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Abstract

Cardiac hypertrophy is a response to increased mechanical demand on the heart. Initially hypertrophy is beneficial by reducing stress on the ventricular wall. However, sustained stress on the heart results in pathological hypertrophy which is characterised by re-induction of the fetal gene program, interstitial fibrosis, cardiomyocyte apoptosis, chamber dilation and eventually, heart failure. In contrast, exercise and pregnancy are considered to cause physiological hypertrophy with preservation of normal cardiac structure. The precise mechanism behind two different hypertrophy types remains unknown.

In the heart mitogen-activated protein kinases (MAPKs) are involved in a complex signalling network. The extracellular-regulated protein kinase (ERK) 2 is one of classical MAPKs and shares with ERK1 an amino acid identity of 84%. Despite having many similarities, studies using total gene knockout mice clearly indicate that ERK1 and ERK2 have different roles and functions. ERK1 deficient knockout mice are fertile, viable and of normal size. Meanwhile, ERK2 knockout mice die during at embryonic day 6.5 to 11.5.

Despite extensive studies the precise role of ERK1 and ERK2 (ERK1/2) in cardiac remodelling remains unclear. Since ERK2 contributes 70% to total ERK1/2 protein content, it is valuable to directly investigate the function of ERK2 in the heart. The use of cardiomyocyte-specific ERK2 deletion (ERK2cko) mice is one such approach compared to models inactivating or overexpressing ERK1/2 inhibitors and activators.

The ERK2cko mice (generated in our lab) were fertile, viable and did not show any change in expression of other MAPKs in the heart or ERK2 in other tissues. To investigate the role of ERK2 in cardiac remodelling ERK2cko mice were exposed to different pathological and physiological hypertrophic stimuli. ERK2fl/o (ERK2f/f) mice were used as a control group.

β-adrenergic stimulation revealed a role of ERK2 in hypertrophy as ERK2cko mice showed less cardiomyocyte growth in comparison to ERK2f/f mice. However, no effect of ERK2 was observed on AngiotensinII-induced hypertrophy.

After 1 week of pressure overload, the mutant mice showed less hypertrophic growth, less interstitial fibrosis and increased apoptosis compared to the controls. Long term pressure overload less increased hypertrophic growth and increased apoptosis in ERK2cko mice, which also showed early signs of heart failure.

In concordance, neonatal rat cardiomyocytes infected with adenovirus expressing dominant negative ERK2 showed blunted hypertrophic growth and were more prone to apoptosis in vitro.

In addition, ERK2 was not involved in physiological hypertrophy induced by 4 weeks swimming exercise.

Taken together, ERK2 played an important role in cardiac hypertrophic remodelling induced by pressure overload and β-adrenergic stimulation where its absence led to blunted hypertrophic growth and sensitised cardiomyocytes to apoptosis. In contrast, ERK2 unlikely played a role in physiological hypertrophy induced by swimming exercise in our experimental setting.
Declaration

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<td>Angiotensin II</td>
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<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
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<td>ASK1</td>
<td>Apoptosis signal-regulated kinase 1</td>
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<td>BMK1</td>
<td>Big mitogen-activated protein kinase 1</td>
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<td>BNP</td>
<td>Brain natriuretic peptide</td>
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<td>CMV</td>
<td>Constitutive cytomegalovirus</td>
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<td>CPE</td>
<td>Cytopathic effect</td>
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<td>Cre</td>
<td>Cyclic recombinase</td>
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<tr>
<td>D-domain</td>
<td>Docking domain</td>
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<tr>
<td>DISC</td>
<td>Death-inducible signalling complex</td>
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<tr>
<td>dIVS</td>
<td>Diastolic interventricular septum thickness</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>dPW</td>
<td>Diastolic left ventricular posterior wall thickness</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ERK1</td>
<td>Extracellular-regulated protein kinase 1</td>
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<td>ERK2</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<td>FS</td>
<td>Fraction shortening</td>
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<td>GATA4</td>
<td>GATA binding protein 4</td>
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<td>GPCRs</td>
<td>G-protein coupled receptors</td>
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<td>HW/BW</td>
<td>Heart weight to body weight</td>
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<tr>
<td>HW/TL</td>
<td>Heart weight to tibia length</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
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<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>Iso</td>
<td>Isoproterenol</td>
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<tr>
<td>JIP</td>
<td>JNK interacting protein</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>LacZ</td>
<td>β-galactosidase</td>
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loxp | Locus of X-over of P1
---|---
LVEDD | Left ventricle end-diastolic diameter
LVESD | Left ventricle end-systolic diameter
MAPK | Mitogen-activated protein kinase
MEK1 | MAPK/ERK kinase 1
MEK2 | MAPK/ERK kinase 2
MEK5 | MAPK/ERK kinase 5
MEKK | MAPK/ERK kinase kinase
MHC | Myosin heavy chain
MKK3 | MAPK kinase 3
MKK4 | MAPK kinase 4
MKK6 | MAPK kinase 6
MKK7 | MAPK kinase 7
MLC2v | Myosin light chain 2 the ventricular specific isoform
NGS | Normal goat serum
NLS | Nuclear localisation sequence
NRSCMs | Neonatal rat cardiomyocytes
p38 MAPK | Mitogen-activated protein kinase p38
PE | Phenylephrine
PFA | Paraformaldehyde
PI3K | Phosphatidylinositol 3-kinase
PW | Posterior wall
S6K | Ribosomal S6 kinase
dIVS | Diastolic interventricular septum thickness
sIVS | Systolic interventricular septum thickness
sPW | Systolic left ventricular posterior wall thickness
TAC | Transverse aortic constriction
TGF-β | Transforming growth factor-β
TUNEL | Terminal-Deoxynucleotidyl-Transferase-Mediated-dUTP-Nick-End-Labeling
VEGF | Vascular endothelia growth factor
β-MHC | β-isoform of myosin heavy chain
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Chapter 1 General Introduction

1.1 The heart

The heart is a vital organ found in all animals with circulatory system, in which its failure leads to death. The heart is divided into four chambers: two atria and two ventricles. The septum of the heart is the dividing wall between the right and the left side of the heart, consisting of the inter-atrial and inter-ventricular parts. Atrioventricular valves connect the atria with respective ventricles. Ventricles have thicker walls capable of generating higher blood pressure. The pulmonary valve separates the right ventricle from the pulmonary artery whereas the aortic valve is situated between the left ventricle (LV) and the aorta. The left ventricle pumps blood into the aorta which then goes into arteries, arterioles and capillaries throughout the organism under high pressure. The left ventricle has approximately twice the wall thickness and 3 times the ventricle mass of the right ventricle. The thinner walled right ventricle generates lower pressure, pumping venous blood from the right atrium into the pulmonary artery, which then passes through the pulmonary veins into the left atrium.

In the heart, the cellular components primarily consist of cardiomyocyte, fibroblasts and the vascular smooth muscle cells\(^1\). Cardiomyocytes make up the largest volume of the heart while representing only a third of the total cell number\(^2\).

Cardiomyocytes are long, cylindrical cells and are composed of myofibrils that contain myofilaments. Myofilaments have repeating units of sarcomeres, the basic contractile unit of the cardiomyocyte that produce muscle contraction. Non-cardiomyocytes account for the remaining two-third cell population including fibroblasts, endothelial cells as well as vascular smooth muscle cells (VSMC)\(^1,2\).
In an early study cardiomyocytes were suggested to proliferate postnataally in humans. At birth, the total number of ventricular cardiomyocytes was estimated at about $1 \times 10^9$ whereas at 20 years of age both male or female hearts showed a significant increase in number of cardiomyocytes ($7.9 \times 10^9$ or $5.9 \times 10^9$ respectively)$^3$. However, a recent study analysing mouse cardiac tissue during 3 developmental periods, foetal, neonatal and adult, has demonstrated contradictory results. The cell cycle progression has been detected by the proliferation marker BrdU. The percentage of BrdU-labelled myocyte nuclei decreases from 23% at embryonic day 14.5 to 8% at neonatal day 7. No BrdU incorporation has been shown from 3 weeks after birth suggesting that cardiomyocytes do not proliferate after that time point$^4$.

1.2 Cardiac hypertrophy

Cardiac hypertrophy is defined as an increase in heart muscle mass without affecting cardiomyocyte cell number. This process involves changes in protein synthesis which leads to increased cardiomyocyte cell size$^5$. Depending on the cause 2 forms of hypertrophy may develop. Pathological hypertrophy occurs in response to stress, such as chronic pressure overload (hypertension), volume overload (valvular heart disease) or myocardial infarction. Physiological hypertrophy is reversible and includes postnatal heart growth, pregnancy- and exercise-induced hypertrophy$^6$ (Figure 1.1).

Pathological and physiological hypertrophies demonstrate increased protein synthesis and altered metabolism.
Protein synthesis during hypertrophy

The ribosomal S6 kinases (S6K1 and S6K2) are considered regulators of protein synthesis in response to hypertrophic stimuli. Pressure overload-induced hypertrophy activates S6K1 in the heart as rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), suppresses S6K1 activation leading to 67% less increased heart weight following pressure overload. This suggests a crucial role of S6K1 in pathological hypertrophy. The role of S6K1 in physiological hypertrophy has been shown in transgenic mice overexpressing insulin-like growth factor-1 receptors (IGF-1R) which display an increased expression of S6K1 in the heart.

Metabolic changes in hypertrophy

In the heart 60 to 80% of the energy metabolism relies on fatty acid oxidation whereas glucose, lactate and ketone provide substrates for the rest ATP production. Depending on nutritional supply and hormonal status, the heart can switch between energy substrates. Physiological hypertrophy induced by exercise in humans results in increased oxidation of glucose. A recent study analysed rat hearts after 7 weeks of treadmill running has shown that as well as the genes involved in glucose metabolism, genes responsible for fatty acid oxidation are upregulated as well.

Pathological hypertrophy is characterised by a metabolic shift from fatty acid oxidation towards glucose utilisation. This metabolism is also found in the foetus during cardiac development which uses glucose as a major substrate.

In pathological hypertrophy this shift in metabolism may be adaptive allowing the heart to produce more ATP per molecule oxygen since ATP production from fatty acids consumes more oxygen than that from glucose.
Gender differences in hypertrophic development

Research has shown that the gender influences the hypertrophic response. In spontaneously hypertensive rats, male rats present heart failure signs at 16 months of age while female rats remain symptom-free up to 22 months of age\textsuperscript{14}. Epidemiological studies show that pre-menopausal women have a lower risk for cardiovascular diseases than men and post-menopausal women, suggesting a cardio-protective role of estrogen\textsuperscript{15}. Estrogen is produced by both men and women, however, circulating levels in pre-menopausal women are 10-20 fold higher whereas post-menopausal women have a similar estrogen level as men\textsuperscript{16}. The cardio-protective role of estrogen in post-menstrual women, on the other hand, remains controversial. Estrogen is considered to lower mortality rate for women with coronary risk factors, such as smoking and high blood pressure\textsuperscript{17}. However, women on combination-therapy, such as estrogen combined with progestin have an increased risk of heart coronary disease\textsuperscript{18}.

Finally, considering the heart morphological changes, hypertrophy may be differentiated into concentric and eccentric remodelling. In concentric hypertrophy, parallel pattern of sarcomere organisation leads to increased wall thickness without changing the chamber volume. Eccentric hypertrophy shows serial organisation of new sarcomeres which results in increased chamber volume without affecting wall thickness\textsuperscript{19}. 

**Figure 1.1: Pathological and physiological hypertrophy**

Depending on the stimulus the normal heart develops either pathological or physiological hypertrophy. Pathological hypertrophy is associated with fetal gene re-expression, development of fibrosis, apoptosis and leads to depressed cardiac function which may result in cardiac dysfunction. Physiological hypertrophy is reversible and associated with normal cardiac function.

### 1.3 Pathological hypertrophic remodelling

Increased mechanical demand on the heart leads to cardiac hypertrophy which initially counterbalances increased stress on the ventricular wall. However, long-term pathological hypertrophy is often associated with a greater risk of heart failure, including loss of cardiomyocytes, increased fibrosis, and ventricular arrhythmias which may lead to sudden death\(^\text{20-22}\).

Different stimuli trigger pathological remodelling, such as mechanical stretch, and release of neurohumoral factors, such as angiotensin II (Ang II), endothelin-1 (ET-1) and cytokine, such as transforming growth factor β (TGF-β)\(^6\). These neurohumoral...
factors bind to respective receptors on cardiac cells to activate intracellular pathways. Several studies have demonstrated the involvement of these activated pathways in pathological hypertrophy.

ET-1 is secreted from cardiomyocytes in response to mechanical stretch and binds to ET<sub>A</sub> and ET<sub>B</sub> receptors with ET<sub>A</sub> representing 90% of ET-1 receptors. Consistently, ET<sub>A</sub> receptor antagonists prevent the development of cardiac hypertrophy and fibrosis in rats following hypertension.

AngII is a member of the renin-angiotensin pathway which is important for blood pressure regulation. The action of AngII to initiate hypertrophy is specifically mediated by the AngII type 1 (AT1) receptor, as AT1 receptor antagonists have been demonstrated to block AngII-induced hypertrophy in cardiomyocytes. In addition, the inhibition of angiotensin converting enzyme (ACE), which converts AngI to AngII has been shown to prevent pressure overload-induced left ventricular hypertrophy in rats.

In ventricular cardiomyocytes AngII is stored in granules which are released in response to stretch-induced growth. Moreover, mechanical stress is able to directly activate the AT-1 receptor in an AngII-independent mechanism.

The renin-angiotenin pathway is also associated with TGF-β pathway. AngII induces mRNA and protein expression of TGF-β1 in neonatal rat cardiomyocytes. Similarly, in hypertensive rat hearts treatment with ACE inhibitor or AT1 receptor antagonists decrease mRNA levels of TGF-β1.

The majority of transmembrane signal transduction in response to neurotransmitters is mediated by G-protein coupled receptors (GPCRs). Binding of heterotrimeric G proteins to GPCR is followed by dissociation of the Ga and Gbγ subunits which leads to activation of downstream targets. Isoforms of the Ga subunit include G<sub>a1</sub>, G<sub>i</sub>, G<sub>q</sub>, G<sub>12</sub>, G<sub>13</sub>.
G12, Gαq and Gα11. Gαq plays an important role as cardiac-specific overexpression or constitutive active Gαq in hearts induce cardiac hypertrophy that lead to cardiac dysfunction.34,35

Signalling through calcium the phosphatase calcineurin increases its activity in response to pressure overload.36 Transgenic mice expressing an activated form of calcineurin develop cardiac hypertrophy which progresses to extensive interstitial fibrosis, heart failure and sudden death.37 Calcineurin is considered to regulate pathological hypertrophic response via dephosphorylating of the nuclear factor of activated T-cell (NFAT), which translocates to the nucleus where it associates with the transcription factors, such as GATA binding protein 4 (GATA4) to regulate the expression of cardiac genes.6

Several genes are upregulated in pathological hypertrophy but not in physiological hypertrophy.5 The cellular response in pathological hypertrophy is often associated with re-induction of the fetal gene program in which gene expression patterns seen during embryonic development are reactivated.5 Fetal genes include the atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α-skeletal actin, and β-myosin heavy chain (β-MHC).38 β-MHC predominates during embryonic and fetal stages while after birth cardiac α-myosin heavy chain (α-MHC) is upregulated. However, within hours after pressure overload α-MHC is downregulated and β-MHC is upregulated.39 The induction of the fetal gene program is followed by the expression of the immediate early genes, such as c-fos, c-myc and c-jun.40 These genes encode transcription factors which regulate a wide range of gene expression.40 Interestingly, the overall hypertrophic response seems to depend on the intensity of stress. It has been shown that intermittent pressure overload results in compensated hypertrophy with little fibrosis without activation of the fetal genes β-MHC, BNP
and ANP. This suggests that there are different degrees of pathological hypertrophy.

The hypertrophic role of the mitogen-activated protein kinases are further discussed in the section 1.5 (Mitogen-activated protein kinases).

1.3.1 Cardiomyocyte apoptosis associated with pathological hypertrophy

Cell death may occur in 3 different pathways: autophagy, necrosis and apoptosis. Autophagy includes degradation of cell compartments by the lysosomal pathway and can be used for energy production. Necrosis is an energy-independent, inflammatory process with rupture of cell membrane. Apoptosis is an evolutionarily conserved, energy-dependent mechanism of controlled cell death. Typical apoptotic morphological changes include cell shrinkage, losing of cell-cell-contact, nuclear shrinkage, chromatin condensation and formation of apoptotic bodies without disruption of the membrane. Macrophages assimilate the final apoptotic bodies that contain fragments of organelles and nuclear material.

The type of cell death response depends on the degree of stress as high levels of oxidative stress result in necrosis while low levels cause apoptosis.

