. (2004) 'Mechanisms of cellular senescence in human and mouse cells', *Biogerontology*, 5(1), pp. 1-10.

Itou, J., Kawakami, H., Burgoyne, T. and Kawakami, Y. (2012a) 'Life-long preservation of the regenerative capacity in the fin and heart in zebrafish', *Biology Open*, 1(8), pp. 739-746.

Itou, J., Oishi, I., Kawakami, H., Glass, T.J., Richter, J., Johnson, A., Lund, T.C. and Kawakami, Y. (2012b) 'Migration of cardiomyocytes is essential for heart regeneration in zebrafish', *Development*, 139(22), pp. 4133-4142.

Izumo, S., Lompr, xE, M., A., Matsuoka, R., Koren, G., Schwartz, K., Nadal-Ginard, B. and Mahdavi, V. (1987) 'Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. Interaction between hemodynamic and thyroid hormone-induced signals', *The Journal of Clinical Investigation*, 79(3), pp. 970-977.

Izumo, S., Nadal-Ginard, B. and Mahdavi, V. (1988) 'Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload', *Proceedings of the National Academy of Sciences*, 85(2), pp. 339-343.

Jaskelioff, M., Muller, F.L., Paik, J.-H., Thomas, E., Jiang, S., Adams, A.C., Sahin, E., Kost-Alimova, M., Protopopov, A., Cadinanos, J., Horner, J.W., Maratos-Flier, E. and DePinho, R.A. (2011) 'Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice', *Nature*, 469(7328), pp. 102-106.

Jay, P.Y., Harris, B.S., Maguire, C.T., Buerger, A., Wakimoto, H., Tanaka, M., Kupershmidt, S., Roden, D.M., Schultheiss, T.M., O'Brien, T.X., Gourdie, R.G., Berul, C.I. and Izumo, S. (2004) 'Nkx2-5 mutation causes anatomic hypoplasia of the cardiac conduction system', *Journal of Clinical Investigation*, 113(8), pp. 1130-1137.

Jean, M.J., deVerteuil, P., Lopez, N.H., Tapia, J.D. and Schoffstall, B. (2012) 'Adult Zebrafish Hearts Efficiently Compensate for Excessive Forced Overload Cardiac Stress with Hyperplastic Cardiomegaly', *BioResearch open access*, 1(2), pp. 88-91.

Jeppesen, J., Jordy, A.B., Sjøberg, K.A., Füllekrug, J., Stahl, A., Nybo, L. and Kiens, B. (2012) 'Enhanced Fatty Acid Oxidation and FATP4 Protein Expression after Endurance Exercise Training in Human Skeletal Muscle', *PloS one*, 7(1), p. e29391.

Jin, H., Yang, R., Li, W., Lu, H., Ryan, A.M., Ogasawara, A.K., Peborgh, J. and Paoni, N.F. (2000) 'Effects of exercise training on cardiac function, gene expression, and apoptosis in rats', *American journal of physiology*, 279(2), pp. H2994-H3002.

Johansen, K. (1965) 'CARDIOVASCULAR DYNAMICS IN FISHES, AMPHIBIANS, AND REPTILES*', *Annals of the New York Academy of Sciences*, 127(1), pp. 414-442.

Jonker, S.S., Giraud, M.K., Giraud, G.D., Chattergoon, N.N., Louey, S., Davis, L.E., Faber, J. and Thornburg, K.L. (2010) 'Cardiomyocyte enlargement, proliferation and maturation during chronic fetal anaemia in sheep', *Experimental physiology*, 95(1), pp. 131-139.

Jonker, S.S., Zhang, L., Louey, S., Giraud, G.D., Thornburg, K.L. and Faber, J.J. (2007) 'Myocyte enlargement, differentiation, and proliferation kinetics in the fetal sheep heart', *Journal of Applied Physiology*, 102(3), pp. 1130-1142.

Jopling, C., Boue, S. and Belmonte, J.C.I. (2011) 'Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration', *Nature Reviews Molecular Cell Biology*, 12(2), pp. 79-89.

Jopling, C., Sleep, E., Raya, M., Marti, M., Raya, A. and Belmonte, J.C.I. (2010) 'Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation', *Nature*, 464(7288), pp. 606-609.

Jopling, C., Suñè, G., Morera, C. and Belmote, J.C.I. (2012) 'p38α MAPK regulates myocardial regeneration in zebrafish', *Cell Cycle*, 11(6), pp. 1195-1201.

Ju, J.-S., Miller, S.E., Jackson, E., Cadwell, K., Piwnica-Worms, D. and Weihl, C.C. (2009) 'Quantitation of selective autophagic protein aggregate degradation in vitro and in vivo using luciferase reporters', *Autophagy*, 5(4), pp. 511-519.

Jugdutt, B.I. (2003) 'Remodeling of the myocardium and potential targets in the collagen degradation and synthesis pathways', *Current Drug Targets - Cardiovascular and Haematological Disorders*, 3(1), pp. 1-30.

Juhaszova, M., Rabuel, C., Zorov, D.B., Lakatta, E.G. and Sollott, S.J. (2005) 'Protection in the aged heart: preventing the heart-break of old age?', *Cardiovascular research*, 66(2), pp. 233-244.

Jung, T., Bader, N. and Grune, T. (2007) 'Lipofuscin', *Annals of the New York Academy of Sciences*, 1119(1), pp. 97-111.

Kaiser, R.A., Liang, Q., Bueno, O., Huang, Y., Lackey, T., Klevitsky, R., Hewett, T.E. and Molkentin, J.D. (2005) 'Genetic inhibition or activation of JNK1/2 protects the myocardium from ischemia-reperfusion-induced cell death in vivo', *Journal of Biological Chemistry*, 280(38), pp. 32602-32608.

Kajstura, J., Cheng, W., Sarangarajan, R., Li, P., Li, B., Nitahara, J.A., Chapnick, S., Reiss, K., Olivetti, G. and Anversa, P. (1996) 'Necrotic and apoptotic myocyte cell death in the aging heart of Fischer 344 rats', *American Journal of Physiology-Heart and Circulatory Physiology*, 271(3), pp. H1215-H1228.

Kajstura, J., Gurusamy, N., Ogórek, B., Goichberg, P., Clavo-Rondon, C., Hosoda, T., D'Amario, D., Bardelli, S., Beltrami, A.P., Cesselli, D., Bussani, R., del Monte, F., Quaini, F., Rota, M., Beltrami, C.A., Buchholz, B.A., Leri, A. and Anversa, P. (2010a) 'Myocyte Turnover in the Aging Human Heart', *Circulation Research*, 107(11), pp. 1374-1386.

Kajstura, J., Leri, A., Finato, N., Di Loreto, C., Beltrami, C.A. and Anversa, P. (1998) 'Myocyte proliferation in end-stage cardiac failure in humans', *Proceedings of the National Academy of Sciences*, 95(15), pp. 8801-8805.

Kajstura, J., Rota, M., Cappetta, D., Ogórek, B., Arranto, C., Bai, Y., Ferreira-Martins, J., Signore, S., Sanada, F. and Matsuda, A. (2012a) 'Cardiomyogenesis in the Aging and Failing Human HeartClinical Perspective', *Circulation*, 126(15), pp. 1869-1881.

Kajstura, J., Rota, M., Hosoda, T., Anversa, P. and Leri, A. (2012b) 'Response to Bergmann et al: Carbon 14 Birth Dating of Human Cardiomyocytes', *Circulation Research*, 110(1), pp. e19-e21.

Kajstura, J., Urbanek, K., Perl, S., Hosoda, T., Zheng, H., Ogórek, B., Ferreira-Martins, J., Goichberg, P., Rondon-Clavo, C. and Sanada, F. (2010b) 'Cardiomyogenesis in the adult human heart', *Circ Res*, 107(2), pp. 305-315.

Kang, P.M., Haunstetter, A., Aoki, H., Usheva, A. and Izumo, S. (2000) 'Morphological and Molecular Characterization of Adult Cardiomyocyte Apoptosis During Hypoxia and Reoxygenation', *Circulation Research*, 87(2), pp. 118-125.

Karamboulas, C., Dakubo, G.D., Liu, J., De Repentigny, Y., Yutzey, K., Wallace, V.A., Kothary, R. and Skerjanc, I.S. (2006) 'Disruption of MEF2 activity in cardiomyoblasts inhibits cardiomyogenesis', *Journal of cell science*, 119(20), pp. 4315-4321.

Kasahara, H., Bartunkova, S., Schinke, M., Tanaka, M. and Izumo, S. (1998) 'Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein', *Circulation Research*, 82(9), pp. 936-946.

Kasahara, H., Ueyama, T., Wakimoto, H., Liu, M.K., Maguire, C.T., Converso, K.L., Kang, P.M., Manning, W.J., Lawitts, J., Paul, D.L., Berul, C.I. and Izumo, S. (2003) 'Nkx2.5 homeoprotein regulates expression of gap junction protein connexin 43 and sarcomere organization in postnatal cardiomyocytes', *Journal of molecular and cellular cardiology*, 35(3), pp. 243-256.

Kaslin, J. and Brand, M. (2013) 'Cerebellar Development and Neurogenesis in Zebrafish', *Handbook of the Cerebellum and Cerebellar Disorders*, pp. 1441-1462.

Kelleher, F.C., O'Sullivan, H., Smyth, E., McDermott, R. and Viterbo, A. (2013) 'Fibroblast growth factor receptors, developmental corruption and malignant disease', *Carcinogenesis*, 34(10), pp. 2198-2205.

Kelly, R.G., Brown, N.A. and Buckingham, M.E. (2001) 'The Arterial Pole of the Mouse Heart Forms from< i> Fgf10</i>-Expressing Cells in Pharyngeal Mesoderm', *Developmental Cell*, 1(3), pp. 435-440.

Kerkelä, R., Pikkarainen, S., Majalahti-Palviainen, T., Tokola, H. and Ruskoaho, H. (2002) 'Distinct roles of mitogen-activated protein kinase pathways in GATA-4 transcription factor-mediated regulation of B-type natriuretic peptide gene', *Journal of Biological Chemistry*, 277(16), pp. 13752-13760.

Kikuchi, K., Holdway, Jennifer E., Major, Robert J., Blum, N., Dahn, Randall D., Begemann, G. and Poss, Kenneth D. (2011a) 'Retinoic Acid Production by

Endocardium and Epicardium Is an Injury Response Essential for Zebrafish Heart Regeneration', *Developmental Cell*, 20(3), pp. 397-404.

Kikuchi, K., Holdway, J.E., Major, R.J., Blum, N., Dahn, R.D., Begemann, G. and Poss, K.D. (2011b) 'Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration', *Dev Cell*, 20(3), pp. 397-404.

Kikuchi, K., Holdway, J.E., Werdich, A.A., Anderson, R.M., Fang, Y., Egnaczyk, G.F., Evans, T., Macrae, C.A., Stainier, D.Y. and Poss, K.D. (2010) 'Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes', *Nature*, 464(7288), pp. 601-5.

Kikuchi, K. and Poss, K.D. (2012) 'Cardiac regenerative capacity and mechanisms', *Annual review of cell and developmental biology*, 28, p. 719.

Kilic, A., Velic, A., De Windt, L.J., Fabritz, L., Voss, M., Mitko, D., Zwiener, M., Baba, H.A., Van Eickels, M., Schlatter, E. and Kuhn, M. (2005) 'Enhanced activity of the myocardial Na+/H+ exchanger NHE-1 contributes to cardiac remodeling in atrial natriuretic peptide receptor-deficient mice', *Circulation*, 112(15), pp. 2307-2317.

Kim, H.-d., Kim, D.-j., Lee, I.-j., Rah, B.-j., Sawa, Y. and Schaper, J. (1992) 'Human fetal heart development after mid-term: morphometry and ultrastructural study', *Journal of molecular and cellular cardiology*, 24(9), pp. 949-965.

Kim, J.-H., Ko, J.-H., Lee, D.-c., Lim, I. and Bang, H. (2012a) 'Habitual physical exercise has beneficial effects on telomere length in postmenopausal women', *Menopause*, 19(10), pp. 1109-1115.