Spontaneous hypertensive rats show a transition from compensated hypertrophy to heart failure, which is associated with increased number of apoptotic cells. Apoptosis occurring in heart failure is very low but abnormal levels can persist for years. In non-failing hearts apoptosis rates of 0.001% to 0.002% have been reported whereas patients with end stage dilated cardiomyopathy show rates of 0.08% to 0.25%. Two different theories might explain why these small percentages lead to heart failure. A chronic loss of small number of cardiomyocytes may result in
dramatic consequences for myocardial function. Alternatively, the apoptotic rate might decrease in the end stage of heart failure\textsuperscript{47}.

The mechanisms of apoptosis can be divided into 3 different pathways: intrinsic and extrinsic pathway as well as the apoptosis pathway in endoplasmatic reticulum (ER pathway).

The key players of the intrinsic pathway are the members of the Bcl-2 and the caspase families (Figure 1.2).

The first group of the Bcl-2 family contains anti-apoptotic members, such as Bcl-2 and Bcl-x\textsubscript{L} (Bcl-x protein long isoform). The main role of this group is the inhibition of pro-apoptotic Bcl-2 members by heterodimerization\textsuperscript{43, 48}. The second group includes pro-apoptotics, such as Bax (Bcl-2-associated X protein) and Bak (Bcl-2-antagonist/killer)\textsuperscript{48, 49}. Another pro-apoptotic group is the BH3-only protein family and members are Bid, Bim (Bcl-2 interacting mediator of death) and Bad (Bcl-2 antagonist of cell death)\textsuperscript{48, 49}. Bim and Bid receive upstream pro-apoptotic signals and activate Bax and Bak\textsuperscript{43,49}.

The pro- and anti-apoptotic Bcl-2 family members regulate cytochrome c and therefore either protect or disturb the mitochondrial membrane. Cytochrome c, usually found in the space between the inner and the outer mitochondrial membrane, is released upon increased membrane permeability\textsuperscript{43}. The increased mitochondrial permeability is followed by release of other intermembrane space proteins, such as the second mitochondrial-derived activator of caspase (SMAC) / direct inhibitor of apoptosis (IAP)-binding protein with low pI (DIABLO) (SMAC/DIABLO)\textsuperscript{43, 50}. Once released, cytochrome c binds to the adapter protein Apaf-1 which oligomerizes to the apoptosom. The apoptosom is crucial for cysteiny1 aspartate-specific protease
(caspase) activation by bringing two or more pro-caspases in close contact thereby allowing their activation⁵¹.

Caspases are subdivided into initiator caspases (8, 9, 10 and 12) and effector caspases (3 and 7)⁴⁹.

A well-studied initiator caspase is the caspase 9 which is activated by proteolytic cleavage of procaspase 9. Within the apoptosom, Apaf-1 facilitates procaspase-9 autoactivation and therefore enables initiation of the intrinsic apoptotic pathway⁵¹–⁵³. Effector caspases are monomers before and after proteolytic activation cleavage⁵¹.

Caspase substrates are crucial for homeostasis, such as α-actin, α-/β-MHC, myosin light chain (MLC) 1/2 and tropomycin⁴⁷. As a negative control mechanism endogenous inhibitors of apoptosis (IAPs) bind and inhibit activated caspases with selective specificity⁵⁴.

The extrinsic pathway is triggered by ligands from the extracellular milieu that bind to death receptors on the cell surface, such as Fas ligand (FasL)⁴⁷,⁴⁹. Stimuli for the extrinsic pathway include radiation, physical stress or nutrient deficiencies⁴⁹. The most studied death receptor is Fas which is bound by FasL⁴³. Binding of FasL to Fas leads to conformational change which initiates the formation of the death-inducing signalling complex (DISC), including pro-caspase 8⁴⁹. Thus, pro-caspase 8 becomes dimerized to caspase 8, which activates the effector caspases 3 or 7 and thereby causes apoptosis⁴³,⁴⁹ (Figure 1.2).

ER stress is caused by accumulation of unfolded and misfolded proteins in the ER lumen that results in the activation of unfolded protein response (UPR). Thereafter, upregulated ER chaperones repair the unfolded and misfolded proteins⁵⁵. However, excessive and prolonged ER stress may initiate ER apoptosis⁵⁵. UPR has been shown
to activate caspase 12 that cleaves pro-caspase 9 independent of the apoptosom formation\textsuperscript{56}.

**Figure 1.2: Schematic Illustration of extrinsic (death receptor mediated) and intrinsic (mitochondria mediated) apoptotic pathway**

DISC consists of FasL, Fas and procaspase 8 or 10 (procaspase 8/10) and leads to caspase activation. Within the intrinsic apoptotic pathway the apoptosom is formed to activate caspase 9. Its components are procaspase 9, cytochrome c, and adapter protein Apaf-1. Caspase 8/10 and 9 activate caspase 3/7 followed by degradation of cellular proteins which finally leads to cell death. Activated caspases are regulated by inhibitors of apoptosis (IAPs) which are negatively regulated by SMAC/DIABLO.

Cytochrome c, SMAC/DIABLO and AIF are released after opening of mitochondrial permeability transition pores (MPTP) which is regulated by pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-x\textsubscript{L}) receptors.
1.3.2 Ventricular fibrosis associated with pathological hypertrophy

An increased workload must be accompanied by a coordinated increase in the surrounding architecture of connective tissue. However, cardiac hypertrophy is not a prerequisite to fibrosis formation, exceptions include physiological and volume overload induced hypertrophy. In pathological hypertrophy and heart failure fibrosis is an important hallmark. However, the exact mechanisms responsible for the switch from normal collagen mass to excessive fibrosis in maladaptive hypertrophy and heart failure are not entirely known. The connective tissue contains collagen and small amounts of elastin, laminin and fibronectin. Collagen in myocardium is composed of collagen type I (80%), collagen type III and collagen type V. During the compensatory phase of cardiac hypertrophy fibrosis may be beneficial as the collagen matrix grows to accommodate the increase in muscle mass.

An important mediator of cardiac fibrosis is TGF-β1 which regulates pro-fibrotic proteins, such as connective tissue growth factor (ctgf). Transgenic mice overexpressing TGF-β1 develop cardiac hypertrophy accompanied by interstitial fibrosis.

A pathological study of human hearts has shown that the volumetric fraction of fibrosis according to heart weight in healthy control hearts is 6.5%, while the volume fraction in hypertrophic hearts increases progressively up to 31.1%.

In summary, pathological hypertrophy induces development of fibrosis and its extent correlates with the severity of cardiac dysfunction.
1.3.3 Heart failure as a consequence of pathological hypertrophy

Initially cardiac hypertrophy is beneficial by normalising the increased wall stress and work load. However, if the chronic increase in wall stress persists, the hypertrophic heart can dilate, which leads to decreased contractile function and failing of the heart. Heart failure compromises the patient’s life and symptoms include fatigue, shortness of breath, drowsiness, insomnia, cough, anxiety and depression. Despite significant therapeutic advances, heart failure affects 1 to 4% of people in modern society. Heart failure incidence increases with age, affecting 3 to 4% of those over 45 years old, 5% of those between 60 and 69 years old, and 10% of those over the age of 70.

Considering the data already described in the previous sections, suppressing pathological hypertrophy and apoptosis may be the key factor to slow or reverse the progression to heart failure.

1.3.4 Pharmaceutical and non-pharmaceutical treatment for pathological hypertrophy and heart failure

For development of LV systolic dysfunction and heart failure hypertension plays a crucial role. Therefore aggressive blood pressure control by inhibiting different signalling pathways and adequate monitoring is important. The age at medical admission and at death seems to be increasing which means that current preventive treatments are delaying the end stage heart failure. Heart failure patients commonly use between 2 to 7 prescribed medications, including side effect treatment. Angiotensin-converting enzyme inhibitors (ACEIs) and Angiotensin receptor inhibitors (ARBs) are typically the first drugs prescribed for patients with heart failure. ACEIs inhibit angiotensin-converting enzymes and therefore prevent
catalyzation of AngII from its precursor AngI. AngII constricts blood vessels and therefore its inhibition opens up blood vessels and lowers blood pressure. ARBs block the AngII receptors instead of blocking AngII conversion. Therefore ARBs affect the blood pressure more specifically with fewer side effects. Another type of drugs, β-blockers have favourable effects when combined with ARB/ACEI\textsuperscript{64}. β-blockers lower blood pressure by blocking noradrenaline which results in lower heart beat and less force of the heart\textsuperscript{65}. In addition, loop diuretics are used which lower blood pressure due to their natriuretic effect by causing 15-25% of sodium to be excreted\textsuperscript{66}. They include drugs such as furosemide and torsemide\textsuperscript{66}.

As a non-pharmacological treatment controlled exercise is recommended for patients with stabilised heart failure. Aerobic and resistance exercise regimes improve the cardiac function and patient’s wellbeing\textsuperscript{67}.

1.4 Physiological hypertrophic remodelling

Physiological hypertrophy features adaptive growth, which is reversible and does not contribute to a disease\textsuperscript{38}. It develops in response to physical conditioning, such as pregnancy and endurance training, including long distance running and swimming. Physiological growth leads to an increase in cardiac mass with normal structure and function and without accumulation of collagen or fetal gene re-activation\textsuperscript{5,38}. Exactly why physiological hypertrophy differs from pathological hypertrophy remains unclear, however, the nature of stress to the heart might play a role\textsuperscript{68}.

Left ventricular hypertrophy found in athletes is predominantly related to the amount but not the type of exercise. In comparison to non-athletes, the heart dimensions in athletes increase on average by 6%\textsuperscript{69}. 
During physiological hypertrophy the phosphatidylinositol 3 kinase (PI3K) signalling pathway is critical. Mice with cardiac expression of constitutively active PI3K (CA-PI3K) show a 6.5-fold increase in PI3K activity compared to the control group \(^7^0\). As a result these CA-PI3K mice display an increase in cardiomyocytes size which causes 20% larger hearts. In addition, in mice with dominant negative PI3K (DN-PI3K) expression the PI3K activity in heart is decreased by 77% compared to the control group resulting in 17% smaller hearts caused by smaller cardiomyocytes \(^7^0\). Thus the findings in both mouse lines indicate a correlation between the heart size and the PI3K activity during physiological postnatal growth. Neither mouse group develops increased apoptosis, interstitial fibrosis and dysfunction of the heart \(^7^0\).

The important role of PI3K(110α) (p110α) in physiological hypertrophy has been clarified by mice expressing cardiac-specific dominant negative form of p110α \(^7^1\). In response to swimming exercise the hypertrophic heart growth is blunted \(^7^1\). Hypertrophy induced by swimming exercise also results in increased IGF-1 mRNA levels \(^7^2\). The hypertrophic stimulus IGF is involved in cardiac growth via activation of the PI3K signalling cascade \(^3^8\). The role of IGF-1 has been specifically analysed in transgenic mice overexpressing IGF-1R \(^8\). These mice have an improved cardiac function as a result of physiological hypertrophy with elevated activation of PI3K \(^8\).

The downstream effector of PI3K is protein kinase B (PKB) which consists of 3 isoforms in mammalian cells: PKBα, PKBβ and PKBγ. PKBα and PKBβ are expressed in cardiomyocytes and both have distinct functions in the heart \(^7^3\). PKBα has been shown to play an important role in development of physiological hypertrophy as PKBα\(^{-/-}\) mice develop impaired physiological hypertrophy and adult cardiomyocytes prepared from PKBα\(^{-/-}\) mice are resistant to IGF-1 stimulation \(^7^4\).
Currently there is no evidence that physiological cardiac remodelling in response to training leads to long-term cardiovascular disease progression or sudden death. A 12 year study in high school athletes in US has reported a sudden death range of 1:200,000 per year among 27 sports. Therefore physiological hypertrophy is considered protective and its progress to sudden death is uncommon.

1.5 Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are protein kinases that convert extracellular signals into a variety of different cellular responses. All eukaryotic cells possess MAPK pathways which play a crucial role in cell differentiation, proliferation, apoptosis and survival. Mammalian MAPKs can be activated by a variety of different stimuli, including growth factors, cytokines and stress. In mammalian cells 14 MAPKs have been identified and are divided into 7 groups. The classical MAPKs consist of the extracellular-regulated kinases 1/2 (ERK1/2), ERK5 (big MAPK, BMK1), c-Jun amino (N)-terminal kinases (JNK) and p38 MAPK. Atypical MAPKs differ from the conventional group and include ERK3, ERK4, ERK6, ERK7, ERK8 and Nemo-like kinase (NLK).

The classical MAPK cascade consists of 3 sequentially acting kinases, namely the upstream MAP kinase kinase kinase (MAPKKK), the MAP kinase kinase (MEK or MKK) and the downstream MAP kinase (MAPK). The MAPKs exert their effects by phosphorylation of cytoplasmatic and nuclear protein substrates. Substrates have specific interaction domains called docking sites and thereby increase the specificity and efficiency of MAPKs. MAPKs are phosphorylated and therefore activated at both Tyr/Thr residues within the conserved Thr-X-Tyr motif in their
activation loop. The residue X can be any of the eukaryotic amino acids and differs between the MAPK groups (glutamic acid in ERK1/2, ERK5; glycine in p38 MAPK; proline in JNK). A single phosphorylation of MAPKs may cause changes in activation; however, the phosphorylation at both residues is crucial for changing the activation loop formation and thereby increases the MAPK activity significantly.

MEKs specifically phosphorylate MAPKs by using dual-specificity. However, MEKs phosphorylate a limited number of MAPKs, often, including no more than 2 members of the MAPK family\textsuperscript{81}. MEKs are activated by MAPKKKs\textsuperscript{82}. MAPKKKs are serine/threonine kinases and activate MEKs through phosphorylation of 2 serine/threonine residues in the conserved S/T-X\textsubscript{3,5}-S/T motif\textsuperscript{83}.

In addition, scaffold proteins provide further specific complex formation between these kinases. They create specific signalling complexes with subcellular localisation by binding 2 or more components of the MAPK family, bringing them into close contact, thus allowing their interaction. Scaffold proteins also affect the duration and efficiency of the signal and organise MAPKs in specific pathways\textsuperscript{76,83}. The duration of a signal is negatively regulated by three different types of protein phosphatases: protein tyrosine phosphatase, serine/threonine protein phosphatase and dual-specificity protein phosphatase (DUSP)\textsuperscript{85}. They remove phosphate from tyrosine, threonine or both residues\textsuperscript{85}.
The MAPK pathway consists of 3 groups, namely mitogen-activated protein kinase (MAPK), MAPK kinase (MAPKK) and MAPK kinase kinase (MAPKKK). MAPKKKs phosphorylate and thus activate MAPKKs which activate MAPKs. Dependent of sequence homology MAPKs are divided into 3 subclasses: c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular-regulated kinase (ERK). The classical ERKs include ERK1, ERK2. ERK5 is a member of big MAPK.

### 1.5.1 ERK2

ERK1 and ERK2 are also termed classical MAPKs and their signalling pathways are the most widely studied ones within the MAPK cascades. ERK1 was firstly discovered and named ERK due to the wide variety of extracellular signals that activate it. Both ERK1 and ERK2 cDNAs were cloned and characterised in the early 1990s. ERK1 and ERK2 are ubiquitously expressed in tissues, such as the heart and brain. They have molecular weights of 44kDa and 42kDa, respectively, an amino acid identity of 84% and therefore, share signalling activities. Thus both are often referred to as ERK1/2. Alternatively spliced isoforms of ERK1/2 may differ in activation, subcellular localisation or tissue distribution. ERK1b behaviour is similar to ERK1 under most conditions, however, in vitro alteration of the

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**Figure 1.3: Overview of mitogen-activated protein kinases (MAPKs)**

<table>
<thead>
<tr>
<th>Stimuli growth factors and cytokine, stress</th>
<th>MEKK2 MEKK1-4</th>
<th>Raf, c-Mos, Tpl2 MLK2, Tpl2, ASK1, TAK1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPKK MEKK (MEKK)</td>
<td>MEK1, 2 MEK5</td>
<td>M KK4, 7 M KK3, 6</td>
</tr>
<tr>
<td>MAPK</td>
<td>ERK1/2 ERK5</td>
<td>JNK p38</td>
</tr>
<tr>
<td>biological</td>
<td>growth, development</td>
<td>apoptosis, inflammation</td>
</tr>
</tbody>
</table>
cytosolic retention region results in a nuclear localisation\textsuperscript{89}. ERK1c acts specifically in the Golgi apparatus during mitosis and is activated by the alternatively spliced isoform MEK1b and thereby creates an ERK1/2 independent pathway\textsuperscript{90–92}. Additional splice variants of ERK1/2 include ERK1d and ERK2b\textsuperscript{92, 93}.

Despite many similarities in gene sequences and expression pattern the functions of ERK1 and ERK2 proteins differ in gene knockout experiments. Homologous ERK1 deficient knockout (ERK1\textsuperscript{−/−}) mice are fertile, viable and of normal size\textsuperscript{95}. Another study has shown a reduced thymocyte maturation in ERK1\textsuperscript{−/−} mice suggesting a role of ERK1 during thymocyte differentiation and proliferation\textsuperscript{96}. Nevertheless, the loss of ERK1 does not affect the expression of ERK2 in all tested tissues, including the sciatic nerve, the only tissue that expresses ERK1 at a higher level than ERK2\textsuperscript{86, 94}.

In contrast, ERK2 knockout mice die during embryogenesis between embryonic day 6.5 to 11.5 due to various reasons depending on the mouse background, including disruption in mesoderm differentiation, trophoblast development as well as an abnormal placental development causing secondary effects, such as a thin heart wall and growth retardation\textsuperscript{97–100}. These results suggest that either the ERK1 level is too low to compensate or that ERK2 binds specifically to substrates which are not activated by ERK1.

In a recent study, more detailed differences between ERK1 and ERK2 have been observed. In fibroblasts ERK1 shows a 3.6 times slower nuclear-cytoplasmic transport due to a unique domain located at the N-terminus. Thus ERK1 is suggested to be mainly controlling cytoplasmic targets while ERK2 interacts with both cytoplasmic and nuclear targets\textsuperscript{101}.

The structure of ERK2 in non-phosphorylated and phosphorylated form was analysed by 2 different groups. In the non-phosphorylated form of ERK2 Thr183 is
situated on the surface of the molecule while Tyr185 is buried in a pocket. Activated ERK2 changes its structure within the activation loop leading to refolding of ERK2. Upon phosphorylation Thr183 forms contact with the N-terminal domain of ERK2 and thereby promotes ERK2 domain closure by positioning Tyr185 at the surface. Phosphorylated Tyr185 is crucial for recognition by substrates. The phosphorylated residues finally allow an optimal orientation of the activation loop to substrates that contain the docking site for ERK (DEF) motif. The DEF motif has been identified in ERK1/2 substrates, such as c-Fos, Elk-1, scaffold kinase suppressor of Ras (KSR), MEK1/2, phosphatases and scaffold proteins which bind to conserved c-terminal common docking (CD) sites in ERK1/2.

1.5.1.1 Activation of ERK2 signalling cascade

ERK1/2 are activated by a wide variety of stress, including growth factors, cytokines and osmotic shock. Growth factors and other stimuli recruit and thereby activate their respective cell surface receptors, such as receptor tyrosine kinases (RTKs). Finally the GDP/GTP exchange factor son of sevenless (SOS) or protein kinases, such as protein kinase C (PKC) activate the G protein Ras. Ras recruits and activates the MAPKKK Raf at the plasma membrane. Raf consists of the isoforms c-Raf (also known as Raf-1), b-Raf and a-Raf that are all expressed in the heart. Once activated Raf phosphorylates the MAPKs MEK1 and MEK2 (often referred to as MEK1/2) on two serine residues within their activation loops. It seems that b-Raf is the main activator of MEK1/2 in cells, whereas a-Raf and c-Raf fine-tune the levels and/or duration of ERK1/2 activity. Interestingly, activated ERK1/2 can either inhibit or activate Raf, however, the precise mechanism remains unclear.
Within the ERK1/2 pathway the MAPKKks c-Mos and Tpl2 (also known as Cot) have been identified as restricted to cell type and stimulus$^{104}$. Activated MEK1/2 phosphorylate ERK1/2 within the conserved Thr-Glu-Tyr (TEY) motif in their activation loops$^{107}$. The activities of the single phosphorylated and non-phosphorylated form of ERK1/2 do not differ significantly. Therefore, phosphorylation of both threonine and tyrosine is crucial to increase the kinase activity well over 1000 fold$^{108}$. Activated ERK1/2 are able to phosphorylate a large number of substrates, such as the MAPK-activated protein kinases (MAPKAPKs); mitogen- and stress activated protein kinase (MSK1/2), MAPK-interacting Ser/Thr kinases (MNK1/2) and 90kDa ribosomal S kinase (p90$^{\text{rsk}}$, RSK) in cytoplasm. However, MSK1/2 and MNK1/2 are also regulated by p38$^{\alpha/\beta}$$^{109}$. In the nucleus ERK1/2 activate many transcription factors, including Elk-1, c-Fos, c-Myc$^{76,109}$. Therefore at the cellular level, ERK1/2 play an important role in many cellular events, including cell cycle progression, proliferation, cytokinesis, differentiation, senescence and apoptosis$^{84}$. Physiologically, ERK1/2 are necessary for memory formation, heart and immune system development. Due to their important roles a disrupted ERK1/2 signalling pathway can lead to various diseases, such as cancer, diabetes, viral infection and cardiovascular diseases$^{84}$. In resting cells ERK1/2 remain in the cytoplasm, although accumulation in nucleus occurs upon overexpression$^{111}$. MEK1/2 use their nuclear export signal (NES) for binding inactive ERK1/2 in the cytoplasm (Figure 1.4). Thus, MEK1/2 function as a cytoplasmatic anchoring protein of ERK1/2$^{111}$. Activated ERK1/2 translocate to the nucleus which is crucial for ERK1/2-dependent gene transcription and cell cycle
However, confinement in the cytoplasm enables ERK1/2 phosphorylation of cytoplasmic substrates. For entering the nucleus, ERK1/2 are involved in both energy-independent and -dependent transport. A study of point mutations of ERK2 has shown that inactive and active ERK2 are transported through the nuclear pore in an energy-independent manner. However, active energy-dependent transport has been proposed during dimerization of ERK2 in a phosphorylation-dependent manner. During dimerization leucine residues of two ERK2 molecules lock together thereby forming a hydrophobic zipper. These leucine residues are exposed on the surface of both non-phosphorylated and phosphorylated ERK2. Thus ERK2 dimers may be composed of two phosphorylated ERK2 or one phosphorylated and one non-phosphorylated ERK2. However, a recent study has challenged this concept. It has been demonstrated that ERK1/2 dimers are crucial for activation of cytoplasmic substrates and not for nuclear substrates.

More recently a study displayed the nuclear translocation sequence (NTS) which allows activated ERK1/2 as monomers to translocate into the nucleus via nuclear pores.

After substrate activation ERK1/2 are exported from the nucleus to cytoplasm through its binding to MEK1/2, which shuttle between cytoplasm and nucleus (Figure 1.4).

ERK1/2 are involved in many cellular events, as already described. Therefore several regulatory mechanisms are crucial to guarantee a specific signalling of ERK1/2.

ERK1/2 have several scaffold proteins which can be restricted to cytoplasm, Golgi apparatus or actin enabling preferential activation of the numerous ERK1/2
substrates (Figure 1.4). Scaffold proteins of ERK1/2 include MEK Partner-1 (MP-1) (binds to MEK1 and ERK1) and IQGAP₁, named for IQ motifs GTPase activating protein (GAP) (binds to b-Raf, MEK1 and ERK1)\textsuperscript{83, 117}. In addition, in Golgi apparatus the protein similar expression to FGF (Sef) binds the activated form of MEK1/2 and thereby blocks the dissociation of ERK1/2 from MEK1/2\textsuperscript{118, 119}. Thus, Sef functions as another cytoplasmic anchor protein, apart from MEK1/2, targeting ERK1/2 to the cytoplasm\textsuperscript{119}. Similarly, the scaffold protein IQGAP₁ retains activated ERK1/2 linked to actin\textsuperscript{121}.

The signalling of ERK1/2 is negatively regulated by various phosphatases, including dual-specificity phosphatases (DUSP), protein serine/threonine phosphatases and protein tyrosine phosphatases that dephosphorylate and thus inactivate ERK1/2\textsuperscript{88}. In the cytoplasm ERK1/2 can be inhibited by DUSP6 (MKP-3), DUSP7 (MKP-X) and DUSP9 (MKP-4) and in the nucleus by DUSP1 (MKP-1), DUSP4 (MPK-2) and DUSP5\textsuperscript{122}. DUSP6 dephosphorylates ERK2 specifically\textsuperscript{123}. In mammalian cells, DUSP5 functions as both an specific inactivator as well as a nuclear anchor protein of ERK2\textsuperscript{124}.

In addition, ERK1/2 are regulated by other MAPKs. For example, p38 MAPK negatively regulates ERK1/2 by activation of the protein phosphatase 2A (PP2A) which dephosphorylates MEK1/2\textsuperscript{125}.

In summary, the activation of the ERK1/2 signalling cascade is the translation of an extracellular signal into a molecular event which includes the change of ERK1/2 in their structure and enzymatic activity followed by interaction with substrates in a subcellular localisation dependent manner.
Figure 1.4: Mechanisms of ERK1/2 regulation

Activation of ERK1/2 by MEK1/2 requires scaffold proteins. Activated ERK1/2 translocate to the nucleus. After substrate activation ERK1/2 are exported to the cytoplasm with the help of MEK1/2. Specific scaffold proteins, such as Sef and IQGAP1 preferentially activate ERK1/2 in subcellular locations (e.g. Golgi apparatus, actin).

1.5.1.2 Role of ERK2 in the heart

Several GPCRs, such as angiotensin receptors, ET receptors and β-, α-adrenergic receptors promote hypertrophy through ERK1/2. Therefore stimuli, such as AngII, ET-1, Isoproterenol (Iso), Phenylephrine (PE) have been used to analyse the role of ERK1/2 in hypertrophy which remains controversial in vitro and in vivo.

In cultured rat cardiomyocytes the inhibition of MEK1/2 by U0126 blocks PE and ET-1 stimulated hypertrophy, suggesting a role of ERK1/2 in hypertrophy.
Consistently, PE stimulation of neonatal rat cardiomyocytes is followed by activation of ERK1/2 which results in expression of ANP. Furthermore, MEK1/ERK2 pathway activation increases ANP promoter activity in response to PE and has been demonstrated using dominant negative MEK1 or ERK2 plasmids. The role of ERK1/2 in hypertrophy is further supported by a study showing that adenovirus-mediated overexpression of constitutively active MEK1 leads to cardiomyocyte hypertrophy, while dominant negative MEK1 inhibits hypertrophic remodelling in neonatal rat culture.

The role of ERK1/2 in hypertrophy has been further studied by various transgenic mouse models. Transgenic mice overexpressing MEK1 display enhanced contractile heart function and blunted apoptosis which indicates a crucial role of MEK/ERK1/2 in hypertrophic remodelling. In transgenic mice with the dominant negative expression of c-Raf pressure overload leads to impaired hypertrophy and these mice are resistant to hypertrophic gene induction. Similarly, cardiac-specific c-Raf knockout mice progress to heart failure without developing hypertrophy.

While many studies report that ERK1/2 contribute to cardiac hypertrophic remodelling a few suggest otherwise. In cultivated cardiomyocytes the inhibitor PD98059 blocks ERK1/2 activity without reducing PE-induced ANP promoter activity. In addition, transgenic mice overexpressing DUSP6 develop hypertrophy despite the inhibition of ERK1/2. In addition, ERK2 heterozygous knockout (ERK2<sup>+/−</sup>) mice do not show reduced cardiac hypertrophy in response to pressure overload.

The contradicting studies in cell culture suggest that the results depend on the specificity of the inhibitor. So far the transgenic mouse models either inactivate
or activate different components of the ERK1/2 pathway which may influence additional signalling cascades and may be the reason for the controversial results.

A recent study has shown that autophosphorylation of ERK2 at residue Thr188 is an important event in ERK1/2-mediated hypertrophy\textsuperscript{137}. Transgenic mice with enhanced ERK2 Thr188 phosphorylation display increased hypertrophic growth while mice with suppressed ERK2 Thr188 phosphorylation are resistant to pressure overload-induced hypertrophy compared to wild-type mice\textsuperscript{137}. \(G_q\)-coupled receptors are bound by either \(G\alpha_q\) or \(G\beta\gamma\) proteins leading to two distinct mechanisms. The Ras/MEK/ERK1/2 cascade is activated by \(G\alpha_q\) proteins resulting in phosphorylation of ERK1/2 within the TEY motif whereas \(G\beta\gamma\) causes autophosphorylation of ERK2 at Thr188 followed by translocation to the nucleus where ERK2 activates hypertrophic substrates, such as Elk-1, MSK1 and c-Myc\textsuperscript{137}.

Despite many studies using animal models, only a few studies have analysed human hearts. In hypertrophic patients the mRNA expression level of Ras correlates with the extent of cardiomyocyte hypertrophy\textsuperscript{138}. Heart failure patients using the left ventricular (LV) assist device (LVAD) for mechanical unloading develop reversed hypertrophy which is accompanied with significantly reduced ERK1/2 activity\textsuperscript{139}. The downregulation of ERK1/2 can be an effect of Sprouty-1 whose mRNA and protein levels increase following the mechanical unloading in LVAD hearts\textsuperscript{140}. The upregulation of Sprouty-1 in isolated cardiomyocytes in response to mechanical stretch is accompanied by a decrease in ERK1/2 phosphorylation\textsuperscript{140}.

Taken together, although the ERK1/2 pathway is widely studied its precise role in hypertrophy is not entirely clear yet.
1.5.1.3 Biological function of ERK1/2 in regulating cell survival

Despite intensive research, the precise mechanisms whereby ERK1/2 regulates apoptosis remain controversial. Early studies in NRCMs showed that ERK1/2 inactivation by MEK1/2 inhibitor PD9805 increased the number of apoptotic cardiomyocytes following treatment with pro-apoptotic agent hydrogen peroxide\(^\text{141}\).

ERK1/2 might influence the apoptotic pathway directly by interactions with several cell death agonists and pro-survival members. ERK1/2 phosphorylates Bim\(_{\text{extra long(EL)}}\) and thereby promotes degradation of the cell death agonist and may also disrupt Bim\(_{\text{EL}}\)-Bax interactions\(^\text{142}\). In non-cardiomyocyte cells ERK1/2 directly interacts with caspase 9 and thereby suppresses the apoptotic pathway\(^\text{143}\). Furthermore, in T-cells ERK1/2 was shown to suppress the activation of pro-apoptotic Bid and caspase 8\(^\text{144}\).

In addition, ERK1/2 have been demonstrated to counteract apoptosis by activation of the transcription factor GATA4, an upstream activator of the anti-apoptotic Bcl-X gene\(^\text{145-147}\). Also, GATA4 protects against DOX-induced cardiac toxicity by activating gene expression of the anti-apoptotic factor Bcl2 \textit{in vitro} and \textit{in vivo}\(^\text{148}\). DOX is an effective chemotherapeutic agent, however, its toxic effects induce cardiac damage, such as extensive fibrosis and apoptosis in the heart\(^\text{149}\). Increased activity of ERK1/2 has been shown to prevent these DOX-effects in rats hearts and in cultured cardiomyocytes\(^\text{149, 150}\). Likewise, ERK1/2 protect against cardiomyocyte apoptosis induced by daunomycin, another chemotherapeutic agent\(^\text{152}\).

Moreover, studies have also shown that in rat hearts ERK1/2 contribute partly to the cardioprotective effects of two widely used classes of drugs, the β-adrenergic receptor blockers and \(\text{Ca}^{2+}\) channel blockers\(^\text{153}\).
In addition, the cardioprotective function of ERK2 has been suggested in ERK2 heterozygous knockout (ERK2+/−) mice that increased apoptosis rate and myocardial injury in response to ischemia/reperfusion (I/R) are observed133. Importantly, these effects are lost in homozygous ERK1−/− mice which suggests a predominant role of ERK2 in cardiac apoptosis133. Likewise, transgenic mice overexpressing MEK1 develop hypertrophy with partial resistance to apoptosis130. In addition, in mice overexpressing DUSP6, the inhibition of ERK1/2 leads to higher apoptosis rates134. Confirming the cardioprotective effects of ERK1/2, cardiac-specific Raf-1 knockout mice have been shown to progress to heart failure at 5 weeks of age132. However, these results are controversial as Raf-1 also mediates its anti-apoptotic function by interaction with the pro-apoptotic, stress-activated apoptosis signal-regulating kinase-1 (ASK1) in an MEK/ERK independent manner153, 154. This suggests that the increased activity of ASK1 observed in Raf-1 knockout mice might contribute to the observed increased apoptosis rate132.

The protective role of ERK1/2 in apoptosis has been challenged by reports that ERK1/2 promote apoptosis in DOX-treated NRCMs or H9C2 cells (derived from embryonic rat heart tissue)156.

Taken together, ERK1/2 pathway seems to have a cardioprotective role, but the specific molecular contributions remain not entirely understood.

1.5.2 ERK5

ERK5 has a bigger molecular weight (~100kDa), twice the size of other MAPKs, and is therefore named big mitogen-activated protein kinase 1 (BMK1). It shares 67% amino acid similarity with ERK1/2 (51% amino acid identity with ERK2)76, 98. Except in the liver ERK5 is ubiquitously expressed99. ERK5 contains a unique 400
amino acid sequence in the C-terminus which includes a transcriptional domain, a MEF2D-interacting motif, a proline-rich region and the NLS\textsuperscript{76, 156}. The transcriptional domain enables ERK5 to function as a transcription activator, unlike other MAPKs\textsuperscript{88}.