Kim, T.H., Yu, S.H., Choi, S.H., Yoon, J.W., Kang, S.M., Chun, E.J., Choi, S.I., Shin, H., Lee, H.K., Park, K.S., Jang, H.C. and Lim, S. (2011) 'Pericardial Fat Amount Is an Independent Risk Factor of Coronary Artery Stenosis Assessed by Multidetector-Row Computed Tomography: The Korean Atherosclerosis Study 2', *Obesity*, 19(5), pp. 1028-1034.

Kim, Y.A., Kim, Y.S. and Song, W. (2012b) 'Autophagic response to a single bout of moderate exercise in murine skeletal muscle', *Journal of physiology and biochemistry*, 68(2), pp. 229-235.

Kimmel, R.A. and Meyer, D. (2010) 'Molecular regulation of pancreas development in zebrafish', *Methods Cell Biol*, 100, pp. 261-280.

Kinsella, T.J. (1992) *Seminars in oncology*. WB SAUNDERS CO INDEPENDENCE SQUARE WEST CURTIS CENTER, STE 300, PHILADELPHIA, PA 19106-3399.

Kishi, S., Bayliss, P.E., Uchiyama, J., Koshimizu, E., Qi, J., Nanjappa, P., Imamura, S., Islam, A., Neuberg, D. and Amsterdam, A. (2008) 'The identification of zebrafish mutants showing alterations in senescence-associated biomarkers', *PLoS genetics*, 4(8), p. e1000152.

Kishi, S., Uchiyama, J., Baughman, A.M., Goto, T., Lin, M.C. and Tsai, S.B. (2003) 'The zebrafish as a vertebrate model of functional aging and very gradual senescence', *Experimental gerontology*, 38(7), pp. 777-786.

Kishimoto, I., Rossi, K. and Garbers, D.L. (2001) 'A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac ventricular myocyte hypertrophy', *Proceedings of the National Academy of Sciences of the United States of America*, 98(5), pp. 2703-2706.

Kisielewska, J., Lu, P. and Whitaker, M. (2005) 'GFP—PCNA as an S‑phase marker in embryos during the first and subsequent cell cycles', *Biology of the Cell*, 97(3), pp. 221-229.

Kitta, K., Clément, S.A., Remeika, J., Blumberg, J.B. and Suzuki, Y.J. (2001) 'Endothelin-1 induces phosphorylation of GATA-4 transcription factor in the HL-1 atrial-muscle cell line', *Biochemical Journal*, 359(2), pp. 375-380.

Knowles, J.W., Esposito, G., Mao, L., Hagaman, J.R., Fox, J.E., Smithies, O., Rockman, H.A. and Maeda, N. (2001) 'Pressure-independent enhancement of cardiac hypertrophy in natriuretic peptide receptor A-deficient mice', *Journal of Clinical Investigation*, 107(8), pp. 975-984.

Koglin, J., Pehlivanli, S., Schwaiblmair, M., Vogeser, M., Cremer, P. and VonScheidt, W. (2001) 'Role of brain natriuretic peptide in risk stratification of patients with congestive heart failure', *Journal of the American College of Cardiology*, 38(7), pp. 1934-1941.

Kohl, P., Camelliti, P., Burton, F.L. and Smith, G.L. (2005) 'Electrical coupling of fibroblasts and myocytes: Relevance for cardiac propagation', *Journal of Electrocardiology*, 38(4 SUPPL.), pp. 45-50.

Komuro, I. and Izumo, S. (1993) 'Csx: A murine homeobox-containing gene specifically expressed in the developing heart', *Proceedings of the National Academy of Sciences of the United States of America*, 90(17), pp. 8145-8149.

Konishi, M., Sugiyama, S., Sugamura, K., Nozaki, T., Matsubara, J., Akiyama, E., Utsunomiya, D., Matsuzawa, Y., Yamashita, Y., Kimura, K., Umemura, S. and Ogawa, H. (2012) 'Accumulation of pericardial fat correlates with left ventricular diastolic dysfunction in patients with normal ejection fraction', *Journal of Cardiology*, 59(3), pp. 344-351.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. and Grosveld, F. (1999) 'The transcription factor GATA6 is essential for early extraembryonic development', *Development*, 126(4), pp. 723-732.

Kranias, E.G. and Hajjar, R.J. (2012) 'Modulation of Cardiac Contractility by the Phopholamban/SERCA2a Regulatome', *Circulation Research*, 110(12), pp. 1646-1660.

Krens, S.F., He, S., Spaink, H.P. and Snaar-Jagalska, B.E. (2006) 'Characterization and expression patterns of the MAPK family in zebrafish', *Gene expression patterns*, 6(8), pp. 1019-1026.

Kroemer, G. and Levine, B. (2008) 'Autophagic cell death: the story of a misnomer', *Nat Rev Mol Cell Biol*, 9(12), pp. 1004-1010.

Krysko, D.V., Vanden Berghe, T., D'Herde, K. and Vandenabeele, P. (2008) 'Apoptosis and necrosis: detection, discrimination and phagocytosis', *Methods*, 44(3), pp. 205-221.

Kuhn, M., Voß, M., Mitko, D., Stypmann, J., Schmid, C., Kawaguchi, N., Grabellus, F. and Baba, H.A. (2004) 'Left ventricular assist device support reverses altered cardiac expression and function of natriuretic peptides and receptors in end-stage heart failure', *Cardiovascular research*, 64(2), pp. 308-314.

Kuma, Y., Sabio, G., Bain, J., Shpiro, N., Márquez, R. and Cuenda, A. (2005) 'BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo', *Journal of Biological Chemistry*, 280(20), pp. 19472-19479.

Kumar, S., Sharma, P., Arora, K., Raje, M. and Guptasarma, P. (2014) 'Calcium Binding to Beta-2-Microglobulin at Physiological Ph Drives the Occurrence of Conformational Changes Which Cause the Protein to Precipitate into Amorphous Forms That Subsequently Transform into Amyloid Aggregates', *PloS one*, 9(4), p. e95725.

Kung, G., Konstantinidis, K. and Kitsis, R.N. (2011) 'Programmed Necrosis, Not Apoptosis, in the Heart', *Circulation Research*, 108(8), pp. 1017-1036.

Kuo, C.T., Morrisey, E.E., Anandappa, R., Sigrist, K., Lu, M.M., Parmacek, M.S., Soudais, C. and Leiden, J.M. (1997) 'GATA4 transcription factor is required for ventral morphogenesis and heart tube formation', *Genes and Development*, 11(8), pp. 1048-1060.

Kwak, H.-B. (2013) 'Aging, exercise, and extracellular matrix in the heart ', *J Exerc Rehabil*, 9(3), pp. 338-347.

Kwak, H.-B., Kim, J.-h., Joshi, K., Yeh, A., Martinez, D.A. and Lawler, J.M. (2011) 'Exercise training reduces fibrosis and matrix metalloproteinase dysregulation in the aging rat heart', *The FASEB journal*, 25(3), pp. 1106-1117.

Kwak, H.-B., Song, W. and Lawler, J.M. (2006) 'Exercise training attenuates age-induced elevation in Bax/Bcl-2 ratio, apoptosis, and remodeling in the rat heart', *The FASEB journal*.

Lafontant, P.J., Behzad, A.R., Brown, E., Landry, P., Hu, N. and Burns, A.R. (2013) 'Cardiac Myocyte Diversity and a Fibroblast Network in the Junctional Region of the Zebrafish Heart Revealed by Transmission and Serial Block-Face Scanning Electron Microscopy', *PloS one*, 8(8), p. e72388.

Lakatta, E.G. (1993) 'Cardiovascular regulatory mechanisms in advanced age', *Physiological Reviews*, 73(2), pp. 413-67.

Lakatta, E.G. (1999) 'Circulatory function in younger and older humans in health', *Principles of Geriatric Medicine and Gerontology*, pp. 645-660.

Lakatta, E.G. (2002) 'Age-associated cardiovascular changes in health: impact on cardiovascular disease in older persons', *Heart Failure Reviews*, 7(1), pp. 29-49.

Lamas, G.A., Vaughan, D.E., Parisi, A.F. and Pfeffer, M.A. (1989) 'Effects of left ventricular shape and captopril therapy on exercise capacity after anterior wall acute myocardial infarction', *The American Journal of Cardiology*, 63(17), pp. 1167-1173.

Laughlin, M.H. (1985) 'Effects of exercise training on coronary transport capacity', *Journal of Applied Physiology*, 58(2), pp. 468-476.

Laughlin, M.H., Overholser, K.A. and Bhatte, M.J. (1989) 'Exercise training increases coronary transport reserve in miniature swine', *Journal of Applied Physiology*, 67(3), pp. 1140-1149.

Lawson, N.D., Vogel, A.M. and Weinstein, B.M. (2002) 'Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation', *Developmental Cell*, 3(1), pp. 127-136.

Lawson, N.D. and Weinstein, B.M. (2002) 'In vivo imaging of embryonic vascular development using transgenic zebrafish', *Developmental biology*, 248(2), pp. 307-318.

Lee, I.-M. and Skerrett, P.J. (2001) 'Physical activity and all-cause mortality: what is the dose-response relation?', *Medicine and Science in Sports and Exercise*, 33(6; SUPP), pp. S459-S471.

Lee, I.M., Shiroma, E.J., Lobelo, F., Puska, P., Blair, S.N. and Katzmarzyk, P.T. (2012) 'Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy', *The Lancet*, 380(9838), pp. 219-229.

Lemasters, J.J., Qian, T., Bradham, C.A., Brenner, D.A., Cascio, W.E., Trost, L.C., Nishimura, Y., Nieminen, A.-L. and Herman, B. (1999) 'Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death', *Journal of bioenergetics and biomembranes*, 31(4), pp. 305-319.

Lemke, L.E., Bloem, L.J., Fouts, R., Esterman, M., Sandusky, G. and Vlahos, C.J. (2001) 'Decreased p38 MAPK Activity in End-Stage Failing Human Myocardium: p38 MAPK< i> α</i> is the Predominant Isoform Expressed in Human Heart', *Journal of molecular and cellular cardiology*, 33(8), pp. 1527-1540.

Lepilina, A., Coon, A.N., Kikuchi, K., Holdway, J.E., Roberts, R.W., Burns, C.G. and Poss, K.D. (2006) 'A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration', *Cell*, 127(3), pp. 607-19.

Leri, A., Kajstura, J. and Anversa, P. (2005) 'Cardiac Stem Cells and Mechanisms of Myocardial Regeneration', *Physiological Reviews*, 85(4), pp. 1373-1416.

Levine, B. and Kroemer, G. (2009) 'Autophagy in aging, disease and death: the true identity of a cell death impostor', *Cell Death Differ*, 16(1), pp. 1-2.

Li, F., Wang, X., Bunger, P.C. and Gerdes, A.M. (1997a) 'Formation of binucleated cardiac myocytes in rat heart: I. Role of actin–myosin contractile ring', *Journal of molecular and cellular cardiology*, 29(6), pp. 1541-1551.

Li, F., Wang, X., Capasso, J.M. and Gerdes, A.M. (1996) 'Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development', *Journal of molecular and cellular cardiology*, 28(8), pp. 1737-1746.

Li, N., Yang, H., Lu, L., Duan, C., Zhao, C. and Zhao, H. (2008) 'Comparison of the labeling efficiency of BrdU, DiI and FISH labeling techniques in bone marrow stromal cells', *Brain research*, 1215, pp. 11-19.

Li, Q., Zhou, L.-Y., Gao, G.-F., Jiao, J.-Q. and Li, P.-F. (2012) 'Mitochondrial network in the heart', *Protein & Cell*, 3(6), pp. 410-418.

Li, Q.Y., Newbury-Ecob, R.A., Terrett, J.A., Wilson, D.I., Curtis, A.R.J., Yi, C.H., Gebuhr, T., Bullen, P.J., Robson, S.C. and Strachan, T. (1997b) 'Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family', *Nature Genetics*, 15(1), pp. 21-29.

Liang, Q., De Windt, L.J., Witt, S.A., Kimball, T.R., Markham, B.E. and Molkentin, J.D. (2001) 'The Transcription Factors GATA4 and GATA6 Regulate Cardiomyocyte Hypertrophy in Vitro and in Vivo', *Journal of Biological Chemistry*, 276(32), pp. 30245-30253.