For shuttling between nucleus and cytoplasm, ERK5 contains both the NES and the constitutive active NLS. Unstimulated ERK5 localises in cytoplasm due to the predominance of NES\textsuperscript{107}.

Stimuli for the ERK5 pathway include various growth factors, oxidative stress and UV radiation\textsuperscript{158}.

Once activated, ERK5 enters the nucleus after structural rearrangements thereby NES loses its influence\textsuperscript{107}. ERK5 is activated by phosphorylation of Thr and Tyr residues within the conserved Thr-Glu-Tyr (TEY) motif sequence in the kinase activation loop\textsuperscript{77}. In addition, activated ERK5 has the ability to increase its transcriptional activity by autophosphorylation of its C-terminal region\textsuperscript{159}. ERK5 is directly phosphorylated by MEK5 on both residues, in turn, MEK5 is activated by MEKK3 and MEKK2\textsuperscript{99}. These MEKK2/3 are not entirely specific since they also activate p38 MAPK and JNK\textsuperscript{103, 159}. Numerous ERK5 substrates have been identified in both the cytoplasm and the nucleus, such as the transcription factors c-myc, c-fos, and myocyte enhancer factor 2 (MEF2) family\textsuperscript{157, 160}. ERK5 also phosphorylates the serum- and glucocorticiod- inducible kinase (SGK) that is required for S-phase entry of the cell\textsuperscript{107}.

ERK5 knockout mice die during embryogenesis between day 9.5 and day 10.5 due to impaired blood vessel development and cardiovascular defects\textsuperscript{162}.

ERK5 has been shown to have several biological roles, such as cell survival, differentiation, proliferation and growth\textsuperscript{88}. Physiological and pathological roles have
been implicated in neuronal survival, breast and prostate cancer as well as cardiac hypertrophy\textsuperscript{157, 162}. However, the role of ERK5 in the heart has been less studied in comparison to ERK1/2. Dominant negative expression of MEK5 in cardiomyocytes results in reduced re-expression of fetal cardiac genes \textit{ANP} and \textit{BNP} in response to PE treatment\textsuperscript{164}. In mice, cardiac expression of activated MEK5 induces hypertrophy that progresses to dilated cardiomyopathy and sudden death between 4 and 18 weeks of age\textsuperscript{164}. Likewise, ERK5 activity is increased in rats during left ventricular hypertrophy\textsuperscript{165}. In humans ERK5 activity has been shown to be decreased in patients with end-stage heart failure\textsuperscript{166}.

Following these indications, the precise role of ERK5 in hypertrophy has been analysed in a recent study using cardiac-specific ERK5 knockout mice\textsuperscript{163}. This study revealed that ERK5 is required for protection against hypertrophic stress, such as pressure overload and β-adrenergic stimulation. ERK5 is particularly responsible for controlling MEF2 activity as inhibition of MEF2 diminished ERK5-regulated hypertrophic response in cultured neonatal cardiomyocytes\textsuperscript{163}.

However, the precise mechanisms to regulate ERK5 pathway is less studied. ERK5 is negatively regulated by specific protein phosphatases, such as MKP-1,-3 and the phosphotyrosine-specific phosphatases PTP-SL\textsuperscript{88}.

Taken together, ERK5 signalling is mainly activated by growth factors and different stress stimuli. In humans ERK5 is involved in cancer formation and similar to ERK1/2 it may play an important role in cardiac hypertrophy.

\subsection*{1.5.2 p38 MAPK}

P38 MAPK is also known as stress-activated protein kinase and the first isoform of p38 MAPK (p38α) has been discovered independently from 3 different lab
groups\textsuperscript{167–169}. Since then further 3 isoforms have been identified as p38 MAPK (p38β, γ and δ)\textsuperscript{169–171}. The p38α and p38β are ubiquitously expressed in tissues, whereas p38γ is mainly expressed in skeletal muscle and p38δ is enriched in lung, kidney, testis, pancreas, and small intestine\textsuperscript{173}. The diversity of the isoforms has been demonstrated by mouse total gene knockout experiments\textsuperscript{173, 174}. Loss of p38α leads to embryonic lethality due to severe defects in placental angiogenesis and erythropoiesis\textsuperscript{173, 174}. In contrast, the knockout mice of any of the other isoforms are viable, fertile and appear normal size without apparent health problems\textsuperscript{99}.

In mammalian cells, p38 isoforms are activated by environmental stress and inflammatory cytokines\textsuperscript{77}. Several MEKKs, such as MEKK1 to -3, apoptosis signal-regulating kinase 1 (ASK1), Tpl2 and TGF β-activated kinase 1 (TAK1) activate MEK3 and MEK6, the major kinases responsible for direct activation of p38\textsuperscript{160}. Upon activation, MKK6 can phosphorylate all isoforms of p38 MAPK whereas MKK3 activates p38α, p38γ, p38δ but not p38β\textsuperscript{160}. MKK4 can activate p38α in some circumstances, such as UV radiation\textsuperscript{176}. All three MEKs are able to perform dual-phosphorylation of p38 MAPK at the Thr-Glu-Tyr motif in the activation loop\textsuperscript{176}. Upon stimulation p38 isoforms phosphorylate numerous substrates in the cell, including MSK1/2 and transcription factors (ATF1, Ets1, Elk-1) in the nucleus, as well as the cytosolic proteins phospholipase A₂ (PLA₂), Bax, MNK1/2 and Tau\textsuperscript{76, 159, 176, 177}.

In the heart p38α is expressed predominantly in comparison to p38β. In NRCMs overexpression of p38α increased apoptosis whereas overexpression of p38β enhanced hypertrophic response\textsuperscript{179}. Consistently, DNA microarray analysis of animals with cardiac-specific overexpression of p38 MAPK showed increased gene
expression associated with inflammation and fibrosis as well as proliferation\textsuperscript{180}. Confirming the pro-apoptotic role of p38, fibroblast lacking p38α are more resistant to stress\textsuperscript{181}. However, other studies have suggested otherwise\textsuperscript{182–184}. Prolonged p38 MAPK activation has been reported to have an anti-apoptotic effect in NRCM\textsuperscript{182}. Similarly, mice with cardiac-specific deletion of p38α showed unaltered hypertrophy following pressure overload\textsuperscript{183}. These mice also displayed cardiac dysfunction accompanied by increased apoptosis, fibrosis and chamber dilation suggesting a role of p38α in survival\textsuperscript{183}. In addition, dominant negative p38α or p38β mice showed no effect on pressure overload-induced hypertrophy without fibrosis development\textsuperscript{184}.

In the heart p38 MAPK plays an important role in cardiomyocyte differentiation. Specifically, p38 MAPK promotes differentiation of human embryonic stem (ES) cells into cardiomyocytes and has a high phosphorylation activity between 3 and 10 days of differentiation\textsuperscript{184, 185}.

The role of p38 MAPK in physiological hypertrophy remains controversial. After 28 days of swimming stress dominant negative p38α and dominant negative p38β mice have been shown to be resistant to cardiac remodelling\textsuperscript{187}. In contrast, a supressing role of p38 MAPK in exercise-induced hypertrophy has been suggested as p38α cardiac-specific knockout mice developed enhanced physiological hypertrophy following swimming\textsuperscript{188}.

In addition, p38 MAPK is a negative regulator of cardiomyocyte proliferation\textsuperscript{189}. Its overexpression blocked proliferation of fetal cardiomyocytes and 7.2% of adult cardiac myocytes re-entered the cell cycle by addition of p38 MAPK inhibitor and the fibroblast growth factor (FGF)\textsuperscript{189}.
Taken together, p38α and p38β seem to be the main contributor of the p38 MAPK family. In the heart p38α is likely a pro-apoptotic regulator and the role of p38 MAPK in physiological hypertrophy remains controversial.

1.5.3 c-Jun N-terminal kinase

The JNK family consists of three isoforms: JNK1, JNK2, JNK3 and includes at least 10 spliced forms with a molecular weight ranging between 46 to 55kDa. The proteins JNK1 and JNK2 are ubiquitously expressed whereas JNK3 protein is primarily found in brain and to a lesser extend in the heart and testis. JNKs are activated in response to inflammatory cytokines and various cellular stresses, including heat shock, oxidative stress, DNA-damaging agents and UV irradiation. JNK isoforms require dual-phosphorylation on Thr and Tyr residues within the conserved Thr-Pro-Tyr sequence in their activation loops. Direct activators of JNKs are MKK4 and MKK7. MKK4 mainly phosphorylates Tyr185 whereas MKK7 preferably phosphorylates Thr183. MKK4/7 are activated by several MEKKs, including MEKK1 to -4 and mixed-lineage kinases (MLK) 1 to -3.

Following stimulation JNKs are able to shuttle from the cytoplasm into the nucleus. JNKs have more than 25 nuclear and cytoplasmatic downstream targets. Many nuclear substrates are transcription factors, such as c-Jun, Jun-D, p53, ATF-2, Elk-1, c-Myc and NFAT. Thus, JNKs are involved in a number of biological processes, such as cell proliferation and apoptosis. None of the JNK deficient knockout mice showed defects in cardiac development. JNK1 and JNK2 play important roles in control of cell proliferation. However, despite their molecular similarities both seem to have distinct functions. In mouse
fibroblasts JNK1 is the major activator of c-Jun. In contrast, JNK2 appears to be responsible for c-Jun degradation as it binds to c-Jun in unstimulated cells, without phosphorylating c-Jun. The phosphorylation of c-Jun leads to formation of AP-1 which is involved in the transcription of a variety of proteins, such as pro-apoptotic proteins Bak and FasL. The pro-apoptotic role of JNK has been confirmed in mouse embryonic fibroblasts (MEFs) derived from JNK1−/− and JNK2−/− mice that are resistant to apoptosis in response to UV irradiation and DNA-damaging agents. However, JNK1 and JNK2 were not required for the Fas-induced apoptosis of fibroblasts.

The role of JNK in physiological and pathological hypertrophy remains unclear. In physiological hypertrophy, JNK is activated in untrained rats following intensity-dependent treadmill exercise, however, no activation was detected in exercise-trained animals.

Initial studies have suggested that JNK induces pathological hypertrophy. NRPMs with constitutive active MKK7 showed a hypertrophic phenotype. Similarly, dominant negative MKK4 leads to less hypertrophic growth in response to ET-1 stimulation.

Controversly, MKK4 cardiac-specific knockout mouse model revealed exacerbated hypertrophy in response to pathological hypertrophy induced by pressure overload and chronic β-adrenergic stimulation. Other studies have confirmed the activation of JNK during pressure overload-induced hypertrophy. During high pressure overload JNK has its activation peak between 10 to 30 minutes post-aortic banding. Mild pressure overload results in an increase of JNK activation after 30 minutes after surgery, with a second phase of increase at day 1 and 2 after...
constriction. Consistently, in rats JNK is activated after 24 hours post-aortic banding.

In addition, a pathological role of JNK was found in neuronal disorders, diabetes and atherosclerosis.

Like all MAPKs, JNK interacts with scaffold proteins, such as JNK-interacting proteins (JIP) 1 and 2, and it is negatively regulated by several phosphatases, including dual-specific phosphatases MKP-1, -2, -5 and -7.

In summary, JNK requires dual-phosphorylation by MKK4 and MKK7. JNK is involved in various processes, such as apoptosis and proliferation. During pathological hypertrophy JNK seems to be a negative regulator.
1.6 Aims and hypothesis of my study

Despite many studies analysing the function of ERK1/2 in several organs the exclusive function of ERK2 in hypertrophy had not been assessed in the heart. The overall aim of my study was to evaluate the signalling regulation of ERK2 in cardiac remodelling in response to physiological and pathological hypertrophic stimuli using in vivo and in vitro experimental approaches. In my study, the role of ERK2 was analysed using a more direct approach in comparison to other studies, that inhibited or activated other components of the ERK1/2 pathway, by using the cardiac ERK2 knockout (ERK2\(^{cko}\)) mouse model as well as the overexpression of dominant negative ERK2 in vitro.

The specific objectives were:

- To characterise ERK2\(^{cko}\) mice without hypertrophic stress stimulation
- To analyse pathological and physiological hypertrophy in ERK2\(^{cko}\) mice and control (ERK2\(^{f/f}\)) mice challenged by either pathological and physiological stress including activation of the β-adrenergic receptors, pressure overload and swimming exercise
- To investigate the hypertrophic and apoptotic role of ERK2 in vitro using neonatal rat cardiomyocytes following β-adrenergic stimulation and pro-apoptotic agent hydrogen peroxide stimulation

It was hypothesised that ERK2 contributed to the development of pathological hypertrophy and counteracted apoptosis; however ERK2 was not involved in physiological hypertrophy.
CHAPTER 2
2.1 In vivo analyses

2.1.1 Cardiomyocyte knockout mouse model

To exclusively study ERK2 signalling pathways in hypertrophic remodelling, cardiomyocyte-specific knockout mice were used in this study. The conditional (tissue-specific) knockout mouse model is a useful method for analysing in vivo functions of specific signalling pathways in a highly controlled manner\textsuperscript{205}.

The cardiomyocytes-specific disruption of the \textit{erk2} gene was achieved by the Cre-loxP system (Figure 2.1). The cyclic recombinase (Cre) is a type I topoisomerase of bateriophage P1 that recognizes loxP (Locus of X-over of P1), a 34bp DNA sequence in the P1 genome. Cre catalyses a deletion of the intervening DNA between two loxP sites in the same direction, leaving one loxP site behind\textsuperscript{206}.

For development of the conditional knockout mouse, two different transgenic mouse lines were generated. The first line contained a transgene that led to a cardiomyocyte-specific expression of Cre under the \textit{myosin light chain} (\textit{MLC2v}) prometer. The heterozygous MLC2v-Cre mice (kindly provided by Dr KR Chien, Massachusetts General Hospital, USA) provided ventricular-specific, efficient Cre activity and were indistinguishable from wild-type mice in cardiac morphology and their response to stress\textsuperscript{207}. To generate the MLC2v-Cre mice, Cre gene was inserted between exon1 and exon 2 of \textit{mlc2v} gene in embryonic cells (ES) derived from 129Sv mice by homologous recombination. The chimera mice generated from these ES cells were crossed with Black Swiss mice to generate MLC2v-Cre mice\textsuperscript{207}. 
The second mouse line (floxed mice) (kindly provided by Dr S Endo, Institute of Science and Technology, Okinawa, Japan) carries two loxP sites in the same orientation flanking exon 2 and 3 of the erk2 gene. Exon 2 codes for the TEY activation motif and exon 3 codes for protein kinase subdomain V and VI\textsuperscript{98,100}. The two loxP sites are placed in introns or non-transcription regions. In these floxed mice (ERK2\textsuperscript{loxP/loxP}), the two loxP sites were inserted into the said region by homologous recombination in ES cells\textsuperscript{208}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.1.png}
\caption{Conditional disruption of \textit{erk2} gene in mice}
\end{figure}

ERK2 was specifically deleted in cardiomyocytes without affecting ERK2 expression in other tissues.

\textit{Breeding scheme}

For this study, mice homozygous for the ERK2\textsuperscript{loxP/loxP} allele (referred to as ERK2\textsuperscript{flof}) were crossed with transgenic MLC2\textsubscript{v}-Cre mouse line to produce heterozygous mice (ERK2\textsuperscript{f/+–MLC2\textsubscript{v}-Cre}). These mice were then crossed with ERK2\textsuperscript{flof} mice to generate the homozygous ERK2\textsuperscript{loxP/loxP–MLC2\textsubscript{v}-Cre} (referred to as ERK2\textsuperscript{cko}) mice which contain a
specific deletion of ERK2 in ventricular cardiomyocytes (Figure 2.2). The mice were hosted in a pathogen-free facility at the University of Manchester. Food and water were provided ad libitum. Dirty cages and bedding were cleaned weekly. The animal studies were carried out according to Home Office and institutional guidelines under the project licence (PPL 40/3069). Available methods to replace, reduce and refine animal use were applied under the existing animal protection legislation.

**Figure 2.2: The breeding scheme for ERK2<sup>cko</sup> mice**

ERK2<sup>f/f</sup> mice were crossed with the MLC2v-Cre mouse line to produce heterozygous mice (ERK2<sup>f/+</sup>-MLC2v-Cre) which were crossed with ERK2<sup>f/f</sup> resulting in homozygous ERK2<sup>cko</sup> mice.

### 2.1.2 Genotyping mice

**Preparation of DNA**

To distinguish between the two genotypes, ERK2<sup>cko</sup> and ERK2<sup>f/f</sup>, tail snips were taken and lysed in 500μl cell lysis buffer (Qiagen, 8335705) with 6μl proteinase K (20mg/ml, Invitrogen, 895533) overnight at 55°C. 500μl phenol/chloroform (25:24 ratio) was added and samples were centrifuged. The DNA containing upper aqueous layer was transferred to a clean Eppendorf tube and 500μl ice-cold isopropanol
(Fisher Scientific) was added. Samples were centrifuged and the supernatant was discarded. The DNA pellet was washed with 70% ethanol, dried and dissolved in injection water.

Polymerase chain reaction

The dissolved DNA was used for polymerase chain reaction (PCR). The PCR mix consisted of: 5μl PCR master-mix (Qiagen, HotStarTaq R Master Mix Kit, 203443), 3.5μl RNase-free water (Quiagen), 1μl (5 units/μl) primer mix and 1μl (100ng/μl) DNA sample. ERK2 primer (forward; 5’GCGGAGAAAGAGGTTGATAAAG-3’; reverse; 5’CTGTTTCACGAGACGATGCCC-3’) or Cre primer (forward; 5’GACGGAATCCATCGCTCGCTCGAC-3’; reverse; 5’GACATGTTCAGGGA TCGCCAGG-3’) were used. The PCR program included: 1) 95ºC for 15 minutes 2) 30 cycles of denaturation [95ºC for 1 minute], annealing [55ºC for 1 minute], elongation [72ºC for 1 minute] 3) 75ºC for 5 minutes. The PCR product was amplified from ERK2f/f and ERK2cko mice containing the ERK2-flox allele (232bp) and from ERK2cko mice, including the Cre transgene (598bp) and visualized by running electrophoretically on a 1.2% agarose gel.

2.1.3 Induction of pathological hypertrophy

2.1.3.1 Transverse aortic constriction (TAC)

TAC induces cardiac hypertrophy as a result of chronic pressure overload. Chronic pressure overload is often believed to induce hypertrophy with maladaptive outcome in humans which can lead to heart failure\(^{38}\). The 8 to 10 weeks old male mice were anesthetized with ketamine (50-100mg/kg, ketamine hydrochloride, FORT Dodge Animal Health Ltd.) and xylazine (5mg/kg, 2%w/v Bayer Healthcare, Animal Health
devision) by intraperitoneal injection. After endotracheal intubation, the chest cavity was exposed at the level of the sternal border through a small incision. The transverse aorta was isolated and constricted by a 7-0 nylon suture ligated firmly against a 27-gauge needle. After the needle removal, a discrete region of stenosis was left. The chest was then closed and the mice were extubated and allowed to recover from anesthesia. Sham operated mice were used as a reference-control and underwent the same surgery in which the aorta was only visualized but not constricted. After surgery, buprenorphine (0.1mg/kg, Alstoe Animal Health Care Ltd) as analgesic and saline (0.1ml/10g) were given by intraperitoneal injection and mice were allowed to recover in a 37°C incubator. After TAC, ERK2f/f and ERK2cko mice showed a similar pressure gradient between the left and right carotid arteries (30 to 50mmHg). Therefore the observed differences in hypertrophy were not caused by a disparity in pressure overload stimulation.

Preparation of the anaesthetic solution:

0.1ml ketamine (100mg/ml) and 0.1ml xylazine (20mg/ml) were mixed with 1.6ml saline and 0.1ml of this solution was used for a 30g mouse.

Preparation of the analgesic solution:

Buprenorphine (3mg/ml) was diluted to 0.3mg/ml with saline and 0.1ml of this solution was used for a 30g mouse.

2.1.3.2 Micro-osmotic Pump

The ALZET® micro-osmotic pump is a drug delivery system and it releases components into the mouse body by continuous infusion maintaining a constant outflow of a drug over a specific time.
Before surgery, 8-10 weeks old male mice were anesthetised with 1.5% isoflurane (Baxter Healthcare Ltd.) in 98.5% oxygen. A small incision was made on the left side of the back and a ALZET® micro-osmotic pump was inserted subcutaneously. The wound was closed using a 6-0 nylon suture. The pumps were implanted into mice to deliver Isoproterenol (Iso, Sigma-Aldrich) at 10mg/kg/day dissolved in water for injections (Braun, 3627594) or AngiotensinII (AngII, Sigma-Aldrich) at 500µg/kg/day dissolved in water for injections. Two different types of ALZET® pumps were used for Iso and AngII. Iso was infused at a rate of 1µl/hour over 1 week using ALZET® micro-osmotic pump (model 2001). AngII was delivered at a rate of 0.25µl/hour for 2 weeks (ALZET® micro-osmotic pump model 1002).

The concentration of the Iso and AngII used was calculated using the following formula:

\[ c = \frac{K_0}{Q} \cdot \text{mouse body weight (g)} \]

\( c \) is the concentration (µg/µl) of Iso and AngII in the vehicle, \( K_0 \) is the mass delivery rate (µg/hour), \( Q \) is the volume delivery rate (µl/hour).

Another group of mice were infused with vehicle of each ALZET® model and served as a control group. Following 1 week (Iso) or 2 weeks (AngII) infusion, animals were anesthetised (Avertin [2, 2, 2-Tribromoethanol], 240mg/kg body weight (Sigma-Aldrich) by intraperitoneal injection) and further analysed.

### 2.1.4 Induction of physiological hypertrophy

Experimental physiological cardiac hypertrophy was induced by swimming exercise. Swimming training was performed on 8 weeks old mice and initiated by placing animals in a water bucket of pre-warmed water, maintained at 34°C. The mice were supervised throughout the exercise and after swimming placed into an incubation
chamber until they were dry. Swimming was performed twice a day for 90 minutes each, with a minimum rest time of 4 hours in between. The mice were acclimated to the exercise gradually, beginning with 10 minutes twice a day and increasing in increments of 10 minutes each day until 90 minutes twice daily was obtained for 20 days without any resting days in between. After a total of 4 weeks of swimming, their cardiac function was determined by echocardiography and mice were killed by cervical dislocalisation for further analysis.

The rest group consisted of mice of both genotypes (ERK2\textsuperscript{f/f} and ERK2\textsuperscript{cko}) and were of the same age as the swimming group. These mice were kept without following the swimming protocol. After 4 weeks the mice were killed by the scheduled method and were subjected to the same analyses as the swimming mice.

2.1.5 Echocardiographic analysis

The cardiac function was evaluated in anesthetized mice by echocardiography using an Acuson Sequoia C256 cardiac ultrasound machine with a 14MHz transducer applied parasternally to the mouse chest wall. The heart was measured in the 2D mode in the parasternal short-axis at aortic root level. For each mouse, the parameter of the left ventricular end-systolic (LV\textsubscript{EsD}) and left ventricular end-diastolic (LV\textsubscript{EdD}) diameter, end-diastolic intraventricular septum thickness (dIVS), end-systolic intraventricular septum thickness and left ventricular posterior wall thickness (PW) were measured (Figure 2.3). From these parameters, LV fraction shortening (%FS) was calculated using the following formula:

\[
%FS = \frac{LV\text{dD} - LV\text{sD}}{LV\text{dD}} \cdot 100^{210}.
\]
Figure 2.3: Echocardiographic views
The M-mode image (bottom) was obtained from short-axis image of left ventricle (LV) to measure the left ventricular properties both at systole and diastole: LV end-diastolic diameter (LVEdD), LV end-systolic diameter (LVEsD), end-diastolic intraventricular septum thickness (dIVS), end-systolic intraventricular septum thickness (sIVS), end-diastolic left ventricular posterior wall thickness (dPW), end-systolic posterior left ventricular wall thickness (sPW).

LV: left ventricle

2.1.6 Histological analyses

Paraffin sectioning

The heart tissue was fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, P6148) for 5 hours and dehydrated with 70%, 90% and 99% ethanol (each 2 hours). It was then cleared in xylene overnight, infiltrated with melted paraffin (Paraplast® embedding media, Sigma, P3683) at 70°C for 1 hour and embedded as a paraffin block. Paraffin sections were cut at a thickness of 5-7μm using a microtome (Leica RM2255) for analysis.
**Cryosectioning**

Heart tissue samples were mounted with Optimum Cutting Temperature (OCT) embedding matrix (Raymond A Lamb, LAMB/OCT) and kept at -80°C. The OCT embedded heart tissue was cryosectioned at 10μm using a Leica Reichert-Jung Cryocut CM 3050 cryostat. The sample was placed onto microscope slides and fixed with ice-cold acetone/methanol (50:50 ratio) for 10 minutes.

**Immunohistochemistry**

After obtaining cardiac ventricular cross-sections via the cryosection method, the cross-sections were washed in PBS (Sigma, P4417) and a wax pen (Vector Laboratories, H-4000) was used to divide sections on the slide. Non-specific staining was blocked with 10% (v/v) horse serum in PBS for 1 hour and ventricular cross-sections were incubated with primary antibodies (diluted in PBS overnight at 4°C, further details below). Sections were washed in PBS followed by incubation with secondary antibodies (1:500, anti-mouse or anti-rabbit, conjugated to Alexa Flour 568, Invitrogen) for 90 minutes. Nuclei were stained by incubating with DAPI (1:1000 diluted in PBS) for 5 minutes. After washing, the sections were mounted using Vectashield (Vector Labs). The slides were analysed using fluorescence microscope and ImageJ software.

To assess the capillary density the endothelial marker PECAM-1 (rabbit, 1:50, sc-1506, Santa Cruz Biotechnology) was applied. The anti-caveolin 3 antibody (mouse, 1:50, 610420, BD Transduction Laboratories™) was used to identify cardiomyocytes.
**Haematoxilin and eosin staining**

The haematoxilin and eosin (H&E) staining was used to analyse myocyte cross-sectional areas. Sections were dewaxed in xylene for 1 hour and hydrated in 100%, 75%, 50% ethanol for 5 minutes each followed by double-distilled (dd) H$_2$O for 5 minutes. The sections were stained in Harris’ haematoxylin solution (ready to use, Raymond A Lamb Ltd., LAMB/230-D) for 5 minutes and rinsed in ddH$_2$O until the nuclei were stained blue. Eosin (Shandon Alcoholic Eosin Y, Thermo Scientific, 6766007) was used to stain the cytoplasm and muscle fibres in pink. The sections were dehydrated in 100% ethanol for 8 minutes, cleared in xylene for 20 minutes and mounted immediately with Eukitt® quick-hardening mounting medium (Fluka, 03989). Images of the cardiac ventricular cross-sections were taken using a snapshot widefield microscope and for each heart the ventricular cross-sectional area of 50 cardiomyocytes were analysed by ImageJ software.

**Fluorescin isothiocyanate (FITC)-conjugated Wheat germ agglutinin (WGA) staining**

To measure the cardiomyocyte cross-sectional area more precisely, the cardiomyocyte plasma membrane was stained using Fluorescin isothiocyanate-conjugated wheat germ agglutinin (FITC-WGA). The paraffin sections were dewaxed in xylene followed by hydration in serial diluted ethanol and distilled water for 5 minutes each. The sections were stained with FITC-WGA (20µg/ml diluted in PBS, Sigma) and DAPI staining (1:1000) at room temperature for 1 hour in the dark. After washing with PBS the cross-sections were mounted using Vectashield (Vector Labs). The cell membrane was stained in green and the nuclei in a blue colour. Images of the ventricular cross-sections were taken using fluorescence microscope.
and for each heart the cross-sectional areas of 50 cardiomyocytes were measured using the ImageJ software.

**Picro-Sirius red staining**

Picro-Sirius red staining was used to detect collagen and reticular fibres. The paraffin sections were de-waxed in xylene and hydrated in serial dilution of ethanol and distilled water. Sections were stained with Harris’ haematoxylin solution and rinsed in ddH$_2$O until nuclei showed a blue colour. The sections were treated with 0.2% (w/v) aquatous phosphomolybdic acid (Fluka, 79560) for 5 minutes to prevent Picro-Sirius red background staining. Picro-Sirius red solution consisted of 0.1% (w/v) Sirius red (Direct Red 80, Sigma-Aldrich, 365548) diluted in saturated aqueous solution of picric acid (Sigma-Aldrich, 23,980-1). After staining in Picro-Sirius red solution for 1 hour, the cross-sections were rinsed in distilled water and dehydrated using 100% ethanol for 8 minutes followed by xylene treatment for 20 minutes. The sections were sealed with mounting medium.

Images of the cross-sections were taken using a snapshot widefield microscope and the interstitial fibrosis areas of 15 randomly chosen fields of each mouse were measured using ImageJ software. The fibrotic tissue was stained as red and the nuclei in a blue colour.

**Masson’s trichrome staining**

Masson’s trichrome staining was used to analyse interstitial fibrosis. Paraffin sections were de-waxed with xylene and hydrated in serial diluted ethanol and distilled water. Sections were treated with Harris’ haematoxylin solution and rinsed in ddH$_2$O until the nuclei showed a blue colour. The cross-sections were then stained
in Biebrich Scarlet-acid Fuchsin solution for 5 minutes (Table 2.1). Treatment with 2.5% (w/v) phosphomolybic acid for 15 minutes removed the Biebrich Scarlet-acid Fuchsin solution sufficiently from the collagen and samples were stained with Light Green-Acetic acid solution for 3 minutes (Table 2.2). Sections were dehydrated with ethanol, cleared by xylene and sealed with mounting medium.

As a result of this staining, 3 different colours could be seen: purple (nuclei), red (cytoplasm, keratin, muscle fibres, intracellular fibres) and green/blue (collagen). Images of the cross-sections were taken using a snapshot widefield microscope and the interstitial fibrosis areas of 15 randomly choses fields were analysed by ImageJ software.

### Table 2.1: Biebrich Scarlet-acid Fuchsin solution for Masson’s Trichrome staining

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% (w/v in ddH₂O) Biebrich Scarlet (Sigma-Aldrich)</td>
<td>90ml</td>
</tr>
<tr>
<td>1% (w/v in ddH₂O) Acid Fuchsin (Sigma-Aldrich)</td>
<td>10ml</td>
</tr>
<tr>
<td>Acetic acid, glacial (≥99.85% acetic acid, Sigma-Aldrich)</td>
<td>1ml</td>
</tr>
</tbody>
</table>

### Table 2.2: Light Green-Acetic acid solution for Masson’s Trichrome staining

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% (w/v in ddH₂O) light green (Sigma-Aldrich)</td>
<td>100ml</td>
</tr>
<tr>
<td>0.8% (v/v in ddH₂O) Acetic acid (Sigma-Aldrich)</td>
<td></td>
</tr>
<tr>
<td>Acetic acid, glacial</td>
<td>1ml</td>
</tr>
</tbody>
</table>

*Tunnel staining of cardiomyocytes*

Triple staining was used to identify apoptosis of ventricular cardiomyocytes by the nonradioactive terminal UTP nick end labelling (TUNEL) staining, nuclei staining
with DAPI and immunofluorescence staining with anti-α-actinin antibody (1:20, Sigma, A7811).

From each mouse two serial 5µm thick cardiac ventricular cross-sections were used. DNA fragments were analysed by using TUNEL system (Roche Applied Science, In situ cell death detection kit, Fluorescine, 11684795910). The paraffin sections were de-waxed in xylene and hydrated in serial diluted ethanol and distilled water. The sections were treated with 20mg/ml Proteinase K for 15 minutes at 37°C and permeabilised using 0.1% (v/v) Triton® X-100 (Sigma-Aldrich, 9002931) and 0.1% (w/v) sodium citrate (Fisher Scientific) diluted in PBS at room temperature for 8 minutes. After washing with PBS, TUNEL was added (TUNEL concentration 1:100) and the cross-sections were incubated in a dark humid chamber for 1 hour at 37°C. Sections were washed with PBS and nuclei were stained with DAPI (1:1000 diluted in PBS) for 5 minutes at room temperature in the dark. After washing in PBS, slides were mounted using Vectashield and analysed using fluorescence microscope and ImageJ software.

For identification of cardiomyocytes sacromeric α-actinin was used. Paraffin sections were deparaffinised and hydrated. After washing with PBS the 4% PFA-fixed tissue required antigen retrieval by heating the sections in 10mM sodium citrate to boiling temperature following cooling of the cross-sections for 20 minutes. Sodium citrate served to break the methylene bridges, which cross-linked proteins and thereby masked antigenic sites. The sections were washed in ddH₂O 3 times and incubated in 1% (v/v) H₂O₂ (Sigma) for 10 minutes. After washing in ddH₂O and PBS, the cardiac-ventricular sections were blocked with blocking solution (10% (v/v) normal goat serum (NGS), 10% (v/v) Triton® X-100, diluted in PBS) for 1 hour. The anti-α-actinin antibody (1:20, diluted in 5% (v/v) NGS) was incubated
overnight at 4°C. The sections were washed in PBS and incubated with secondary antibody (anti-rabbit, 1:500 in 5% (v/v) NGS, conjugated to Alexa Fluor 568, Invitrogen) for 1 hour followed by washing in PBS and mounting with Vectashield. The slides were analysed using fluorescence microscope.

Triple fluorescence staining resulted in sections stained with 3 colours: TUNEL staining appeared in green, α-actinin staining in red and nucleus staining in blue colour. TUNEL staining was considered positive when nucleus and TUNEL staining occurred in the same area. Images of cardiac-ventricular cross-sections were taken using a snapshot fluorescence microscope and 15 randomly choses frames were analysed by ImageJ software. A total number of 500 cells were analysed for each mouse. To determine the ratio of TUNEL-positive cardiomyocytes the number of TUNEL-positive cardiomyocyte nuclei was divided by the total number of cardiomyocyte nuclei.