Liao, P., Wang, S.-Q., Wang, S., Zheng, M., Zheng, M., Zhang, S.-J., Cheng, H., Wang, Y. and Xiao, R.-P. (2002) 'p38 Mitogen-activated protein kinase mediates a negative inotropic effect in cardiac myocytes', *Circulation Research*, 90(2), pp. 190-196.

Liebl, J.C. (2011) *C-14 bomb peak dating of human DNA samples at the microgram level*. uniwien.

Lin, J., Lopez, E.F., Jin, Y., Van Remmen, H., Bauch, T., Han, H.-C. and Lindsey, M.L. (2008) 'Age-related cardiac muscle sarcopenia: Combining experimental and mathematical modeling to identify mechanisms', *Experimental gerontology*, 43(4), pp. 296-306.

Lin, Q., Schwarz, J., Bucana, C. and Olson, E.N. (1997) 'Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C', *Science*, 276(5317), pp. 1404-1407.

Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I. and Harvey, R.P. (1993) 'Nkx-2.5: A novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants', *Development*, 119(2), pp. 419-431.

Liu, J., Bressan, M., Hassel, D., Huisken, J., Staudt, D., Kikuchi, K., Poss, K.D., Mikawa, T. and Stainier, D.Y.R. (2010) 'A dual role for ErbB2 signaling in cardiac trabeculation', *Development*, 137(22), pp. 3867-3875.

Liu, L., Azhar, G., Gao, W., Zhang, X. and Wei, J.Y. (1998) 'Bcl-2 and Bax expression in adult rat hearts after coronary occlusion: age-associated differences', *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 275(1), pp. R315-R322.

Liu, M., Zhang, P., Chen, M., Zhang, W., Yu, L., Yang, X.-C. and Fan, Q. (2012) 'Aging might increase myocardial ischemia/reperfusion-induced apoptosis in humans and rats', *Age*, 34(3), pp. 621-632.

Livak, K.J. and Schmittgen, T.D. (2001) 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method', *Methods*, 25(4), pp. 402-408.

Lloyd-Jones, D.M., Larson, M.G., Leip, E.P., Beiser, A., D'Agostino, R.B., Kannel, W.B., Murabito, J.M., Vasan, R.S., Benjamin, E.J. and Levy, D. (2002) 'Lifetime Risk for Developing Congestive Heart Failure: The Framingham Heart Study', *Circulation*, 106(24), pp. 3068-3072.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M. and Kroemer, G. (2013) 'The Hallmarks of Aging', *Cell*, 153(6), pp. 1194-1217.

Lopez, M.J., Garbers, D.L. and Kuhn, M. (1997) 'The guanylyl cyclase-deficient mouse defines differential pathways of natriuretic peptide signaling', *Journal of Biological Chemistry*, 272(37), pp. 23064-23068.

Lyons, I., Parsons, L.M., Hartley, L., Li, R., Andrews, J.E., Robb, L. and Harvey, R.P. (1995) 'Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5', *Genes and Development*, 9(13), pp. 1654-1666.

MacLellan, W.R., Garcia, A., Oh, H., Frenkel, P., Jordan, M.C., Roos, K.P. and Schneider, M.D. (2005) 'Overlapping roles of pocket proteins in the myocardium are unmasked by germ line deletion of p130 plus heart-specific deletion of Rb', *Molecular and cellular biology*, 25(6), pp. 2486-2497.

MacLellan, W.R. and Schneider, M.D. (2000) 'Genetic dissection of cardiac growth control pathways', *Annual Review of Physiology*, 62(1), pp. 289-320.

Malliaras, K., Zhang, Y., Seinfeld, J., Galang, G., Tseliou, E., Cheng, K., Sun, B., Aminzadeh, M. and Marbán, E. (2013) 'Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart', *EMBO molecular medicine*, 5(2), pp. 191-209.

Mann, D.L. and Spinale, F.G. (1998) 'Activation of matrix metalloproteinases in the failing human heart: Breaking the tie that binds', *Circulation*, 98(17), pp. 1699-1702.

Manson, J.E., Greenland, P., LaCroix, A.Z., Stefanick, M.L., Mouton, C.P., Oberman, A., Perri, M.G., Sheps, D.S., Pettinger, M.B. and Siscovick, D.S. (2002) 'Walking Compared with Vigorous Exercise for the Prevention of Cardiovascular Events in Women', *New England Journal of Medicine*, 347(10), pp. 716-725.

Maron, B.J., Roberts, W.C., Arad, M. and et al. (2009) 'CLinical outcome and phenotypic expression in lamp2 cardiomyopathy', *JAMA*, 301(12), pp. 1253-1259.

Matz, D.G., Oberpriller, J.O. and Oberpriller, J.C. (1998) 'Comparison of mitosis in binucleated and mononucleated newt cardiac myocytes', *The Anatomical Record*, 251(2), pp. 245-255.

Maulik, N., Sasaki, H., Addya, S. and Das, D.K. (2000) 'Regulation of cardiomyocyte apoptosis by redox-sensitive transcription factors', *FEBS letters*, 485(1), pp. 7-12.

Maulik, N., Yoshida, T., Engelman, R.M., Deaton, D., Flack Iii, J.E., Rousou, J.A. and Das, D.K. (1998) 'Ischemic preconditioning attenuates apoptotic cell death associated with ischemia/reperfusion', in *Myocardial Ischemia and Reperfusion*. Springer, pp. 139-145.

Mays, P.K., Bishop, J.E. and Laurent, G.J. (1988) 'Age-related changes in the proportion of types I and III collagen', *Mechanisms of ageing and development*, 45(3), pp. 203-212.

McClelland, G.B., Craig, P.M., Dhekney, K. and Dipardo, S. (2006) 'Temperature‐and exercise‐induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (Danio rerio)', *The Journal of physiology*, 577(2), pp. 739-751.

McCurley, A.T. and Callard, G.V. (2008) 'Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment', *BMC molecular biology*, 9(1), p. 102.

McMullen, J.R. (2008) 'ROLE OF INSULIN-LIKE GROWTH FACTOR 1 AND PHOSPHOINOSITIDE 3-KINASE IN A SETTING OF HEART DISEASE', *Clinical and Experimental Pharmacology and Physiology*, 35(3), pp. 349-354.

McMullen, J.R., Shioi, T., Zhang, L., Tarnavski, O., Sherwood, M.C., Kang, P.M. and Izumo, S. (2003) 'Phosphoinositide 3-kinase(p110α) plays a critical role for the induction of physiological, but not pathological, cardiac hypertrophy', *Proceedings of the National Academy of Sciences*, 100(21), pp. 12355-12360.

Messier, B.L., C.P.; Smart, I (1958) 'Presence of DNA synthesis and mitosis in the brain of young adult mice', *Exp. Cell Res.*, 14, pp. 224-226.

Michalopoulos, G.K. (2011) 'Liver regeneration: alternative epithelial pathways', *The international journal of biochemistry & cell biology*, 43(2), pp. 173-179.

Mignotte, B. and Vayssiere, J.-L. (1998) 'Mitochondria and apoptosis', *European Journal of Biochemistry*, 252(1), pp. 1-15.

Mizushima, N., Levine, B., Cuervo, A.M. and Klionsky, D.J. (2008) 'Autophagy fights disease through cellular self-digestion', *Nature*, 451(7182), pp. 1069-1075.

Mjaatvedt, C.H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R.A., Kern, M.J., Eisenberg, C.A., Turner, D. and Markwald, R.R. (2001) 'The outflow tract of the heart is recruited from a novel heart-forming field', *Developmental biology*, 238(1), pp. 97-109.

Moe, G.W. (2006) 'B-type natriuretic peptide in heart failure', *Current Opinion in Cardiology*, 21(3), pp. 208-214.

Molkentin, J.D. (2000) 'The zinc finger-containing transcription factors GATA-4, -5, and -6: Ubiquitously expressed regulators of tissue-specific gene expression', *Journal of Biological Chemistry*, 275(50), pp. 38949-38952.

Molkentin, J.D., Lin, Q., Duncan, S.A. and Olson, E.N. (1997) 'Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis', *Genes and Development*, 11(8), pp. 1061-1072.

Molkentin, J.D. and Markham, B.E. (1993) 'Myocyte-specific enhancer-binding factor (MEF-2) regulates α-cardiac myosin heavy chain gene expression in vitro and in vivo', *Journal of Biological Chemistry*, 268(26), pp. 19512-19520.

Molkentin, J.D. and Olson, E.N. (1996) 'Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors', *Proceedings of the National Academy of Sciences of the United States of America*, 93(18), pp. 9366-9373.

Mommsen, T. (1998) *The Physiology of Fishes*.

Montani, J.P., Carroll, J.F., Dwyer, T.M., Antic, V., Yang, Z. and Dulloo, A.G. (2004) 'Ectopic fat storage in heart, blood vessels and kidneys in the pathogenesis of cardiovascular diseases', *Int J Obes Relat Metab Disord*, 28(S4), pp. S58-S65.

Morales, R., Herrera, M.T., Arenal, A., Cruz, A., Hernández, O., Pimentel, R., Guillén, I., Martínez, R. and Estrada, M.P. (2001) 'Tilapia chromosomal growth hormone gene expression accelerates growth in transgenic zebrafish (Danio rerio)', *Electronic Journal of Biotechnology*, 4(2), pp. 5-6.

Moretti, A., Caron, L., Nakano, A., Lam, J.T., Bernshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M. and Martin-Puig, S. (2006) 'Multipotent Embryonic< i> Isl1</i>< sup>+</sup> Progenitor Cells Lead to Cardiac, Smooth Muscle, and Endothelial Cell Diversification', *Cell*, 127(6), pp. 1151-1165.

Mori, T., Chen, Y.F., Feng, J.A., Hayashi, T., Oparil, S. and Perry, G.J. (2004) 'Volume overload results in exaggerated cardiac hypertrophy in the atrial natriuretic peptide knockout mouse', *Cardiovascular research*, 61(4), pp. 771-779.

Morimoto, T., Hasegawa, K., Kaburagi, S., Kakita, T., Wada, H., Yanazume, T. and Sasayama, S. (2000) 'Phosphorylation of GATA-4 is involved in α1-adrenergic agonist- responsive transcription of the endothelin-1 gene in cardiac myocytes', *Journal of Biological Chemistry*, 275(18), pp. 13721-13726.

Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S. and Parmacek, M.S. (1998) 'GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo', *Genes and Development*, 12(22), pp. 3579-3590.

Moskalev, A.A., Aliper, A.M., Smit-McBride, Z., Buzdin, A. and Zhavoronkov, A. (2014) 'Genetics and epigenetics of aging and longevity', *Cell Cycle*, 13(7), pp. 1063-1077.

Motiwale, L., Ingle, A.D. and Rao, K.V.K. (2005) 'Mouse skin tumor promotion by sodium arsenate is associated with enhanced PCNA expression', *Cancer Letters*, 223(1), pp. 27-35.

Mudgett, J.S., Ding, J., Guh-Siesel, L., Chartrain, N.A., Yang, L., Gopal, S. and Shen, M.M. (2000) 'Essential role for p38α mitogen-activated protein kinase in placental angiogenesis', *Proceedings of the National Academy of Sciences of the United States of America*, 97(19), pp. 10454-10459.

Mughal, W., Dhingra, R. and Kirshenbaum, L. (2012) 'Striking a Balance: Autophagy, Apoptosis, and Necrosis in a Normal and Failing Heart', *Current Hypertension Reports*, 14(6), pp. 540-547.

Mukherjee, D. and Sen, S. (1993) 'Alteration of cardiac collagen phenotypes in hypertensive hypertrophy: role of blood pressure', *Journal of molecular and cellular cardiology*, 25(2), pp. 185-196.

Murry, C.E., Jennings, R.B. and Reimer, K.A. (1986) 'Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium', *Circulation*, 74(5), pp. 1124-36.

Muskhelishvili, L., Latendresse, J.R., Kodell, R.L. and Henderson, E.B. (2003) 'Evaluation of cell proliferation in rat tissues with BrdU, PCNA, Ki-67 (MIB-5) immunohistochemistry and in situ hybridization for histone mRNA', *Journal of Histochemistry & Cytochemistry*, 51(12), pp. 1681-1688.