2.1.7 Immunoblotting

*Preparation of protein samples*

Tissue samples were washed in ice-cold PBS (phosphate buffered saline, Sigma) and homogenised on ice in 1xTLB (1x triton lysis buffer). The homogenate was centrifuged at 13000rpm at 4°C and the supernatant was transferred to a clean Eppendorf tube. The Bradford assay (Bio-Rad, ready to use, 500-0006) as a spectrometric analytical method was used to determine the protein concentration. The protein sample and dye reagent of the protein assay (1:5 in ddH₂O) were mixed, the absorption was measured at 595nm in a spectrophotometer (WPA Lightwave UV/Vis Spectrophotometer S2000) and samples were normalized for Western blotting.
**SDS-PAGE and Western blotting**

The resolving and stacking gels were prepared to make the Sodium Dodecyl Sulfate Polyacrylamide gel (SDS-PAGE) (Table 2.3 and Table 2.4). Protein samples were denaturated by heating at 95°C with 6xLaemmli buffer and resolved at 20mA, 50W and 400V.

**Table 2.3: Resolving gel**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>8%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Protogel [ml] (National Diagnostics)</td>
<td>1.33</td>
<td>1.67</td>
</tr>
<tr>
<td>1.5M pH8.8 Tris-HCl [ml] (Fisher Bioreagents)</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>10% Ammonium persulfate [µl] (Sigma)</td>
<td>20.83</td>
<td>20.83</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED) [µl] (Sigma)</td>
<td>1.95</td>
<td>1.95</td>
</tr>
<tr>
<td>H₂O [ml]</td>
<td>2.4</td>
<td>2.06</td>
</tr>
</tbody>
</table>

**Table 2.4: Stacking gel**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Protogel [ml]</td>
<td>0.75</td>
</tr>
<tr>
<td>1.5M pH6.8 Tris-HCl [ml]</td>
<td>1.25</td>
</tr>
<tr>
<td>10% Ammonium persulfate [µl]</td>
<td>50</td>
</tr>
<tr>
<td>TEMED [µl]</td>
<td>5</td>
</tr>
<tr>
<td>H₂O [ml]</td>
<td>3</td>
</tr>
</tbody>
</table>

For analysis the protein (50µg per sample) was transferred to Polyvinylidene fluoride (PVDF) membrane (Millipore corporation) by semi-dry Western blot transfer (at 12V for 1 hour 45 minutes). Wet transfer was used for ERK5 (100kDa) and was performed at 50V for 3 hours. The membrane was blocked with blocking buffer and incubated overnight at 4°C with the primary antibody (diluted in blocking buffer)
The membrane was washed with 1xTBS-T (5 minutes/wash) and incubated with secondary antibody (anti-mouse, anti-rabbit or anti-goat immunoglobulin G-coupled to horseradish peroxidise, Amersham-Pharmacia) (Table 2.5). After washing the membrane with 1xTBS-T the secondary antibody was detected by enhanced chemiluminescence (ECL) kit and captured by BioMaxMRFilm (Kodak).

**Table 2.5: Solutions and buffers for Immunoblotting**

<table>
<thead>
<tr>
<th>Solution/Buffer</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1xTLB</td>
<td>20mM Tris pH 7.4, 137mM NaCl, 2mM EDTA pH 7.4, 1% Triton X-100, 25mM b-glycerophosphate, 1mM Na$_3$VO$_4$, 1mM phenylmethanesulphonylfluoride (PMSF), 1.54μM Aprotinin, 21.6μM Leupeptin, 10% Glycerol</td>
</tr>
<tr>
<td>(1xtriton lysis buffer)</td>
<td></td>
</tr>
<tr>
<td>1xRunning buffer</td>
<td>25mM Tris, 192mM glycine, 347mM SDS</td>
</tr>
<tr>
<td>1xSemi-dry transfer buffer</td>
<td>80% 1xRunning buffer, 20% Methanol</td>
</tr>
<tr>
<td>1xWet transfer buffer</td>
<td>80% 1xRunning buffer, 20% Methanol, 347mM SDS</td>
</tr>
<tr>
<td>1xTBS-T</td>
<td>150mM NaCl, 500mM Tris Base, 0.5% Tween-20 (pH 7.4)</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>3% skimmed milk in 1xTBS-T</td>
</tr>
<tr>
<td>6xLaemmli loading buffer</td>
<td>208mM SDS, 0.18M Tris, 15% β-mercaptoethanol, 50% Glycerol, 0.75mM Bromophenol blue, pH6.8</td>
</tr>
</tbody>
</table>
Table 2.6: Primary antibodies for Immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MEK1</td>
<td>1:1000</td>
<td>sc-219, Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-MEK2</td>
<td>1:1000</td>
<td>Sc-524, Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-ERK1/2</td>
<td>1:1000</td>
<td>9102, Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-phospho-ERK1/2</td>
<td>1:1000</td>
<td>4370, Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-ERK1</td>
<td>1:1000</td>
<td>sc-93, Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-ERK2</td>
<td>1:500</td>
<td>sc-154, Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-ERK5/BMK1</td>
<td>1:1000</td>
<td>Upstate (Millipore)</td>
</tr>
<tr>
<td>Anti-phospho-ERK5</td>
<td>1:1000</td>
<td>44612G, Invitrogen</td>
</tr>
<tr>
<td>Anti-p38 MAPK (C20)</td>
<td>1:1000</td>
<td>sc535-G, Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-phospho-p38 MAPK</td>
<td>1:1000</td>
<td>9211, Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-JNK</td>
<td>1:1000</td>
<td>9252, Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-phospho-JNK</td>
<td>1:1000</td>
<td>9251, Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-PKB</td>
<td>1:1000</td>
<td>9272, Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-phospho-PKB (Thr-308, Ser-473)</td>
<td>1:1000</td>
<td>9275, 9271 Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-cleaved-Caspase 3 (Asp175)</td>
<td>1:1.000</td>
<td>9661, Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-α-Tubulin</td>
<td>1:2000</td>
<td>T6199, Sigma-Aldrich</td>
</tr>
</tbody>
</table>

2.1.8 Quantitative real-time PCR

Preparation of mRNA samples

Tissue samples were washed in ice-cold PBS and homogenised on ice in Trizol. After adding chloroform (Sigma), samples were vigorously shaked and the aqueous layer containing RNA was separated by centrifugation at 13000rpm at 4°C. The pellet was discarded and RNA was precipitated with isopropanol added for 30 minutes followed by centrifugation at 12000rpm at 4°C. The RNA pellet was washed
in 70% ethanol, dried at room temperature and resuspended in nuclease-free water (Ambion Inc.).

RNA samples were treated with rDNase1 (Ambion Inc.) dissolved in DNase I buffer (Ambion Inc.) and incubated at 37°C for 1 hour to remove any contaminating genomic DNA. DNase inactivation reagent (Ambion Inc.) was used to inactivate the rDNase1 followed by centrifugation. The RNA concentration and quality of the supernatant was measured with Nanodrop (Thermo). An optical density (OD)_{260} to OD_{280} ratio of 1.8 to 2 was considered good quality as pure RNA (without significant amounts of contaminants, such as protein, phenol and nucleic acids) has an OD_{260} to OD_{280} ratio of ~2 while the optical absorbance of protein is measured at 280nm. Nanodrop RNA quality check was confirmed by running 500ng of each sample on a 1% agarose gel.

**Reverse transcriptase PCR**

The mRNA was reversed to cDNA using Superscript II kit (Invitrogen). 2µg of total RNA was mixed with 2µl 1:1 Oligo(dT)\textsubscript{15} primer (0.5g/l in water, Promega) / dNTP mix (Roche) and pure water was added to a total volume of 12µl. The mixture was heated at 72°C for 10 minutes and immediately chilled on ice for 2 minutes, followed by adding 8µl RT-PCR master-mix (5xFirst Strand buffer (Invitrogen), pure water, RNase inhibitor SuperaseIn (20U/µl, Ambion Inc.), SuperScript® II Reverse Transcriptase (200U/µl, Invitrogen)). The reverse transcriptase PCR reaction was performed at 42°C for 60 minutes followed by 72°C for 15 minutes. The PCR product was further diluted appropriately for real-time PCR reaction.
Real-time PCR

The real-time (RT) PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, 1102265) according to manufacturer’s recommendations. SYBR Green binds to any double stranded DNA and provides a fluorescent signal. Each 25µl reaction contained 80ng/µl of complementary DNA (cDNA) sample, 2.5µl of primers (10x QuantiTect Primer Assay, QIAGEN), 12.5µl of SYBR Green PCR master mix, 9µl RNase and DNase free water (Sigma). The reporter signal was normalised to reactions performed using GAPDH primers (QIAGEN). Primers used were ANP (QT00250922), BNP (QT00107541), Col1α2 (QT01055516), Col3α1 (QT01055572) and GAPDH (QT01658692).

The PCR product was detected with the 7500 real-time PCR System (Applied Biosystems). Thermal cycling was performed with the program: 95ºC for 15 seconds; 55ºC for 30 seconds; 72ºC for 33 seconds; for 40 cycles.

The fold change was determined using the comparative threshold method \(^{211}\).

Calculation: \(2^{-\Delta\Delta C_T}\)

with \(\Delta\Delta C_T = (C_{T,\text{Target}} - C_{T,\text{GAPDH}})_{\text{Treatment X}} - (\text{mean } C_{T,\text{Target}} - \text{mean } C_{T,\text{GAPDH}})_{\text{Calibrator X}}\)

Calibrator X: negative control of ERK2\(^{ff}\) mice for treatment X
2.2 In vitro analyses

2.2.1 Isolation of neonatal rat cardiomyocytes

Sprague Dawley rats were used for preparation of neonatal rat cardiomyocytes (NRCMs). 2 to 3 day-old rats were killed by cervical dislocalisation and hearts were collected into ice-cold filter-sterilised Aspirate Digestion (ADS) buffer (Table 2.7). The atria and extraneous tissue were removed; each heart was cut into 2 pieces and incubated in 7ml filter-sterilised Digestion solution for 7 minutes at 37°C in a shaking water bath (Table 2.7). After each digestion step the detached cardiomyocytes suspended in the Digestion solution were passed through a cell strainer (70µm, Falcon™, BD Bioscience) into a new bottle and 2ml of foetal calf serum (FCS, Invitrogen) was added to prevent further action of collagenase. The first cell harvest was discarded, exclusively containing connective tissue. The heart digestion was continued 7 times for 7 minutes each at 37°C. The isolated cell suspension was collected by centrifugation at 1200rpm for 5 minutes, the pellet was re-suspended in 40ml Pre-plating medium and plated onto Falcon tissue culture dishes at 37°C allowing fibroblasts to attach on the dishes (4 plates, 10ml each) (Table 2.7). After 1 hour the remaining floating cardiomyocytes were removed and counted using Trypan Blue stain and cell counting chamber. Cardiomyocytes were diluted in plating medium to $1 \cdot 10^6$ cells/ml into 6 or 12 well plates (Table 2.7). The 12 well plates were prepared the day before the cell preparation. Coverslips (10mm diameter) were treated with 70% ethanol for 30 minutes followed by washing in PBS and were finally placed into 12 well plates (using sterile forceps). Laminin buffer (Invitrogen, 1:120 diluted in PBS) was added and the plates were sealed at room temperature until further use. Directly before adding NRCMs, the Laminin buffer was removed. BrdU (5-bromo-2-deoxyuridine) was added to the Plating medium.
NRClMs were further incubated at 37°C overnight. The next day, medium was removed and replaced by Plating medium and the cells were kept in the incubator for another day prior to further treatment.

**Table 2.7: Buffer/Medium for isolation of neonatal rat cardiomyocytes**

<table>
<thead>
<tr>
<th>Buffer/Medium</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADS buffer</td>
<td>116mM NaCl, 20mM HEPES (Fisher BioReagents, BP310), 1mM NaH₂PO₄ (Sodium phosphate monobasic monohydrate, Sigma-Aldrich, S9638) 6mM Glucose (Fisher Scientific), 5mM KCl (Fisher Scientific), 0.8mM MgSO₄, pH 7.35</td>
</tr>
<tr>
<td>Digestion solution</td>
<td>75ml ADS buffer, 100mg Collagenase A (0.21 Units/mg, Roche), 100mg/ml Pancreatin (Sigma-Aldrich)</td>
</tr>
<tr>
<td>Pre-plating medium</td>
<td>68% Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), 17% M199 (Invitrogen), 10% horse serum (Invitrogen), 5% FCS, 250µg/ml Fungizone (Ampotericin B, Invitrogen)</td>
</tr>
<tr>
<td>Plating medium</td>
<td>Pre-plating medium with added 1µM BrdU (Sigma-Aldrich)</td>
</tr>
</tbody>
</table>

### 2.2.2 Preparation of adenovirus

The provided recombinant adenovirus Ad5-CMV-ERK2 DN (Seven Hills Bioreagents, JMAd-57) contains a specific overexpression of dominant negative (DN) mutant of rat ERK2 protein (Ad-DNERK2) and was used at MOI 25. The Ad-DNERK2 contains an AEF mutation yielding a nonactivatable form of ERK2 in which the phosphoacceptors threonine and tyrosine had been mutated to alanine (T183A) and phenylalanine (Y185F) respectively. The purchased recombinant adenovirus containing the cDNA encoding DNERK2 had been prepared as previously described. Briefly, the pAC-CMV-pLpA vector was prepared by insertion of the constitutive cytomegalovirus (CMV) early gene promoter/enhancer, the pUC 18 polylinker, and a fragment of the SV40 genome into a pAC vector. A rat cDNA for DNERK2 was then inserted into the pAC-CMV-pLpA vector. The
resulting plasmid was cotransfected with pJM17 into 293 HEK cells. pJM17 encodes the full length adenovirus 5 genome interrupted by the insertion of the bacterial plasmid pBRX, thereby exceeding the packaging limit for adenovirus. Homologous recombination between the two plasmids resulted in replacement of the Adenovirus 5 early gene region 1 with the DNEKR2 cDNA, rendering the recombinant virus replication defective. As a virus control the recombinant adenovirus β-galactosidase (Ad-LacZ) was used.

2.2.3 Adenovirus amplification, titration and infection

Human embryonic kidney (HEK293) cells were used for virus amplification and titration. In T75 flask (Corning) HEK293 cells were cultivated to 80 to 100% confluence. The medium was changed to 2% serum containing medium and the primer adenoviral stock was added. The virus-containing cells were incubated at 37°C until the full cytopathic effect (CPE) was observed (morphological changes in host cells resulting from viral infection; HEK293 cells were round shaped and the majority of cells were detached). The suspension was collected by centrifugation at 1,500rpm at room temperature and kept at -80°C. The virus lysate was suspended in 2% serum-maintenance medium and mixed with 16 T225 flasks of HEK 293 cells at 90% confluence. Cells were incubated at 37°C in an atmosphere of 5% CO₂ in air. Once plaques of 50% confluence appeared after 2 to 5 days, the cells were harvested, followed by centrifugation and re-suspension of the pellet in PBS. Three cycles of freeze/thaw in liquid nitrogen/37°C water bath was performed following centrifugation.

The adenovirus was purified by CsCl gradient centrifugation. Density gradient was prepared in a Beckman centrifuge tube and consisted of 8.6M CsCl diluted in CsCl
buffer, 7.9M CsCl diluted in CsCl buffer and cell suspension (Table 2.9). The
gradient was centrifuged in a pre-cooled 4°C ultracentrifuge (SW40 TI swing-out
rotor) for 2 hours at 22,500rpm. The virus band (lowest white band) was removed,
diluted with half of its volume in Tris EDTA (TE) buffer and loaded onto second
gradient consisting of 8.6M CsCl, 7.9M CsCl to further purify the virus (Table 2.9).
The samples were centrifuged at 23,800rpm for 18 hours at 4°C. The virus band was
removed and placed into a closed dialysis membrane (molecular weight cut off
(MOCW): 12k-14kDa, Medicell International Ltd.) followed by dialysis for 1 hour
in buffer A and 2 hours against buffer B (Table 2.9). The virus was removed,
aliquoted into Eppendorf tubes and stored at -80°C.
To assess the titration, 1·10^3 HEK293 cells were plated into each well of a 96 well
plate. 24 hours after inoculation the medium was removed and replaced with 100µl
of serially diluted virus in triplicate from 10^{-2} to 7.63·10^{-12} or normal maintenance
medium (control). 24 hours later another 100µl of fresh medium was added into the
wells. The plate was maintained in a 37°C incubator for another 6 days. The plaque
formation was observed each day using a microscope. At the final day, the final
dilution showing CPE in the inoculated cells was identified as the end-point well.
The test was valid if the most concentrated wells showed complete CPE and the
control wells showed no signs. According to the dilution in the end-point well, the
plaque forming units per millilitre (pfu/ml) was calculated using Table 2.8.
Table 2.8: Adenovirus pfu/ml from titration

<table>
<thead>
<tr>
<th>Dilution</th>
<th>pfu/ml</th>
<th>Dilution</th>
<th>pfu/ml</th>
<th>Dilution</th>
<th>pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-2}$</td>
<td>$1\times10^3$</td>
<td>$6.25\times10^{-8}$</td>
<td>$1.6\times10^8$</td>
<td>$2.44\times10^{-10}$</td>
<td>$4.1\times10^{10}$</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>$1\times10^4$</td>
<td>$3.12\times10^{-8}$</td>
<td>$3.2\times10^8$</td>
<td>$1.22\times10^{-10}$</td>
<td>$8.19\times10^{10}$</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>$1\times10^5$</td>
<td>$1.56\times10^{-8}$</td>
<td>$6.4\times10^8$</td>
<td>$6.1\times10^{-11}$</td>
<td>$6.1\times10^{11}$</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>$1\times10^6$</td>
<td>$7.8\times10^{-9}$</td>
<td>$1.28\times10^9$</td>
<td>$3.05\times10^{-11}$</td>
<td>$3.28\times10^{11}$</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>$1\times10^7$</td>
<td>$3.91\times10^{-9}$</td>
<td>$2.56\times10^9$</td>
<td>$1.53\times10^{-11}$</td>
<td>$6.55\times10^{11}$</td>
</tr>
<tr>
<td>$5\times10^{-7}$</td>
<td>$2\times10^7$</td>
<td>$1.95\times10^{-9}$</td>
<td>$5.12\times10^9$</td>
<td>$7.63\times10^{-11}$</td>
<td>$1.31\times10^{12}$</td>
</tr>
<tr>
<td>$2.5\times10^{-7}$</td>
<td>$4\times10^7$</td>
<td>$9.77\times10^{-10}$</td>
<td>$1.02\times10^{10}$</td>
<td>$3.81\times10^{-11}$</td>
<td>$2.62\times10^{12}$</td>
</tr>
<tr>
<td>$1.25\times10^{-7}$</td>
<td>$8\times10^7$</td>
<td>$4.88\times10^{-10}$</td>
<td>$2.05\times10^{10}$</td>
<td>$3.81\times10^{-11}$</td>
<td>$2.62\times10^{12}$</td>
</tr>
</tbody>
</table>

The multiplicity of infection (MOI) was 25 for the infection of cardiomyocytes and the amount of virus required for 100% infection was calculated the following:

\[
virus (\mu l) = MOI \cdot \text{cell number} \left(2.5\cdot10^6/\text{well in 6 well plate}\right) / \text{Titre (pfu/\mu l)}
\]

Table 2.9: Buffer for virus amplification and titration

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsCl</td>
<td>5mM Tris (Fisher Bioreagent), 1mM EDTA, pH 7.8</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris, 1mM EDTA (Fisher Scientific), pH 7.8</td>
</tr>
<tr>
<td>Buffer A</td>
<td>10mM Tris, 1mM MgCl$_2$ (Fisher Scientific), 135mM NaCl (Fisher Scientific)</td>
</tr>
<tr>
<td>Buffer B</td>
<td>10mM Tris, 1mM MgCl$_2$, 135mM NaCl, 10% glycerol (Fisher Scientific)</td>
</tr>
</tbody>
</table>

2.2.4 Experimental procedures in NRCM

2.2.4.1 TUNEL staining of NRCM

NRCMs were infected with Ad-DNERK2 or the control virus (Ad-LacZ) for 24 hours. Apoptosis was induced in infected NRCMs by treatment with 100µM H$_2$O$_2$ for 4 or 24 hours. The medium was removed and the cells were fixed with 4% PFA
for 30 minutes at 4°C. After washing with PBS cells were permeabilised with 0.1% (v/v) Triton X-100 and 0.1% (w/v) sodium citrate (diluted in PBS) on ice for 2 minutes. TUNEL (Roche Applied Science, In Situ Cell Death Detection Kit, Fluorescine) was added for 1 hour in a dark humid chamber at 37°C. DAPI was added for 5 minutes followed by washing in PBS. The slides were mounted using Vectashield (Vector Labs) and analysed using a fluorescence microscope and ImageJ software. Nuclei were stained with DAPI in blue and apoptotic nuclei showed green fluorescence. Nuclei with merged colour (blue and green) were considered as positive apoptotic cells.

2.2.4.2 Cellular hypertrophy experiments

*Immunocytochemistry*

To evaluate the hypertrophic growth, NRCMs were plated over laminin-coated coverslips and infected with Ad-DNERK2 (MOI 25) or Ad-LacZ (MOI 25) in 2% serum-medium for 24 hours, followed by further 48 hours treatment with phenylephrine (PE, 100mM, Sigma-Aldrich). Thereafter, NRCMs were fixed in 4% PFA for 30 minutes at 4°C. Cells were washed in PBS and permeabilised with 0.1% Triton X-100 for 10 minutes at room temperature. After blocking in 0.5% bovine serum albumin (BSA) NRCMs were incubated with α-actinin antibody (mouse, 1:20, Sigma, A7811, diluted in 0.5% BSA overnight). NRCMs were washed in 0.5% BSA and incubated with secondary anti-mouse antibody conjugated to Alexa Flour 568 (Invitrogen) for 1 hour at room temperature. After washing in PBS, the nuclei were stained with DAPI (1:1000) for 5 minutes at room temperature followed by washing.
in PBS. The slides were mounted using Vectashield (Vector Labs). With ImageJ software the surface area of 150 cells per group was measured.

The hypertrophic response was further analysed by staining of ANP expression. NRCMs were infected with adenovirus (Ad-DNER2 or Ad-LacZ) and treated with PE for 48 hours. Thereafter NRCMs were used for immunocytochemistry as described above. NRCM were incubated with anti-ANP antibody (rabbit, 1:500, Peninsula Laboratories, T-4015) and anti-α-actinin antibody (1:100) following incubation with second antibodies (conjugated to Alexa Flour 568) and the nuclei staining with DAPI (1:1000). The slides were mounted using Vectashield (Vector Labs). Total of 300 NRCMs from random fields per experimental group were selected to count ANP-expressing cells using fluorescence microscopy and ImageJ software.

**Luciferase reporter assay**

NRCM were prepared and transferred into 24 well plates (1x10⁶ cells/well) (as described in 2.2.1). To analyse the *BNP* promoter activity NRCM were co-infected with either Ad-DNERK2 or Ad-LacZ and with recombinant adenovirus encoding BNP-luciferase reporter gene (Ad-BNP-Luc) at MOI 25 in serum-free medium for 24 hours.

The *BNP* promoter-luciferase construct was generated by cloning rat *BNP* promoter region upstream of firefly luciferase reporter gene. The construct was thereafter cloned to pAdEasy using the shuttle system which contained the CMV promoter as recommended by manufacturer (240007, Stratagene).

The viruses were removed and NRCMs were treated with PE (30µM) for 24 hours. *BNP* luciferase activity was analysed using luciferase assay kit (Promega). Briefly,
the cells were rinsed in PBS and 100µl passive lysis buffer was added. The culture plate was placed on a rocking platform for 30 minutes at room temperature. Aliquots (40µl) were transferred into an Eppendorf tube and 40µl Luciferase Reagent was added. The firefly luciferase activity of the cell lysate (560nm peak emission wavelength) was measured with luminometer (wavelength: 300-650nm, TD-20/20 Turner designs, Steptech Instrument Service Limited).

2.3 Analysis of data and statistical analysis

Data were collected using blind analysis and were expressed as means ± SEM (standard error of the mean). Statistical comparisons between 2 groups were analyzed using the paired student’s t test, for multiple comparisons 1- or 2-way ANOVA followed by Bonferroni t-test were used where appropriate. A p value of <0.05 was considered to be statistically significant between groups.
CHAPTER 3
Chapter 3 Results

3.1 Characterisation of ERK2\textsuperscript{cko} mice

In order to investigate the role of ERK2 in the heart, we generated mice with cardiomyocyte-specific deletion of \textit{erk2} (ERK2\textsuperscript{cko}) using the Cre-loxP system. The cardiomyocyte-specific deletion in ERK2\textsuperscript{cko} mice was analysed at the age of 8 weeks. The mRNA levels of \textit{erk2} in ERK2\textsuperscript{cko} left ventricle were significantly reduced (73.34\%), whereas these mRNA levels in brain, liver and skeletal muscle (SM) were indifferent in comparison to ERK2\textsuperscript{ff} mice (Figure 3.1).

In ERK2\textsuperscript{cko} mice, immunoblot analysis of the cardiac left ventricles showed 75.14\% protein reduction of ERK2. In contrast, ERK2 reached similar expression levels as ERK2\textsuperscript{ff} mice in brain, liver and SM. Despite the high affinity of ERK2 with ERK1, the protein expression of ERK1 was unaltered in all these tissues between both genotypes (Figure 3.1).

In addition, the protein expression of other MAPKs was analysed to detect any changes that could compensate for the loss of ERK2. In ERK2\textsuperscript{cko} mice, the absence of ERK2 in ventricles did not affect other MAPK pathways as the expression levels of ERK5, p38 MAPK and JNK were comparable to the control group (Figure 3.1).

The cardiac structure was further analysed by histological analysis using haematoxylin and eosin (H&E), Masson’s trichome staining and Picro-Sirius red staining. In ventricles from ERK2\textsuperscript{cko} mice, the cardiomyocyte cross-sectional areas were similar to ERK2\textsuperscript{ff} mice (202.81±4.70\(\mu\)m\(^2\) versus 203.10±3.69\(\mu\)m\(^2\), respectively) (Figure 3.2). Signs of interstitial fibrosis were not found in either genotypes (Figure 3.3).

In addition, TUNEL assay revealed no apoptotic left ventricular cardiomyocytes in both ERK2\textsuperscript{cko} and ERK2\textsuperscript{ff} mice (Figure 3.4).
The heart function was examined by echocardiography. Both genotype groups showed comparable basic function (Table 3.1).

Taken together, *erk2* was specifically and efficiently deleted in left ventricular cardiomyocytes of ERK2*cko* mice without changing the expression levels of other MAPKs in comparison to the control group. Both genotypes displayed similar cardiac structure and function. These data showed that despite the cardiomyocyte-specific knockout of ERK2 in the ERK2*cko* heart ventricles, no compensatory changes occurred on protein synthesis of ERK2 in other tissues, the expression of other MAPKs in cardiomyocytes as well as on tissue structure of the heart. Therefore the differences between ERK2*cko* mice and the control group (ERK2*ff*) resulted from the lack of ERK2 alone.
Figure 3.1: Characterisation of cardiomyocyte-specific deletion of erk2.

A. Quantitative real-time PCR analysis of mRNA levels of erk2 in the left ventricle (LV), brain, liver and skeletal muscle (SM), showed a 73% decrease in LV of ERK2\textsuperscript{cko} mice compared to ERK2\textsuperscript{f/f} mice. The data were derived from 3 independent experiments performed in triplicate and were normalised to GAPDH content. B, Immunoblot analysis showed specific deletion of ERK2 in LV in ERK2\textsuperscript{cko} mice compared to ERK2 expression in brain, liver and SM. ERK1 expression was similar in both genotypes. Tubulin served as protein loading control. The ratio of ERK1 and ERK2 expression to tubulin is shown in the bar graph. C, Immunoblot analyses demonstrated similar expression levels of MEK1, MEK2, ERK5, p38 MAPK and JNK in both genotypes. Data are presented as means ± SEM (n = 3). n.s.: no statistically significant difference found between ERK2\textsuperscript{f/f} and ERK2\textsuperscript{cko} groups.
Figure 3.2: Similar cardiomyocyte size in \textit{ERK2}^{cko} and \textit{ERK2}^{f/f} mice

\textbf{A}, Hematoxylin/eosin (H&E) staining of cardiac-ventricular cross-sections (scale bar = 20\textmu m) \textbf{B}, The quantification of the mean cross-sectional areas of LV was similar in \textit{ERK2}^{cko} and \textit{ERK2}^{f/f} mice. Data are presented as means ± SEM (n = 5). n.s.: no statistically significant difference found.
Figure 3.3: No interstitial fibrosis was found in both genotypes
Masson’s trichome staining and Picro-Sirius red staining of cross-sections showed no fibrosis in both genotypes (scale bar = 50µm); Representative image of 5 animals per group.
Figure 3.4: Cardiomyocyte apoptosis was not detected in both genotypes. ERK2<sup>f/f</sup> and ERK2<sup>cko</sup> hearts showed no apoptosis. Sections of LV were analysed by TUNEL assay. Triple staining was performed: TUNEL: green; DAPI: blue; α-actinin: red. (scale bar = 50µm); TAC mice were used as a positive control, Representative image of 5 animals per group.
Table 3.1: Echocardiographic characterisation of ERK2<sup>f/f</sup> and ERK2<sup>cko</sup> mice

<table>
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<tr>
<th>Parameters</th>
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<td>Heart rate (bpm)</td>
<td>432.40±12.60</td>
<td>445.50±12.72</td>
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<td>LVEdD (mm)</td>
<td>3.79±0.17</td>
<td>3.99±0.19</td>
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<td>LVEsD (mm)</td>
<td>2.71±0.18</td>
<td>2.88±0.16</td>
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<td>dIVS (mm)</td>
<td>0.85±0.12</td>
<td>0.95±0.08</td>
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<td>sIVS (mm)</td>
<td>1.18±0.04</td>
<td>1.24±0.06</td>
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<td>dPW (mm)</td>
<td>0.86±0.10</td>
<td>0.80±0.10</td>
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<tr>
<td>sPW (mm)</td>
<td>1.17±0.12</td>
<td>1.12±0.11</td>
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<tr>
<td>FS (%)</td>
<td>29.12±1.95</td>
<td>28.10±1.49</td>
</tr>
</tbody>
</table>

ERK2<sup>f/f</sup> and ERK2<sup>cko</sup> mice showed similar cardiac function by echocardiographic assessment. Data are means ± SEM (n = 10).

LVEdD: left ventricular end-diastolic diameter; LVEsD: left ventricular end-systolic diameter; dIVS: diastolic interventricular septum thickness; sIVS: systolic interventricular septum thickness; dPW: diastolic left ventricular posterior wall thickness; sPW: systolic left ventricular posterior wall thickness; FS: fraction shortening.
3.2 ERK2 activation *in vivo* and *in vitro* following hypertrophic stimulation

Following the characterisation of ERK2\(^{cko}\) mice, the activation of ERK2 in response to various stimuli was analysed prior to further experiments to investigate pathological hypertrophic remodelling in the ERK2\(^{cko}\) mice.

Firstly, the activation of ERK2 was determined *in vitro* using NRCMs following hypertrophic stimulation with Isoproterenol (Iso, 10µM), AngiotensinII (AngII, 10µM) and Phenylephrine (PE, 100µM). The immunoblot analysis showed increased phosphorylation of ERK2 upon Iso, AngII or PE treatment (Figure 3.5). The ERK2 total protein content was similar to the vehicle group.

To characterise the ERK2 phosphorylation *in vivo*, wild-type mice were treated with pressure overload (1 week of TAC) or Iso (subcutaneous injection (10mg/kg), after 30 minutes mice were killed and heart ventricles were analysed). Immunoblot analysis clearly showed ERK2 activation in ventricles of wild-type mice upon stress (Figure 3.5).

The activation of ERK2 might suggest an important regulatory role during pathological hypertrophy following stimulation by PE, AngII, Iso and pressure overload.

Interestingly, the *in vivo* data showed a more significant ERK2 activation in response to hypertrophic stimuli compared to the *in vitro* data. Therefore further experiments mainly concentrated on using the ERK2 cardiac knockout mice.

Subsequently, different results following hypertrophic stimulation in ERK2\(^{cko}\) mice were due to the cardiomyocyte-specific knockout of ERK2 in ventricles.

Taken together, *in vitro* and *in vivo* pathological hypertrophic stimulation led to ERK2 phosphorylation and thereby showed an activation of ERK2 in response to these stimuli.
**Figure 3.5:** ERK2 was activated *in vitro* and *in vivo* in response to various hypertrophic stimuli

**A**, NRCMs were analysed for the expression and phosphorylation levels of ERK2. The hypertrophic stimulation by PE (100µM), AngII (10µM) and Iso (10µM) led to ERK2 activation, n=2.

**B**, Iso (10mg/kg) and **C**, pressure overload (1 week of TAC) induced-activation of ERK2 in wild-type hearts was examined by immunoblotting. 1 week of TAC or Iso-treatment (30 minutes, intraperitoneal) resulted in an increase of ERK2 phosphorylation in ventricles of wild type mice, n=2 to 3.

P-ERK1: phosphorylated ERK1; P-ERK2: phosphorylated ERK2
3.3 Attenuated cardiac hypertrophic response to chronic β-adenergic stimulation

First, the role of ERK2 in pathological hypertrophic signalling induced by β-adenergic stimulation was analysed. ERK2\textsuperscript{cko} and ERK2\textsuperscript{ff} mice were treated with Iso (10mg/kg/day) using micro-osmotic pump for 1 week.