Muslin, A. (2008) 'MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets', *Clinical Science*, 115, pp. 203-218.

Nadruz Jr, W., Corat, M.A.F., Marin, T.M., Guimarães Pereira, G.A. and Franchini, K.G. (2005) 'Focal adhesion kinase mediates MEF2 and c-Jun activation by stretch: Role in the activation of the cardiac hypertrophic genetic program', *Cardiovascular research*, 68(1), pp. 87-97.

Nadruz Jr, W., Kobarg, C.B., Constancio, S.S., Corat, P.D.C. and Franchini, K.G. (2003) 'Load-induced transcriptional activation of c-jun in rat myocardium: Regulation by myocyte enhancer factor 2', *Circulation Research*, 92(2), pp. 243-251.

Najjar, S.S., Scuteri, A. and Lakatta, E.G. (2005) 'Arterial Aging: Is It an Immutable Cardiovascular Risk Factor?', *Hypertension*, 46(3), pp. 454-462.

Nakajima, S., Lan, L., Wei, L., Hsieh, C.-L., Rapić-Otrin, V., Yasui, A. and Levine, A.S. (2014) 'Ubiquitin-Specific Protease 5 Is Required for the Efficient Repair of DNA Double-Strand Breaks', *PloS one*, 9(1), p. e84899.

Nakajima, Y. (2010) 'Second lineage of heart forming region provides new understanding of conotruncal heart defects', *Congenital Anomalies*, 50(1), pp. 8-14.

Nakanishi, R., Rajani, R., Cheng, V.Y., Gransar, H., Nakazato, R., Shmilovich, H., Otaki, Y., Hayes, S.W., Thomson, L.E.J., Friedman, J.D., Slomka, P.J., Berman, D.S. and Dey, D. (2011) 'Increase in epicardial fat volume is associated with greater coronary artery calcification progression in subjects at intermediate risk by coronary calcium score: A serial study using non-contrast cardiac CT', *Atherosclerosis*, 218(2), pp. 363-368.

Nakatogawa, H., Suzuki, K., Kamada, Y. and Ohsumi, Y. (2009) 'Dynamics and diversity in autophagy mechanisms: lessons from yeast', *Nat Rev Mol Cell Biol*, 10(7), pp. 458-467.

Naya, F.J., Black, B.L., Wu, H., Bassel-Duby, R., Richardson, J.A., Hill, J.A. and Olson, E.N. (2002) 'Mitochondrial deficiency and cardiac sudden death in mice lacking the MEF2A transcription factor', *Nature medicine*, 8(11), pp. 1303-1309.

Nelson, M.E., Rejeski, W.J., Blair, S.N., Duncan, P.W., Judge, J.O., King, A.C., Macera, C.A. and Castaneda-Sceppa, C. (2007) 'Physical activity and public health in older adults: recommendation from the American College of Sports Medicine and the American Heart Association', *Circulation*, 116(9), p. 1094.

Nemer, G., Qureshi, S.T., Malo, D. and Nemer, M. (1999) 'Functional analysis and chromosomal mapping of Gata5, a gene encoding a zinc finger DNA-binding protein', *Mammalian Genome*, 10(10), pp. 993-999.

Nicholls, C., Li, H., Wang, J.-Q. and Liu, J.-P. (2011) 'Molecular regulation of telomerase activity in aging', *Protein & Cell*, 2(9), pp. 726-738.

Nishida, K., Yamaguchi, O., Hirotani, S., Hikoso, S., Higuchi, Y., Watanabe, T., Takeda, T., Osuka, S., Morita, T., Kondoh, G., Uno, Y., Kashiwase, K., Taniike, M., Nakai, A., Matsumura, Y., Miyazaki, J.-i., Sudo, T., Hongo, K., Kusakari, Y., Kurihara, S., Chien, K.R., Takeda, J., Hori, M. and Otsu, K. (2004) 'p38α Mitogen-Activated Protein Kinase Plays a Critical Role in Cardiomyocyte Survival but Not in Cardiac Hypertrophic Growth in Response to Pressure Overload', *Molecular and Cellular Biology*, 24(24), pp. 10611-10620.

Nishikimi, T., Maeda, N. and Matsuoka, H. (2006) 'The role of natriuretic peptides in cardioprotection', *Cardiovascular research*, 69(2), pp. 318-328.

Nitahara, J.A., Cheng, W., Liu, Y., Li, B., Leri, A., Li, P., Mogul, D., Gambert, S.R., Kajstura, J. and Anversa, P. (1998) 'Intracellular Calcium, DNase Activity and Myocyte Apoptosis in Aging Fischer 344 Rats', *Journal of molecular and cellular cardiology*, 30(3), pp. 519-535.

Nualnim, N., Parkhurst, K., Dhindsa, M., Tarumi, T., Vavrek, J. and Tanaka, H. (2012) 'Effects of Swimming Training on Blood Pressure and Vascular Function in Adults >50 Years of Age', *The American journal of cardiology*, 109(7), pp. 1005-1010.

O'Callaghan, N. and Fenech, M. (2011) 'A quantitative PCR method for measuring absolute telomere length', *Biological Procedures Online*, 13(1), pp. 1-10.

Oberpriller, J.O. and Oberpriller, J.C. (1974) 'Response of the adult newt ventricle to injury', *Journal of Experimental Zoology*, 187(2), pp. 249-259.

Ogawa, Y., Itoh, H., Tamura, N., Suga, S.-i., Yoshimasa, T., Uehira, M., Matsuda, S., Shiono, S., Nishimoto, H. and Nakao, K. (1994) 'Molecular cloning of the complementary DNA and gene that encode mouse brain natriuretic peptide and generation of transgenic mice that overexpress the brain natriuretic peptide gene', *Journal of Clinical Investigation*, 93(5), p. 1911.

Ogura, Y., Iemitsu, M., Naito, H., Kakigi, R., Kakehashi, C., Maeda, S. and Akema, T. (2011) 'Single bout of running exercise changes LC3-II expression in rat cardiac muscle', *Biochemical and biophysical research communications*, 414(4), pp. 756-760.

Oh, M., Dey, A., Gerard, R.D., Hill, J.A. and Rothermel, B.A. (2010) 'The CCAAT/Enhancer Binding Protein β (C/EBPβ) Cooperates with NFAT to Control Expression of the Calcineurin Regulatory Protein RCAN1–4', *Journal of Biological Chemistry*, 285(22), pp. 16623-16631.

Ohba, H., Takada, H., Musha, H., Nagashima, J., Mori, N., Awaya, T., Omiya, K. and Murayama, M. (2001) 'Effects of prolonged strenuous exercise on plasma levels of atrial natriuretic peptide and brain natriuretic peptide in healthy men', *American Heart Journal*, 141(5), pp. 751-758.

Oka, T., Maillet, M., Watt, A.J., Schwartz, R.J., Aronow, B.J., Duncan, S.A. and Molkentin, J.D. (2006) 'Cardiac-specific deletion of gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability', *Circulation Research*, 98(6), pp. 837-845.

Oka, T., Xu, J. and Molkentin, J.D. (2007a) 'Re-employment of developmental transcription factors in adult heart disease', *Seminars in Cell & Developmental Biology*, 18(1), pp. 117-131.

Oka, T., Xu, J. and Molkentin, J.D. (2007b) *Seminars in cell & developmental biology*. Elsevier.

Oliver, P.M., Fox, J.E., Kim, R., Rockman, H.A., Kim, H.S., Reddick, R.L., Pandey, K.N., Milgram, S.L., Smithies, O. and Maeda, N. (1997) 'Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A', *Proceedings of the National Academy of Sciences of the United States of America*, 94(26), pp. 14730-14735.

Olivetti, G., Abbi, R., Quaini, F., Kajstura, J., Cheng, W., Nitahara, J.A., Quaini, E., Di Loreto, C., Beltrami, C.A. and Krajewski, S. (1997) 'Apoptosis in the failing human heart', *New England Journal of Medicine*, 336(16), pp. 1131-1141.

Olivetti, G., Cigola, E., Maestri, R., Corradi, D., Lagrasta, C., Gambert, S.R. and Anversa, P. (1996) 'Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart', *Journal of molecular and cellular cardiology*, 28(7), pp. 1463-1477.

Olivetti, G., Melissari, M., Capasso, J.M. and Anversa, P. (1991) 'Cardiomyopathy of the aging human heart. Myocyte loss and reactive cellular hypertrophy', *Circulation Research*, 68(6), pp. 1560-1568.

Olzinski, A., Bao, W., DeLong, D., Lenhard, S., Wright, F., Coatney, R., Jucker, B., Eisennagel, S., Needle, S. and Wang, T. (2013) 'Cardiac fibrosis and diastolic dysfunction are highly correlated in a mouse chronic pressure-overload model', *The FASEB Journal*, 27, p. 1129.6.

Orlandi, A., Francesconi, A., Marcellini, M., Ferlosio, A. and Spagnoli, L.G. (2004) 'Role of ageing and coronary atherosclerosis in the development of cardiac fibrosis in the rabbit', *Cardiovascular research*, 64(3), pp. 544-552.

Palstra, A.P., Schaaf, M. and Planas, J.V. (2013) 'Exercise Physiology of Zebrafish: Swimming Effects on Skeletal and Cardiac Muscle Growth, on the Immune

System, and the Involvement of the Stress Axis', in *Swimming Physiology of Fish*. Springer, pp. 323-342.

Pashmforoush, M., Lu, J.T., Chen, H., St. Amand, T., Kondo, R., Pradervand, S., Evans, S.M., Clark, B., Feramisco, J.R., Giles, W., Ho, S.Y., Benson, D.W., Silberbach, M., Shou, W. and Chien, K.R. (2004) 'Nkx2-5 pathways and congenital heart disease: Loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block', *Cell*, 117(3), pp. 373-386.

Pasumarthi, K.B.S., Nakajima, H., Nakajima, H.O., Soonpaa, M.H. and Field, L.J. (2005) 'Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice', *Circulation research*, 96(1), pp. 110-118.

Pawley, J. (2010) *Handbook of biological confocal microscopy*. Springer.

Pérez-Pomares, J.M. and de la Pompa, J.L. (2011) 'Signaling during epicardium and coronary vessel development', *Circulation Research*, 109(12), pp. 1429-1442.

Perlmutter, D.H. (2008) 'Autophagic disposal of the aggregation-prone protein that causes liver inflammation and carcinogenesis in [alpha]-1-antitrypsin deficiency', *Cell Death Differ*, 16(1), pp. 39-45.

Pescatello, L., Franklin, B., Fagard, R., Farquhar, W., Kelley, G. and Ray, C. (2004) 'Exercise and hypertension: American College of Sports Medicine Position Stand', *Med Sci Sports Exerc*, 36(3), pp. 533-53.

Petersen, R.O. and Baserga, R. (1965) 'Nucleic acid and protein synthesis in cardiac muscle of growing and adult mice', *Experimental Cell Research*, 40(2), pp. 340-352.

Phaneuf, S. and Leeuwenburgh, C. (2002) 'Cytochrome c release from mitochondria in the aging heart: a possible mechanism for apoptosis with age', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 282(2), pp. R423-R430.

Pierce, E., Doshi, R. and Deane, R. (1998) 'PCNA and Ki-67 labelling indices in pre-irradiated and post-irradiated astrocytomas: a comparative immunohistochemical analysis for evaluation of proliferative activity', *Molecular Pathology*, 51(2), pp. 90-95.

Pikkarainen, S., Tokola, H., Kerkelä, R. and Ruskoaho, H. (2004) 'GATA transcription factors in the developing and adult heart', *Cardiovascular research*, 63(2), pp. 196-207.

Poddar, D., Basu, A., Baldwin, W.M., Kondratov, R.V., Barik, S. and Mazumder, B. (2013) 'An Extraribosomal Function of Ribosomal Protein L13a in Macrophages Resolves Inflammation', *The Journal of Immunology*, 190(7), pp. 3600-3612.

Pollack, M., Phaneuf, S., Dirks, A. and Leeuwenburgh, C. (2002) 'The Role of Apoptosis in the Normal Aging Brain, Skeletal Muscle, and Heart', *Annals of the New York Academy of Sciences*, 959(1), pp. 93-107.

Porrello, E.R., Mahmoud, A.I., Simpson, E., Hill, J.A., Richardson, J.A., Olson, E.N. and Sadek, H.A. (2011) 'Transient regenerative potential of the neonatal mouse heart', *Science*, 331(6020), pp. 1078-1080.

Poss, K.D. (2007) 'Getting to the heart of regeneration in zebrafish', *Semin Cell Dev Biol*, 18(1), pp. 36-45.

Poss, K.D., Wilson, L.G. and Keating, M.T. (2002) 'Heart regeneration in zebrafish', *Science*, 298(5601), pp. 2188-2190.

Potthoff, M.J. and Olson, E.N. (2007) 'MEF2: a central regulator of diverse developmental programs', *Development*, 134(23), pp. 4131-4140.

Poupa, O., Gesser, H., Jonsson, S. and Sullivan, L. (1974) 'Coronary-supplied compact shell of ventricular myocardium in salmonids: growth and enzyme pattern', *Comparative Biochemistry and Physiology Part A: Physiology*, 48(1), pp. 85-95.

Poupa, O. and Lindström, L. (1983) 'Comparative and scaling aspects of heart and body weights with reference to blood supply of cardiac fibers', *Comparative Biochemistry and Physiology Part A: Physiology*, 76(3), pp. 413-421.

Powers, S.K., Lennon, S.L., Quindry, J. and Mehta, J.L. (2002) 'Exercise and cardioprotection', *Current Opinion in Cardiology*, 17(5), pp. 495-502.

Prasad, A.M. and Inesi, G. (2011) 'Silencing calcineurin A subunit reduces SERCA2 expression in cardiac myocytes', *American Journal of Physiology-Heart and Circulatory Physiology*, 300(1), pp. H173-H180.

Preibisch, S., Saalfeld, S. and Tomancak, P. (2009) 'Globally optimal stitching of tiled 3D microscopic image acquisitions', *Bioinformatics*, 25(11), pp. 1463-1465.

Prevedello, D.M., Jagannathan, J., Jane Jr, J.A., Lopes, M.B.S. and Laws Jr, E.R. (2005) 'Relevance of high Ki-67 in pituitary adenomas: Case report and review of the literature', *Neurosurgical Focus*, 19(5), pp. 1-9.

Primeau, A.J., Adhihetty, P.J. and Hood, D.A. (2002) 'Apoptosis in Heart and Skeletal Muscle', *Canadian Journal of Applied Physiology*, 27(4), pp. 349-395.

Pu, W.T., Ishiwata, T., Juraszek, A.L., Ma, Q. and Izumo, S. (2004) 'GATA4 is a dosage-sensitive regulator of cardiac morphogenesis', *Developmental biology*, 275(1), pp. 235-244.

Quindry, J.C., Miller, L., McGinnis, G., Kliszczewicz, B., Irwin, J.M., Landram, M., Urbiztondo, Z., Nanayakkara, G. and Amin, R. (2012) 'Ischemia reperfusion injury, KATP channels, and exercise-induced cardioprotection against apoptosis', *Journal of Applied Physiology*, 113(3), pp. 498-506.

Raya, Á., Koth, C.M., Büscher, D., Kawakami, Y., Itoh, T., Raya, R.M., Sternik, G., Tsai, H.-J., Rodríguez-Esteban, C. and Izpisúa-Belmonte, J.C. (2003) 'Activation of Notch signaling pathway precedes heart regeneration in zebrafish', *Proceedings of the National Academy of Sciences of the United States of America*, 100(Suppl 1), pp. 11889-11895.

Reiken, S., Gaburjakova, M., Guatimosim, S., Gomez, A.M., D'Armiento, J., Burkhoff, D., Wang, J., Vassort, G., Lederer, W.J. and Marks, A.R. (2003) 'Protein Kinase A Phosphorylation of the Cardiac Calcium Release Channel (Ryanodine Receptor) in Normal and Failing Hearts ROLE OF PHOSPHATASES AND RESPONSE TO ISOPROTERENOL', *Journal of Biological Chemistry*, 278(1), pp. 444-453.

Reiter, J.F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N. and Stainier, D.Y.R. (1999) 'Gata5 is required for the development of the heart and endoderm in zebrafish', *Genes and Development*, 13(22), pp. 2983-2995.

Rengo, G., Lymperopoulos, A., Zincarelli, C., Femminella, G.D., Liccardo, D., Pagano, G., de Lucia, C., Cannavo, A., Gargiulo, P., Ferrara, N., Perrone Filardi, P.,

Koch, W.J. and Leosco, D. (2012a) 'Blockade of β-adrenoceptors restores the GRK2-mediated adrenal α2-adrenoceptor–catecholamine production axis in heart failure', *British Journal of Pharmacology*, 166(8), pp. 2430-2440.

Rengo, G., Perrone-Filardi, P., Femminella, G.D., Liccardo, D., Zincarelli, C., de Lucia, C., Pagano, G., Marsico, F., Lymperopoulos, A. and Leosco, D. (2012b) 'Targeting the β-Adrenergic Receptor System Through G-Protein–Coupled Receptor Kinase 2: A New Paradigm for Therapy and Prognostic Evaluation in Heart Failure: From Bench to Bedside', *Circulation: Heart Failure*, 5(3), pp. 385-391.

Rengo, G., Zincarelli, C., Femminella, G.D., Liccardo, D., Pagano, G., de Lucia, C., Altobelli, G.G., Cimini, V., Ruggiero, D., Perrone-Filardi, P., Gao, E., Ferrara, N., Lymperopoulos, A., Koch, W.J. and Leosco, D. (2012c) 'Myocardial β2-adrenoceptor gene delivery promotes coordinated cardiac adaptive remodelling and angiogenesis in heart failure', *British Journal of Pharmacology*, 166(8), pp. 2348-2361.

Richards, A.M. (2007) 'Natriuretic peptides: Update on peptide release, bioactivity, and clinical use', *Hypertension*, 50(1), pp. 25-30.

Robert, V., Besse, S., Sabri, A., Silvestre, J.-S., Assayag, P., Nguyen, V.T., Swynghedauw, B. and Delcayre, C. (1997) 'Differential regulation of matrix metalloproteinases associated with aging and hypertension in the rat heart', *Laboratory investigation; a journal of technical methods and pathology*, 76(5), pp. 729-738.

Rodier, F., Kim, S.-H., Nijjar, T., Yaswen, P. and Campisi, J. (2005) 'Cancer and aging: the importance of telomeres in genome maintenance', *The international journal of biochemistry & cell biology*, 37(5), pp. 977-990.

Rosenkranz, A.C., Woods, R.L., Dusting, G.J. and Ritchie, R.H. (2003) 'Antihypertrophic actions of the natriuretic peptides in adult rat cardiomyocytes: Importance of cyclic GMP', *Cardiovascular research*, 57(2), pp. 515-522.

Rosenkranz, S., Flesch, M., Amann, K., Haeuseler, C., Kilter, H., Seeland, U., Schlüter, K.-D. and Böhm, M. (2002) 'Alterations of β-adrenergic signaling and cardiac hypertrophy in transgenic mice overexpressing TGF-β1', *American Journal of Physiology-Heart and Circulatory Physiology*, 283(3), pp. H1253-H1262.

Rosito, G.A., Massaro, J.M., Hoffmann, U., Ruberg, F.L., Mahabadi, A.A., Vasan, R.S., O'Donnell, C.J. and Fox, C.S. (2008) 'Pericardial Fat, Visceral Abdominal Fat, Cardiovascular Disease Risk Factors, and Vascular Calcification in a Community-Based Sample: The Framingham Heart Study', *Circulation*, 117(5), pp. 605-613.

Rosner, B. (2010) *Fundamentals of biostatistics*. Cengage Learning.

Rota, M., Hosoda, T., De Angelis, A., Arcarese, M.L., Esposito, G., Rizzi, R., Tillmanns, J., Tugal, D., Musso, E. and Rimoldi, O. (2007) 'The young mouse heart is composed of myocytes heterogeneous in age and function', *Circulation research*, 101(4), pp. 387-399.

Rothen-Rutishauser, B.M., Ehler, E., Perriard, E., Messerli, J.M. and Perriard, J.C. (1998) 'Different behaviour of the non-sarcomeric cytoskeleton in neonatal and adult rat cardiomyocytes', *Journal of molecular and cellular cardiology*, 30(1), pp. 19-31.

Rothermel, B.A. and Hill, J.A. (2008) 'Autophagy in Load-Induced Heart Disease', *Circulation Research*, 103(12), pp. 1363-1369.

Rowlerson, A., Radaelli, G., Mascarello, F. and Veggetti, A. (1997) 'Regeneration of skeletal muscle in two teleost fish: Sparus aurata and Brachydanio rerio', *Cell and tissue research*, 289(2), pp. 311-322.

Rubattu, S., Bigatti, G., Evangelista, A., Lanzani, C., Stanzione, R., Zagato, L., Manunta, P., Marchitti, S., Venturelli, V., Bianchi, G., Volpe, M. and Stella, P. (2006) 'Association of Atrial Natriuretic Peptide and Type A Natriuretic Peptide Receptor Gene Polymorphisms With Left Ventricular Mass in Human Essential Hypertension', *Journal of the American College of Cardiology*, 48(3), pp. 499-505.

Rudolph, K.L., Chang, S., Lee, H.-W., Blasco, M., Gottlieb, G.J., Greider, C. and DePinho, R.A. (1999) 'Longevity, Stress Response, and Cancer in Aging Telomerase-Deficient Mice', *Cell*, 96(5), pp. 701-712.

Ruivo, R., Anne, C., Sagné, C. and Gasnier, B. (2009) 'Molecular and cellular basis of lysosomal transmembrane protein dysfunction', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1793(4), pp. 636-649.

Ryckebusch, L., Wang, Z., Bertrand, N., Lin, S.-C., Chi, X., Schwartz, R., Zaffran, S. and Niederreither, K. (2008) 'Retinoic acid deficiency alters second heart field formation', *Proceedings of the National Academy of Sciences*, 105(8), pp. 2913-2918.

Ryo, A., Suzuki, Y., Ichiyama, K., Wakatsuki, T., Kondoh, N., Hada, A., Yamamoto, M. and Yamamoto, N. (1999) 'Serial analysis of gene expression in HIV-1-infected T cell lines', *FEBS Letters*, 462(1–2), pp. 182-186.

Saadane, N., Alpert, L. and Chalifour, L.E. (1999) 'Expression of immediate early genes, GATA-4, and Nkx-2.5 in adrenergic-induced cardiac hypertrophy and during regression in adult mice', *British Journal of Pharmacology*, 127(5), pp. 1165-1176.

Sabio, G., Arthur, J.S.C., Kuma, Y., Peggie, M., Carr, J., Murray‐Tait, V., Centeno, F., Goedert, M., Morrice, N.A. and Cuenda, A. (2005) 'p38γ regulates the localisation of SAP97 in the cytoskeleton by modulating its interaction with GKAP', *The EMBO Journal*, 24(6), pp. 1134-1145.

Sakai, H., Noda, A., Shirai, N., Iidaka, T., Yanai, T. and Masegi, T. (2002) 'Proliferative activity of canine mast cell tumours evaluated by bromodeoxyuridine incorporation and Ki-67 expression', *Journal of comparative pathology*, 127(4), pp. 233-238.

Salazar, N.C., Vallejos, X., Siryk, A., Rengo, G., Cannavo, A., Liccardo, D., De Lucia, C., Gao, E., Leosco, D. and Koch, W.J. (2013) 'GRK2 blockade with betaARKct is essential for cardiac beta2-adrenergic receptor signaling towards increased contractility', *Cell Commun. Signal*, 11(64.10), p. 1186.

Sales, V.L., Engelmayr, G.C., Mettler, B.A., Johnson, J.A., Sacks, M.S. and Mayer, J.E. (2006) 'Transforming growth factor-β1 modulates extracellular matrix production, proliferation, and apoptosis of endothelial progenitor cells in tissue-engineering scaffolds', *Circulation*, 114(1 suppl), pp. I-193-I-199.

Salic, A. and Mitchison, T.J. (2008) 'A chemical method for fast and sensitive detection of DNA synthesis in vivo', *Proceedings of the National Academy of Sciences*, 105(7), pp. 2415-2420.

Sanada, S. and Kitakaze, M. (2004) 'Ischemic preconditioning: emerging evidence, controversy, and translational trials', *International Journal of Cardiology*, 97(2), pp. 263-276.

Sanchez-Quintana, D., García‑Martínez, V., Climent, V. and Hurlé, J.M. (1996) 'Myocardial fiber and connective tissue architecture in the fish heart ventricle', *Journal of Experimental Zoology*, 275(2‑3), pp. 112-124.

Saraste, Pulkki, Kallajoki, Heikkilä, Laine, Mattila, Nieminen, Parvinen and Voipio, P. (1999) 'Cardiomyocyte apoptosis and progression of heart failure to transplantation', *European journal of clinical investigation*, 29(5), pp. 380-386.

Saraste, A. (1999) 'Morphologic criteria and detection of apoptosis', *Herz*, 24(3), pp. 189-195.

Saraste, A. and Pulkki, K. (2000) 'Morphologic and biochemical hallmarks of apoptosis', *Cardiovascular Research*, 45(3), pp. 528-537.

Sarkar, S., Ravikumar, B., Floto, R.A. and Rubinsztein, D.C. (2008) 'Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies', *Cell Death & Differentiation*, 16(1), pp. 46-56.

Sastre, J., Pallardó, F.V. and Viña, J. (2000) 'Mitochondrial Oxidative Stress Plays a Key Role in Aging and Apoptosis', *IUBMB Life*, 49(5), pp. 427-435.

Savarese, G., Prastaro, M., Rengo, G., Leosco, D., Trimarco, B. and Perrone-Filardi, P. (2013) 'NATRIURETIC PEPTIDE-GUIDED THERAPY IN CHRONIC HEART FAILURE: A META-ANALYSIS OF 2,876 PATIENTS IN 13 RANDOMIZED TRIALS', *Journal of the American College of Cardiology*, 61(10_S).

Scharhag, J., Löllgen, H. and Kindermann, W. (2013) 'Competitive Sports and the Heart: Benefit or Risk?', *Dtsch Arztebl International*, 110(1-2), pp. 14-24.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012) 'Fiji: an open-source platform for biological-image analysis', *Nat Meth*, 9(7), pp. 676-682.

Schlesinger, J., Schueler, M., Grunert, M., Fischer, J.J., Zhang, Q., Krueger, T., Lange, M., Tönjes, M., Dunkel, I. and Sperling, S.R. (2011) 'The Cardiac Transcription Network Modulated by Gata4, Mef2a, Nkx2.5, Srf, Histone Modifications, and MicroRNAs', *PLoS Genet*, 7(2), p. e1001313.

Schlüter, C., Duchrow, M., Wohlenberg, C., Becker, M.H., Key, G., Flad, H.-D. and Gerdes, J. (1993) 'The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins', *The Journal of cell biology*, 123(3), pp. 513-522.

Schmid, G. and Pfitzer, P. (1985) 'Mitoses and binucleated cells in perinatal human hearts', *Virchows Archiv B*, 48(1), pp. 59-67.

Schnabel, K., Wu, C.-C., Kurth, T. and Weidinger, G. (2011) 'Regeneration of Cryoinjury Induced Necrotic Heart Lesions in Zebrafish Is Associated with Epicardial Activation and Cardiomyocyte Proliferation', *PloS one*, 6(4), p. e18503.

Schoenebeck, J.J. and Yelon, D. (2007) 'Illuminating cardiac development: Advances in imaging add new dimensions to the utility of zebrafish genetics', *Seminars in Cell and Developmental Biology*, 18(1), pp. 27-35.

Schott, J.J., Benson, D.W., Basson, C.T., Pease, W., Silberbach, G.M., Moak, J.P., Maron, B.J., Seidman, C.E. and Seidman, J.G. (1998) 'Congenital heart disease caused by mutations in the transcription factor NKX2-5', *Science*, 281(5373), pp. 108-111.

Schwartz, J.B.a.Z., D. P. (ed.) (2007) *Braunwald's Heart Disease: a Textbook of Cardiovascular Medicine*.

Schwartz, R.S., Shuman, W.P., Larson, V., Cain, K.C., Fellingham, G.W., Beard, J.C., Kahn, S.E., Stratton, J.R., Cerqueira, M.D. and Abrass, I.B. (1991) 'The effect of intensive endurance exercise training on body fat distribution in young and older men', *Metabolism*, 40(5), pp. 545-551.

Sciarretta, S., Volpe, M. and Sadoshima, J. (2014) 'Mammalian Target of Rapamycin Signaling in Cardiac Physiology and Disease', *Circulation Research*, 114(3), pp. 549-564.

Sedmera, D., Reckova, M., DeAlmeida, A., Sedmerova, M., Biermann, M., Volejnik, J., Sarre, A., Raddatz, E., McCarthy, R.A., Gourdie, R.G. and Thompson, R.P. (2003) 'Functional and morphological evidence for a ventricular conduction system in zebrafish and Xenopus hearts', *American Journal of Physiology - Heart and Circulatory Physiology*, 284(4 53-4), pp. H1152-H1160.

Seeland, U., Haeuseler, C., Hinrichs, R., Rosenkranz, S., Pfitzner, T., Scharffetter‑Kochanek, K. and Böhm, M. (2002) 'Myocardial fibrosis in transforming growth factor‑β 1 (TGF‑β 1) transgenic mice is associated with inhibition of interstitial collagenase', *European journal of clinical investigation*, 32(5), pp. 295-303.

Senyo, S.E., Steinhauser, M.L., Pizzimenti, C.L., Yang, V.K., Cai, L., Wang, M., Wu, T.-D., Guerquin-Kern, J.-L., Lechene, C.P. and Lee, R.T. (2013) 'Mammalian heart renewal by pre-existing cardiomyocytes', *Nature*, 493(7432), pp. 433-436.

Sepulveda, J.L., Belaguli, N., Nigam, V., Chen, C.-Y., Nemer, M. and Schwartz, R.J. (1998) 'GATA-4 and Nkx-2.5 Coactivate Nkx-2 DNA Binding Targets: Role for Regulating Early Cardiac Gene Expression', *Molecular and Cellular Biology*, 18(6), pp. 3405-3415.

Sharma, G.D., Nguyen, H.T., Antonov, A.S., Gerrity, R.G., von Geldern, T. and Pandey, K.N. (2002) 'Expression of atrial netriuretic peptide receptor-A antagonizes the mitogen-activated protein kinases (Erk2 and P38MAPK) in cultured human vascular smooth muscle cells', *Molecular and Cellular Biochemistry*, 233(1-2), pp. 165-173.

Sheydina, A., Riordon, D.R. and Boheler, K.R. (2011) 'Molecular mechanisms of cardiomyocyte aging', *Clinical Science*, 121(8), pp. 315-329.

Shih, H., Lee, B., Lee, R.J. and Boyle, A.J. (2011) 'The Aging Heart and Post-Infarction Left Ventricular Remodeling', *Journal of the American College of Cardiology*, 57(1), pp. 9-17.

Shiojima, I., Komuro, I., Oka, T., Hiroi, Y., Mizuno, T., Takimoto, E., Monzen, K., Aikawa, R., Akazawa, H., Yamazaki, T., Kudoh, S. and Yazaki, Y. (1999) 'Context-dependent transcriptional cooperation mediated by cardiac transcription factors Csx/Nkx-2.5 and GATA-4', *Journal of Biological Chemistry*, 274(12), pp. 8231-8239.

Shyu, K.G., Ko, W.H., Yang, W.S., Wang, B.W. and Kuan, P. (2005) 'Insulin-like growth factor-1 mediates stretch-induced upregulation of myostatin expression in neonatal rat cardiomyocytes', *Cardiovascular research*, 68(3), pp. 405-414.

Simonsen, A., Cumming, R.C., Brech, A., Isakson, P., Schubert, D.R. and Finley, K.D. (2008) 'Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila', *Autophagy*, 4(2), pp. 176-184.

Simpson, P., Peterson, B., Hughes, N. and Schelling, G.T. (2000) 'Recombinant bovine somatotropin enhances growth rates in two species of ornamental fish; giant danios (Danio aequipinnatus) and zebrafish (Danio rerio)', *J Anim Sci*, 78, p. 139.

Siu, P.M., Bryner, R.W., Martyn, J.K. and Alway, S.E. (2004) 'Apoptotic adaptations from exercise training in skeletal and cardiac muscles', *The FASEB journal*, 18(10), pp. 1150-1152.

Siwik, D.A., Chang, D.L.F. and Colucci, W.S. (2000) 'Interleukin-1β and tumor necrosis factor-α decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro', *Circulation Research*, 86(12), pp. 1259-1265.

Smogorzewska, A., Van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M.R., Schnapp, G. and De Lange, T. (2000) 'Control of human telomere length by TRF1 and TRF2', *Molecular and Cellular Biology*, 20(5), pp. 1659-1668.

Smuder, A.J., Kavazis, A.N., Min, K. and Powers, S.K. (2011) 'Exercise protects against doxorubicin-induced markers of autophagy signaling in skeletal muscle', *Journal of Applied Physiology*, 111(4), pp. 1190-1198.

Snider, P., Olaopa, M., Firulli, A.B. and Conway, S.J. (2007) 'Cardiovascular Development and the Colonizing Cardiac Neural Crest Lineage', *TheScientificWorldJOURNAL*, 7, pp. 1090-1113.

Solanas, M., Moral, R. and Escrich, E. (2001) 'Improved non-radioactive Northern blot protocol for detecting low abundance mRNAs from mammalian tissues', *Biotechnology letters*, 23(4), pp. 263-266.

Song, K., Backs, J., McAnally, J., Qi, X., Gerard, R.D., Richardson, J.A., Hill, J.A., Bassel-Duby, R. and Olson, E.N. (2006) 'The Transcriptional Coactivator

CAMTA2 Stimulates Cardiac Growth by Opposing Class II Histone Deacetylases', *Cell*, 125(3), pp. 453-466.

Song, K., Nam, Y.-J., Luo, X., Qi, X., Tan, W., Huang, G.N., Acharya, A., Smith, C.L., Tallquist, M.D. and Neilson, E.G. (2012) 'Heart repair by reprogramming non-myocytes with cardiac transcription factors', *Nature*, 485(7400), pp. 599-604.

Song, W., Lu, X. and Feng, Q. (2000) 'Tumor necrosis factor-α induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes', *Cardiovascular research*, 45(3), pp. 595-602.

Song, Y., Yao, Q., Zhu, J., Luo, B. and Liang, S. (1999) 'Age-related variation in the interstitial tissues of the cardiac conduction system; and autopsy study of 230 Han Chinese', *Forensic science international*, 104(2), pp. 133-142.

Soonpaa, M. and Field, L.J. (1997) 'Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts', *American Journal of Physiology-Heart and Circulatory Physiology*, 272(1), pp. H220-H226.

Soonpaa, M.H., Kim, K.K., Pajak, L., Franklin, M. and Field, L.J. (1996) 'Cardiomyocyte DNA synthesis and binucleation during murine development', *American Journal of Physiology-Heart and Circulatory Physiology*, 271(5), pp. H2183-H2189.

Soonpaa, M.H., Rubart, M. and Field, L.J. (2013) 'Challenges measuring cardiomyocyte renewal', *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1833(4), pp. 799-803.

Soufi, F.G., Farajnia, S., Aslanabadi, N., Ahmadiasl, N., Alipour, M., Doustar, Y., Abdolalizadeh, J. and Sheikhzadeh, F. (2008) 'Long-term exercise training affects age-induced changes in HSP70 and apoptosis in rat heart', *General physiology and biophysics*, 27(4), p. 263.

Stainier, D.Y.R. (2001) 'Zebrafish genetics and vertebrate heart formation', *Nature Reviews Genetics*, 2(1), pp. 39-48.

Stanley, E.G., Biben, C., Elefanty, A., Barnett, L., Koentgen, F., Robb, L. and Harvey, R.P. (2002) 'Efficient cre-mediated deletion in cardiac progenitor cells

conferred by a 3′ UTR-ires-Cre allele of the homeobox gene Nkx2-5', *International Journal of Developmental Biology*, 46(4 SPEC.), pp. 431-439.

Starnes, J.W., Taylor, R.P. and Park, Y. (2003) 'Exercise improves postischemic function in aging hearts', *American Journal of Physiology - Heart and Circulatory Physiology*, 285(1), pp. H347-H351.

Steinhauser, M.L., Bailey, A.P., Senyo, S.E., Guillermier, C., Perlstein, T.S., Gould, A.P., Lee, R.T. and Lechene, C.P. (2012) 'Multi-isotope imaging mass spectrometry quantifies stem cell division and metabolism', *Nature*, 481(7382), pp. 516-519.

Sucov, H.M. and Evans, R.M. (1995) 'Retinoic acid and retinoic acid receptors in development', *Molecular neurobiology*, 10(2-3), pp. 169-184.

Suh, Y., Lee, K.-A., Kim, W.-H., Han, B.-G., Vijg, J. and Park, S.C. (2002) 'Aging alters the apoptotic response to genotoxic stress', *Nat Med*, 8(1), pp. 3-4.

Sullivan, K.E. and Black, L.D. (2013) 'The Role of Cardiac Fibroblasts in Extracellular Matrix-Mediated Signaling During Normal and Pathological Cardiac Development', *Journal of biomechanical engineering*, 135(7), p. 071001.

Sumida, T., Otani, H., Kyoi, S., Okada, T., Fujiwara, H., Nakao, Y., Kido, M. and Imamura, H. (2005) 'Temporary blockade of contractility during reperfusion elicits a cardioprotective effect of the p38 MAP kinase inhibitor SB-203580', *American Journal of Physiology-Heart and Circulatory Physiology*, 288(6), pp. H2726-H2734.

Sun, X., Hoage, T., Bai, P., Ding, Y., Chen, Z., Zhang, R., Huang, W., Jahangir, A., Paw, B. and Li, Y.-G. (2009) 'Cardiac hypertrophy involves both myocyte hypertrophy and hyperplasia in anemic zebrafish', *PLoS One*, 4(8), p. e6596.

Sun, Y., Liang, X., Najafi, N., Cass, M., Lin, L., Cai, C.-L., Chen, J. and Evans, S.M. (2007) 'Islet 1 is expressed in distinct cardiovascular lineages, including pacemaker and coronary vascular cells', *Developmental biology*, 304(1), pp. 286-296.

Sun, Y. and Weber, K.T. (2000) 'Infarct scar: A dynamic tissue', *Cardiovascular research*, 46(2), pp. 250-256.

Suzuki, K., Smolenski, R.T., Jayakumar, J., Murtuza, B., Brand, N.J. and Yacoub, M.H. (2000) 'Heat shock treatment enhances graft cell survival in skeletal myoblast transplantation to the heart', *Circulation*, 102(suppl 3), pp. Iii-216-Iii-221.

Swynghedauw, B. (1999) 'Molecular mechanisms of myocardial remodeling', *Physiological Reviews*, 79(1), pp. 215-262.

Taber, L.A., Hu, N., Pexieder, T., Clark, E.B. and Keller, B.B. (1993) 'Residual strain in the ventricle of the stage 16-24 chick embryo', *Circulation research*, 72(2), pp. 455-462.

Tacar, O., Sriamornsak, P. and Dass, C.R. (2013) 'Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems', *Journal of Pharmacy and Pharmacology*, 65(2), pp. 157-170.

Takimoto, E., Mizuno, T., Terasaki, F., Shimoyama, M., Honda, H., Shiojima, I., Hiroi, Y., Oka, T., Hayashi, D., Hirai, H., Kudoh, S., Toko, H., Kawamura, K., Nagai, R., Yazaki, Y. and Komuro, I. (2000) 'Up-regulation of natriuretic peptides in the ventricle of Csx/Nkx2-5 transgenic mice', *Biochemical and biophysical research communications*, 270(3), pp. 1074-1079.

Tamura, K., Sudo, T., Senftleben, U., Dadak, A.M., Johnson, R. and Karin, M. (2000a) 'Requirement for p38α in Erythropoietin Expression: A Role for Stress Kinases in Erythropoiesis', *Cell*, 102(2), pp. 221-231.

Tamura, N., Ogawa, Y., Chusho, H., Nakamura, K., Nakao, K., Suda, M., Kasahara, M., Hashimoto, R., Katsuura, G., Mukoyama, M., Itoh, H., Saito, Y., Tanaka, I., Otani, H. and Katsuki, M. (2000b) 'Cardiac fibrosis in mice lacking brain natriuretic peptide', *Proceedings of the National Academy of Sciences of the United States of America*, 97(8), pp. 4239-4244.

Tanaka, H. and Seals, D.R. (2003) 'Invited review: dynamic exercise performance in masters athletes: insight into the effects of primary human aging on physiological functional capacity', *Journal of Applied Physiology*, 95(5), pp. 2152-2162.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. and Izumo, S. (1999) 'The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development', *Development*, 126(6), pp. 1269-1280.

Tanasescu, M., Leitzmann, M.F., Rimm, E.B., Willett, W.C., Stampfer, M.J. and Hu, F.B. (2002) 'Exercise type and intensity in relation to coronary heart disease in men', *Jama*, 288(16), pp. 1994-2000.

Tang, R., Dodd, A., Lai, D., McNabb, W.C. and Love, D.R. (2007) 'Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization', *Acta biochimica et biophysica Sinica*, 39(5), pp. 384-390.

Tannous, P., Zhu, H., Johnstone, J.L., Shelton, J.M., Rajasekaran, N.S., Benjamin, I.J., Nguyen, L., Gerard, R.D., Levine, B., Rothermel, B.A. and Hill, J.A. (2008) 'Autophagy is an adaptive response in desmin-related cardiomyopathy', *Proceedings of the National Academy of Sciences*, 105(28), pp. 9745-9750.

Taupin, P. (2007) 'BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation', *Brain research reviews*, 53(1), pp. 198-214.

Taylor, J.H., Woods, P.S. and Hughes, W.L. (1957) 'The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labeled thymidinee', *Proceedings of the National Academy of Sciences of the United States of America*, 43(1), p. 122.

Taylor, R.P. and Starnes, J.W. (2003) 'Age, cell signalling and cardioprotection', *Acta Physiologica Scandinavica*, 178(2), pp. 107-116.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M. and Nguyen, M. (2010) 'A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines', *Methods*, 50(4), pp. S1-S5.

Tchkonia, T., Zhu, Y., van Deursen, J., Campisi, J. and Kirkland, J.L. (2013) 'Cellular senescence and the senescent secretory phenotype: therapeutic opportunities', *The Journal of Clinical Investigation*, 123(3), pp. 966-972.

Terman, A., Dalen, H., Eaton, J.W., Neuzil, J. and Brunk, U.T. (2003) 'Mitochondrial recycling and aging of cardiac myocytes: the role of autophagocytosis', *Experimental gerontology*, 38(8), pp. 863-876.

Terracio, L., Miller, B. and Borg, T.K. (1988) 'Effects of cyclic mechanical stimulation of the cellular components of the heart: in vitro', *In vitro cellular & developmental biology*, 24(1), pp. 53-58.

Thijssen, D.H., Torella, D., Hopman, M.T. and Ellison, G.M. (2009) 'The role of endothelial progenitor and cardiac stem cells in the cardiovascular adaptations to age and exercise', *Frontiers in bioscience: a journal and virtual library*, 14, p. 4685.

Thomas, D.P., Zimmerman, S.D., Hansen, T.R., Martin, D.T. and McCormick, R.J. (2000) 'Collagen gene expression in rat left ventricle: interactive effect of age and exercise training', *Journal of Applied Physiology*, 89(4), pp. 1462-1468.

Thomas, P.D., Cotter, T.A., Li, X., McCormick, R.J. and Gosselin, L.E. (2001) 'Exercise training attenuates aging-associated increases in collagen and collagen crosslinking of the left but not the right ventricle in the rat', *European Journal of Applied Physiology*, 85(1-2), pp. 164-169.

Thompson, D., Karpe, F., Lafontan, M. and Frayn, K. (2012) 'Physical activity and exercise in the regulation of human adipose tissue physiology', *Physiological Reviews*, 92(1), pp. 157-191.

Thompson, J.T., Rackley, M.S. and O'Brien, T.X. (1998) 'Upregulation of the cardiac homeobox gene Nkx2-5 (CSX) in feline right ventricular pressure overload', *American Journal of Physiology - Heart and Circulatory Physiology*, 274(5 43-5), pp. H1569-H1573.

Thornburg, K., Jonker, S., O'Tierney, P., Chattergoon, N., Louey, S., Faber, J. and Giraud, G. (2011) 'Regulation of the cardiomyocyte population in the developing heart', *Progress in biophysics and molecular biology*, 106(1), pp. 289-299.

Toko, H., Zhu, W., Takimoto, E., Shiojima, I., Hiroi, Y., Zou, Y., Oka, T., Akazawa, H., Mizukami, M., Sakamoto, M., Terasaki, F., Kitaura, Y., Takano, H., Nagai, T., Nagai, R. and Komuro, I. (2002) 'Csx/Nkx2-5 is required for homeostasis and survival of cardiac myocytes in the adult heart', *Journal of Biological Chemistry*, 277(27), pp. 24735-24743.

Tomás-Loba, A., Flores, I., Fernández-Marcos, P.J., Cayuela, M.L., Maraver, A., Tejera, A., Borrás, C., Matheu, A., Klatt, P., Flores, J.M., Viña, J., Serrano, M. and

Blasco, M.A. (2008) 'Telomerase Reverse Transcriptase Delays Aging in Cancer-Resistant Mice', *Cell*, 135(4), pp. 609-622.

Tota, B. (1978) 'Functional cardiac morphology and biochemistry in Atlantic bluefin tuna', *The Physiological Ecology of Tunas*, pp. 89-112.

Tóth, M.L., Sigmond, T., Borsos, É., Barna, J., Erdélyi, P., Takács-Vellai, K., Orosz, L., Kovács, A.L., Csikós, G. and Sass, M. (2008) 'Longevity pathways converge on autophagy genes to regulate life span in Caenorhabditis elegans', *Autophagy*, 4(3), pp. 330-338.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly-Y, M., Gidlof, S., Oldfors, A., Wibom, R., Tornell, J., Jacobs, H.T. and Larsson, N.-G. (2004) 'Premature ageing in mice expressing defective mitochondrial DNA polymerase', *Nature*, 429(6990), pp. 417-423.

Tsai, S.B., Tucci, V., Uchiyama, J., Fabian, N.J., Lin, M.C., Bayliss, P.E., Neuberg, D.S., Zhdanova, I.V. and Kishi, S. (2007) 'Differential effects of genotoxic stress on both concurrent body growth and gradual senescence in the adult zebrafish', *Aging Cell*, 6(2), pp. 209-224.

Tümpel, S. and Rudolph, K.L. (2012) 'The role of telomere shortening in somatic stem cells and tissue aging: lessons from telomerase model systems', *Annals of the New York Academy of Sciences*, 1266(1), pp. 28-39.

Tuttle, A.H., Rankin, M.M., Teta, M., Sartori, D.J., Stein, G.M., Kim, G.J., Virgilio, C., Granger, A., Zhou, D., Long, S.H., Schiffman, A.B. and Kushner, J.A. (2010) 'Immunofluorescent Detection of Two Thymidine Analogues (CldU and IdU) in Primary Tissue', (46), p. e2166.

Valenzuela, H. (2013) 'Longer Telomeres Associated With Endurance Exercise'.

Van Dantzig, J.M., Delemarre, B.J., Koster, R.W., Bot, H. and Visser, C.A. (1996) 'Pathogenesis of mitral regurgitation in acute myocardial infarction: Importance of changes in left ventricular shape and regional function', *American Heart Journal*, 131(5), pp. 865-871.

van der Meer, S., Zwerink, M., van Brussel, M., van der Valk, P., Wajon, E. and van der Palen, J. (2012) 'Effect of outpatient exercise training programmes in patients

with chronic heart failure: a systematic review', *European Journal of Preventive Cardiology*, 19(4), pp. 795-803.

Van der Meulen, T., Schipper, H., van den Boogaart, J.G.M., Huising, M.O., Kranenbarg, S. and Van Leeuwen, J.L. (2006) 'Endurance exercise differentially stimulates heart and axial muscle development in zebrafish (Danio rerio)', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 291(4), pp. R1040-R1048.

Van Oijen, M., Medema, R.H., Slootweg, P. and Rijksen, G. (1998) 'Positivity of the proliferation marker Ki-67 in noncycling cells', *American journal of clinical pathology*, 110(1), pp. 24-31.

Van Raamsdonk, W., Maslam, S., De Jong, D.H., Smit-Onel, M.J. and Velzing, E. (1998) 'Long term effects of spinal cord transection in zebrafish: swimming performances, and metabolic properties of the neuromuscular system', *Acta histochemica*, 100(2), pp. 117-131.

van Wijk, B., Gunst, Q.D., Moorman, A.F.M. and van den Hoff, M.J.B. (2012) 'Cardiac Regeneration from Activated Epicardium', *PloS one*, 7(9), p. e44692.

Vande Velde, C., Cizeau, J., Dubik, D., Alimonti, J., Brown, T., Israels, S., Hakem, R. and Greenberg, A.H. (2000) 'BNIP3 and Genetic Control of Necrosis-Like Cell Death through the Mitochondrial Permeability Transition Pore', *Molecular and Cellular Biology*, 20(15), pp. 5454-5468.

Vandenbossche, J.L., Massie, B.M., Schiller, N.B. and Karliner, J.S. (1988) 'Relation of left ventricular shape to volume and mass in patients with minimally symptomatic chronic aortic regurgitation', *American Heart Journal*, 116(4), pp. 1022-1027.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) 'Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes', *Genome biology*, 3(7), p. research0034.

Vanlangenakker, N., Berghe, T.V., Krysko, D.V., Festjens, N. and Vandenabeele, P. (2008) 'Molecular mechanisms and pathophysiology of necrotic cell death', *Current molecular medicine*, 8(3), pp. 207-220.

Vigorito, C. and Giallauria, F. (2014) 'Effects of exercise on cardiovascular performance in the elderly', *Frontiers in Physiology*, 5, p. 51.

Vliegen, H.W., Van Der Laarse, A., Cornelisse, C.J. and Eulderink, F. (1991) 'Myocardial changes in pressure overload-induced left ventricular hypertrophy. A study on tissue composition, polyploidization and multinucleation', *European Heart Journal*, 12(4), pp. 488-494.

von Harsdorf, R., Li, P.-F. and Dietz, R. (1999) 'Signaling Pathways in Reactive Oxygen Species–Induced Cardiomyocyte Apoptosis', *Circulation*, 99(22), pp. 2934-2941.

Vong, L.H., Ragusa, M.J. and Schwarz, J.J. (2005) 'Generation of conditional Mef2cloxP/loxP mice for temporal‐and tissue‐specific analyses', *Genesis*, 43(1), pp. 43-48.

Waldo, K.L., Kumiski, D.H., Wallis, K.T., Stadt, H.A., Hutson, M.R., Platt, D.H. and Kirby, M.L. (2001) 'Conotruncal myocardium arises from a secondary heart field', *Development*, 128(16), pp. 3179-3188.

Waller, B.F. (1988) 'The old-age heart: Normal aging changes which can produce or mimic cardiac disease', *Clinical Cardiology*, 11(8), pp. 513-517.

Wang, D., Oparil, S., Feng, J.A., Li, P., Perry, G., Chen, L.B., Dai, M., John, S.W.M. and Chen, Y.F. (2003) 'Effects of pressure overload on extracellular matrix expression in the heart of the atrial natriuretic peptide-null mouse', *Hypertension*, 42(1), pp. 88-95.

Wang, J., Panáková, D., Kikuchi, K., Holdway, J.E., Gemberling, M., Burris, J.S., Singh, S.P., Dickson, A.L., Lin, Y.-F. and Sabeh, M.K. (2011) 'The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion', *Development*, 138(16), pp. 3421-3430.

Wang, X., Osinska, H., Klevitsky, R., Gerdes, A.M., Nieman, M., Lorenz, J., Hewett, T. and Robbins, J. (2001) 'Expression of R120G–αB-Crystallin Causes Aberrant Desmin and αB-Crystallin Aggregation and Cardiomyopathy in Mice', *Circulation Research*, 89(1), pp. 84-91.

Warrington, J.A., Nair, A., Mahadevappa, M. and Tsyganskaya, M. (2000) 'Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes', *Physiological genomics*, 2(3), pp. 143-147.

Waxman, J.S., Keegan, B.R., Roberts, R.W., Poss, K.D. and Yelon, D. (2008) 'Hoxb5b acts downstream of retinoic acid signaling in the forelimb field to restrict heart field potential in zebrafish', *Developmental cell*, 15(6), pp. 923-934.

Weber, K.T. (2004) 'Fibrosis in hypertensive heart disease: Focus on cardiac fibroblasts', *Journal of Hypertension*, 22(1), pp. 47-50.

Wei, Y., Mizzen, C.A., Cook, R.G., Gorovsky, M.A. and Allis, C.D. (1998) 'Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena', *Proceedings of the National Academy of Sciences*, 95(13), pp. 7480-7484.

Weinbrenner, C., Liu, G.-S., Cohen, M.V. and Downey, J.M. (1997) 'Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart', *Journal of molecular and cellular cardiology*, 29(9), pp. 2383-2391.

Weinert, B.T. and Timiras, P.S. (2003) 'Invited review: theories of aging', *Journal of Applied Physiology*, 95(4), pp. 1706-1716.

Whelan, R.S., Kaplinskiy, V. and Kitsis, R.N. (2010) 'Cell death in the pathogenesis of heart disease: mechanisms and significance', *Annual Review of Physiology*, 72, pp. 19-44.

White, F.C., Bloor, C.M., McKirnan, M.D. and Carroll, S.M. (1998) 'Exercise training in swine promotes growth of arteriolar bed and capillary angiogenesis in heart', *Journal of Applied Physiology*, 85(3), pp. 1160-1168.

Wilkins, B.J., Dai, Y.-S., Bueno, O.F., Parsons, S.A., Xu, J., Plank, D.M., Jones, F., Kimball, T.R. and Molkentin, J.D. (2004) 'Calcineurin/NFAT coupling participates

in pathological, but not physiological, cardiac hypertrophy', *Circulation Research*, 94(1), pp. 110-118.

Williams, D.A., Delbridge, L.M., Cody, S.H., Harris, P.J. and Morgan, T.O. (1992) 'Spontaneous and propagated calcium release in isolated cardiac myocytes viewed by confocal microscopy', *American Journal of Physiology-Cell Physiology*, 262(3), pp. C731-C742.

Wills, A.A., Holdway, J.E., Major, R.J. and Poss, K.D. (2008) 'Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish', *Development*, 135(1), pp. 183-192.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L. and Bockamp, E. (2008) 'Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair', *Cell*, 135(6), pp. 1118-1129.

Wohlgemuth, S.E., Julian, D., Akin, D.E., Fried, J., Toscano, K., Leeuwenburgh, C. and Dunn, J.W.A. (2007) 'Autophagy in the heart and liver during normal aging and calorie restriction', *Rejuvenation research*, 10(3), pp. 281-292.

Wohlgemuth, S.E., Seo, A.Y., Marzetti, E., Lees, H.A. and Leeuwenburgh, C. (2010) 'Skeletal muscle autophagy and apoptosis during aging: Effects of calorie restriction and life-long exercise', *Experimental gerontology*, 45(2), pp. 138-148.

Wong, C.X., Abed, H.S., Molaee, P., Nelson, A.J., Brooks, A.G., Sharma, G., Leong, D.P., Lau, D.H., Middeldorp, M.E. and Roberts-Thomson, K.C. (2011) 'Pericardial fat is associated with atrial fibrillation severity and ablation outcome', *Journal of the American College of Cardiology*, 57(17), pp. 1745-1751.

Woodiwiss, A.J., Oosthuyse, T. and Norton, G.R. (1998) 'Reduced cardiac stiffness following exercise is associated with preserved myocardial collagen characteristics in the rat', *European Journal of Applied Physiology and Occupational Physiology*, 78(2), pp. 148-154.

Wright, W.E. and Shay, J.W. (2001) 'Cellular senescence as a tumor-protection mechanism: The essential role of counting', *Current Opinion in Genetics and Development*, 11(1), pp. 98-103.

Wright, W.E. and Shay, J.W. (2005) 'Telomere-binding factors and general DNA repair', *Nature Genetics*, 37(2), pp. 116-118.

Xiao, J., Xu, T., Li, J., Lv, D., Chen, P., Zhou, Q. and Xu, J. (2014) 'Exercise-induced physiological hypertrophy initiates activation of cardiac progenitor cells', *Int J Clin Exp Pathol*, 7(2), pp. 663-669.

Xiao, Z.-Q., Moragoda, L., Jaszewski, R., Hatfield, J.A., Fligiel, S.E.G. and Majumdar, A.P.N. (2001) 'Aging is associated with increased proliferation and decreased apoptosis in the colonic mucosa', *Mechanisms of ageing and development*, 122(15), pp. 1849-1864.

Xu, J., Gong, N.L., Bodi, I., Aronow, B.J., Backx, P.H. and Molkentin, J.D. (2006) 'Myocyte enhancer factors 2A and 2C induce dilated cardiomyopathy in transgenic mice', *Journal of Biological Chemistry*, 281(14), pp. 9152-9162.

Yan, L., Vatner, D.E., Kim, S.-J., Ge, H., Masurekar, M., Massover, W.H., Yang, G., Matsui, Y., Sadoshima, J. and Vatner, S.F. (2005) 'Autophagy in chronically ischemic myocardium', *Proceedings of the National Academy of Sciences of the United States of America*, 102(39), pp. 13807-13812.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T.L. (2012) 'Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction', *BMC bioinformatics*, 13(1), p. 134.

Yokochi, T. and Gilbert, D.M. (2007) 'Replication labeling with halogenated thymidine analogs', *Current Protocols in Cell Biology*, pp. 22.10. 1-22.10. 14.

Younes, A., Boluyt, M.O., O'Neill, L., Meredith, A.L., Crow, M.T. and Lakatta, E.G. (1995) 'Age-associated increase in rat ventricular ANP gene expression correlates with cardiac hypertrophy', *American Journal of Physiology-Heart and Circulatory Physiology*, 269(3), pp. H1003-H1008.

Zhang, G., Gurtu, V., Kain, S.R. and Yan, G. (1997) 'Early detection of apoptosis using a fluorescent conjugate of annexin V', *Biotechniques*, 23(3), pp. 525-531.

Zhang, Y., Chong, E. and Herman, B. (2002) 'Age-associated increases in the activity of multiple caspases in Fisher 344 rat organs', *Experimental gerontology*, 37(6), pp. 777-789.

Zhou, B., Ma, Q., Rajagopal, S., Wu, S.M., Domian, I., Rivera-Feliciano, J., Jiang, D., von Gise, A., Ikeda, S. and Chien, K.R. (2008) 'Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart', *Nature*, 454(7200), pp. 109-113.

Zhu, H., Tannous, P., Johnstone, J.L., Kong, Y., Shelton, J.M., Richardson, J.A., Le, V., Levine, B., Rothermel, B.A. and Hill, J.A. (2007) 'Cardiac autophagy is a maladaptive response to hemodynamic stress', *Journal of Clinical Investigation*, 117(7), pp. 1782-1793.

Zou, S., Tian, C., Ge, S. and Hu, B. (2013) 'Neurogenesis of Retinal Ganglion Cells Is Not Essential to Visual Functional Recovery after Optic Nerve Injury in Adult Zebrafish', *PloS one*, 8(2), p. e57280.

Zou, Y., Evans, S., Chen, J., Kuo, H.C., Harvey, R.P. and Chien, K.R. (1997) 'CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway', *Development*, 124(4), pp. 793-804.