Following Iso treatment, the heart weight to tibia length (HW/TL) ratio was similar in both genotypes. Therefore histological experiments were performed to analyse the role of ERK2 in hypertrophic growth at the cellular level.

Cardiac ventricular cross-sections analysis revealed a less increased cardiomyocyte cross-sectional area in ERK2\textsuperscript{cko} mice following Iso administration (264.75±2.80µm\textsuperscript{2}), whereas ERK2\textsuperscript{ff} mice showed significantly increased hypertrophic growth (300.71±4.92µm\textsuperscript{2}) (Figure 3.6). Consistently, the transcript levels of the hypertrophic biomarkers, ANP and BNP, in ERK2\textsuperscript{cko} ventricles were significantly lower in comparison to that in ERK2\textsuperscript{ff} hearts (Figure 3.7).

In addition, Iso-treated ERK2\textsuperscript{cko} mice showed minimal development of interstitial myocardial fibrosis (Figure 3.8).

The structural changes in Iso-treated mice led to differences in cardiac function (Table 3.2). Echocardiographic assessment showed a significantly less increased contractility in ERK2\textsuperscript{cko} mice compared to the Iso-treated ERK2\textsuperscript{ff} mice (FS: 37.40±1.38% versus 44.22±3.05%).

These results clearly showed that ERK2 was involved in hypertrophic remodelling caused by β-adrenergic stimulation. Following 1 week of Iso treatment, the loss of ERK2 resulted in blunted hypertrophic growth of left ventricular cardiomyocytes and the increase of cardiac contractility was depressed in comparison to the control group. The biomarkers ANP and BNP reflected the less increased hypertrophic
growth in ERK2^{cko} ventricles. Both hormones are secreted in response to stretching of the heart muscle and have cardioprotective functions due to their diuretic, vasodilating and natriuretic effects\textsuperscript{216}. Both ERK2^{cko} and ERK2^{f/f} mice developed hypertrophy by increasing the heart size at two different levels to adapt to the inotropic effects of Iso treatment.

Despite the hypertrophic growth of ventricular cardiomyocytes in both genotypes no significant increase in fibrosis was observed suggesting absence of significant apoptosis. Thus at that stage of hypertrophy the ventricles of ERK2^{cko} and ERK2^{f/f} mice developed compensated hypertrophic remodelling without adverse effects on heart structure resulting from Iso stimulation for 1 week.

To summarise, Iso treatment activated the β-adrenergic pathway leading to development of hypertrophy in the ventricles. In particular, ERK2 played an important role in that hypertrophic remodelling as ERK2^{cko} mice showed blunted hypertrophic growth and less increased heart contractility in comparison to the control group.
Figure 3.6: ERK2^{cko} mice showed blunted Iso-induced cardiac hypertrophy.

**A**, Following 1 week of chronic Iso treatment ERK2^{cko} mice showed a significantly blunted cardiac hypertrophic growth compared to the control group (top panel, scale bar = 5mm). H&E staining of heart cross-sections displayed smaller cardiomyocytes in ERK2^{cko} left ventricles (bottom panel, scale bar = 20µm).  

**B**, The quantification of the mean cross-sectional areas of left ventricular cardiomyocytes revealed a significantly increased hypertrophic growth in ERK2^{f/f} mice compared to the knockout mice. Data are presented as means ± SEM (n = 5 to 6). # statistical significance to respective control group.
Figure 3.7: Quantitative real-time PCR analyses showed less increased expression of hypertrophic markers in ERK2^{cko} mice following chronic Iso stimulation.

Quantitative real-time PCR revealed the role of ERK2 in β-adenergic-induced hypertrophy by showing the less increased re-activation of hypertrophic gene markers, ANP and BNP compared to the control group. The data were derived from 5 independent experiments performed in triplicate and normalised to GAPDH content. Data are presented as means ± SEM (n = 5). # statistical significance to respective control group.
Figure 3.8: Iso treatment did not induce significantly increased fibrosis in both groups.

A, Picro-Sirius red staining of ventricular cross-sections showed minimal level of interstitial ventricular fibrosis in both genotypes (fibrotic tissue: red, scale bar = 50muş). B, Quantification of the relative area of fibrosis was expressed as percentage of the fibrosis area in the microscope views. Data are presented as means ± SEM (n = 3 to 5). n.s.: no statistically significant difference found.
**Table 3.2: Echocardiographic parameters of mice following Iso infusion**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ERK2(^{f/f})</th>
<th></th>
<th>ERK2(^{c\text{ko}})</th>
<th></th>
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<tr>
<td>Heart rate (bpm)</td>
<td>vehicle</td>
<td>458.83±9.64</td>
<td>Iso</td>
<td>582.71±37.42*</td>
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<td>LVEdD (mm)</td>
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<td>vehicle</td>
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<td>LVEsD (mm)</td>
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<td>dIVS (mm)</td>
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<td>1.21±0.15*</td>
<td>vehicle</td>
<td>0.97±0.06</td>
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<tr>
<td>sIVS (mm)</td>
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<td>1.41±0.1*</td>
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<tr>
<td>FS (%)</td>
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<td>44.22±3.05*</td>
<td>vehicle</td>
<td>30.72±1.7</td>
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ERK2\(^{f/f}\) and ERK2\(^{c\text{ko}}\) mice were treated with Iso for 1 week (10mg/kg/day) followed by echocardiographic assessment. Data are means ± SEM (n = 5 to 8 per group). *p<0.05, Iso-treated versus vehicle-treated mice within the same genotype; #p<0.05, ERK2\(^{c\text{ko}}\) versus ERK2\(^{f/f}\) after Iso treatment.
3.4 Similar cardiac hypertrophic response found to chronic renin-angiotensin stimulation

To further examine the role of ERK2 in cardiac hypertrophy induced by the renin-angiotensin pathway mice were treated with AngII using micro-osmotic pump. After 2 weeks of AngII administration (500µg/kg/day), ERK2\textsuperscript{cko} mice developed similar HW/TL ratios compared to the treated control group (7.28±0.23g/mm versus 7.22±0.35g/mm, respectively) (Figure 3.9). The cardiomyocyte cross-sectional areas were similar in ERK2\textsuperscript{cko} ventricles (230.57±3.14µm\textsuperscript{2}) compared to ERK2\textsuperscript{f/f} mice (240.08±7.90µm\textsuperscript{2}) (Figure 3.9). No interstitial myocardial fibrosis was observed in either genotype (Figure 3.10).

Finally, AngII induced hypertrophy resulted in similar cardiac function in ERK2\textsuperscript{cko} mice (FS: 30.92±1.33%) and ERK2\textsuperscript{f/f} mice (FS: 33.09±2.2%) in comparison to their vehicle groups (FS: 30.43±1.6% [ERK2\textsuperscript{cko}] and 29.85±2.9% [ERK2\textsuperscript{f/f}], respectively) (Table 3.3). In addition, echocardiographic analyses revealed hypertrophic growth by increases in interventricular septum thickness and left ventricular posterior wall thickness in both AngII-treated groups. These changes suggested initiation of hypertrophy in response to AngII (Table 3.3).

AngII specifically activates α\textsubscript{1}-adrenergic receptors leading to hypertrophic remodelling. In contrast to Iso stimulation, the activation did not show significant changes in heart function by analysing FS. However, small differences were observed as increased cardiomyocyte cross-sectional areas following treatment in comparison to the vehicle groups. Therefore histological experiments are crucial for analysing changes in more detail compared to echocardiography. In contrast, echocardiography is useful for analysing the heart in the living organism allowing more crude observations. Despite similar FS, both ERK2\textsuperscript{cko} and ERK2\textsuperscript{f/f} ventricles
showed a tendency of hypertrophic growth by enlarged diastolic PW and IVS further confirming histological data.

Taken together, AngII-treated ERK2^{cko} and ERK2^{f/f} mice showed a comparable cardiac structure, including hypertrophic growth and the lack of interstitial fibrosis in ventricles.

Considering the activation of ERK2 in NRCM following AngII stimulation (Figure 3.5) the similar hypertrophic growth in both genotypes was unexpected. However, the analyses of the cross-sectional areas showed a non-significant tendency of blunted cardiac hypertrophic growth in ERK2^{cko} mice. Therefore using either a higher dose of AngII or extending the period of the experiment might reveal a role of ERK2 in the renin-angiotensin pathway.
Figure 3.9: No difference in hypertrophic growth was found between ERK2<sup>fl</sup>f and ERK2<sup>cko</sup> mice following AngII treatment

A, Following 2 weeks of AngII treatment (500µg/kg/day) ERK2<sup>cko</sup> mice showed similar cardiac hypertrophy compared to the control group (top panel, scale bar = 5mm). Histological analysis of ventricular cardiomyocyte cross-sections using H&E staining (bottom panel, scale bar = 20µm). B, ERK2<sup>cko</sup> and ERK2<sup>fl</sup>f mice displayed similar HW/TL ratios. C, Mean cross-sectional areas of cardiomyocytes in ERK2<sup>cko</sup> and ERK2<sup>fl</sup>f LV were increased similarly. Data are presented as means ± SEM (n = 5 to 8). # statistical significance to respective control group. n.s.: no statistically significant difference found.
Figure 3.10: No fibrosis after chronic AngII administration was detected in ERK2<sup>cko</sup> and ERK2<sup>f/f</sup> mice

Picro-Sirius red staining of cardiomyocyte cross-sections showed lack of interstitial fibrosis in both genotypes following 2 weeks of AngII administration (500µg/kg/day) using micro-osmotic pump (scale bar = 50µm), Representative image of 5 to 8 animals per group.
Table 3.3: Echocardiographic characteristics of ERK2\textsuperscript{f/f} and ERK2\textsuperscript{cko} mice following AngII stimulation

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<th>ERK2\textsuperscript{cko}</th>
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<td>vehicle</td>
<td>AngII</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>458.83±9.64</td>
<td>472.40±21.05</td>
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<td>LVEdD (mm)</td>
<td>3.5±0.05</td>
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<td>LVEsD (mm)</td>
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<td>dIVS (mm)</td>
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<td>sIVS (mm)</td>
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<td>dPW (mm)</td>
<td>0.76±0.05</td>
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<tr>
<td>sPW (mm)</td>
<td>0.96±0.12</td>
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</tr>
<tr>
<td>FS (%)</td>
<td>29.85±2.90</td>
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ERK2\textsuperscript{f/f} and ERK2\textsuperscript{cko} mice were treated with AngII for 2 weeks (500µg/kg/day) followed by echocardiographic assessment. Data are means ± SEM (n = 5 to 8 per group). *p<0.05, AngII-treated versus vehicle-treated mice within the same genotype.
3.5 Pressure overload induced less hypertrophic response with higher apoptosis in ERK2\textsuperscript{cko} mice

After investigating the role of ERK2 in hypertrophy following Iso or AngII treatment, pressure overload by TAC, a clinically relevant stress, was used to analyse the importance of ERK2 in hypertrophic remodelling. In contrast to the previous specific hypertrophic stimuli, pressure overload induced several pathways such as stretch-induced hypertrophic remodelling which enabled analyses of the apoptotic mechanisms.

To determine the role of ERK2 during pressure overload-induced cardiac hypertrophy 1 week of TAC was performed on ERK2\textsuperscript{cko} and ERK2\textsuperscript{ff} mice.

ERK2\textsuperscript{cko} mice demonstrated significantly blunted HW/TL ratio (11% increase, 7.47±0.28mg/mm), compared to their sham group (Figure 3.11). In contrast, in ERK2\textsuperscript{ff} mice the hypertrophic growth increased by 30% following pressure overload (8.87±0.32mg/mm). Consistent with these results, cardiomyocyte cross-sectional areas were smaller in ERK2\textsuperscript{cko}-TAC ventricles (259.91±3.27µm\textsuperscript{2}), in comparison to the greater hypertrophied growth in ERK2\textsuperscript{ff}-TAC mice (298.80±2.33µm\textsuperscript{2}) (Figure 3.11). Analysis of cardiomyocyte cross-sectional areas using FITC-WGA staining confirmed these results (Figure 3.11).

Following 1 week of pressure overload, the transcripts of hypertrophic gene markers ANP and BNP were significantly less expressed in ERK2\textsuperscript{cko} mice in comparison to the ERK2\textsuperscript{ff} TAC group (Figure 3.12). These data were consistent with the histological analyses of hypertrophic growth using H&E and FITC-WGA staining.

Besides cardiac hypertrophy, pressure overload also induced interstitial fibrosis. Picro-Sirius red staining showed 62% less ventricular fibrosis in ERK2\textsuperscript{cko}-TAC
ventricles (1.42±0.42%) compared to the control TAC group (Figure 3.13). Masson’s trichome staining confirmed the results by Picro-Sirius red staining (Figure 3.13). In concordance with the fibrosis staining, fibrosis marker genes *procollagen type 1α2 (Col1a2)* and *procollagen type 3α3 (Col3a3)* were remarkably less upregulated in ERK2<sup>cko</sup>-TAC mice whereas their expression levels were significantly upregulated in ERK2<sup>ff</sup>-TAC mice (Figure 3.14).

The activation of other hypertrophic regulators, including ERK1, ERK5, protein kinase B (PKB), p38 MAPK and JNK was determined. Notably, ERK1 levels were similar in all groups. Meanwhile, the activation of the other hypertrophic regulators was also comparable between the two genotypes (Figure 3.16).

To detect apoptosis, a hallmark for the transition from pathological hypertrophy to heart failure, TUNEL staining assay was performed. After 1 week of TAC, ERK2<sup>cko</sup> ventricles had 38.40% enhanced TUNEL-positive nuclei compared to the control-TAC group (14.06±2.13% versus 8.66±0.41%) (Figure 3.15).

In addition, the role of ERK2 in hypertrophy-induced angiogenesis was analysed. The blood vessel to nucleus ratio was increased in ERK2<sup>ff</sup> ventricles, but not in ERK2<sup>cko</sup> mice (Figure 3.17). In addition, a blood vessel to cardiomyocyte ratio was determined as a single blood vessel might be shared by several neighbouring cardiomyocytes. Therefore, this ratio precisely represented angiogenesis in its natural scenario. The blood vessels to cardiomyocyte ratio were significantly less increased in ventricles of ERK2<sup>cko</sup> mice whereas in ERK2<sup>ff</sup> mice angiogenesis in response to pressure overload was increased (Figure 3.17).

Echocardiographic analysis showed that after 1 week of TAC the FS was similar and ERK2<sup>cko</sup> mice displayed a relatively normal overall cardiac function (Table 3.4).
However, echocardiographic parameters like the increases in PW and IVS (both systolic and diastolic) indicated hypertrophic growth.

TAC induced stretch-induced hypertrophy which activates several different pathways of the MAPK family. Despite the broader potential to stimulate additional pathways, no compensatory changes occurred. That emphasised the crucial role of ERK2 in pressure overload-induced hypertrophy.

Despite the significantly increased cardiomyocyte cross-sectional areas as well as the significantly increased interstitial fibrosis, no difference in FS occurred in both genotypes following TAC. That observation was different from the Iso-induced hypertrophy that showed elevated FS with similar increased cardiomyocyte cell size in ERK2^{ff} ventricles (Iso: 300.71±4.92µm² versus 1 week of TAC: 298.80±2.33µm²). These data might be explained in several ways. The echocardiographic analyses were performed without using a consistent orientation point such as measurement in relation to the occurrence of the aorta within the M-mode analyses. In addition, the time frame might be not suitable for observing echocardiographic changes of the heart following pressure overload. Despite the similar cross-sectional areas in ERK2^{ff} ventricles following 1 week of TAC and 2 weeks of Iso both stimuli activate different pathways which makes a direct comparison difficult. Further analyses following 5 weeks of pressure overload might show elevated levels of FS and therefore confirm the histological data.

Taken together, these results showed that 1 week of pressure overload induced cardiac hypertrophic remodelling in ERK2^{ff} mice. The blunted hypertrophic response in ERK2^{cko} mice was due to the absence of ERK2 in the ventricles because other MAPKs were unable to compensate for the loss of ERK2 during hypertrophic growth. Moreover, in response to 1 week of TAC, ERK2 was required for
cardiomyocyte survival and its absence led to increased apoptosis. In addition, ERK2 influenced pressure overload-induced angiogenesis. ERK2^{cko} mice developed less microvascularisation in their ventricles compared to ERK2^{fl/fl} mice.
Figure 3.11: ERK2<sup>cko</sup> mice demonstrated blunted hypertrophic response to 1 week of TAC.

A. ERK2<sup>cko</sup> mice developed significant less hypertrophy after 1 week of TAC (top panel, scale bar = 5mm), H&E staining and FITC-WGA staining of heart ventricular cross-sections (scale bar = 20µm). B. HW/TL ratios of ERK2<sup>fl</sup> and ERK2<sup>cko</sup> mice were measured following TAC or sham operation. C. Following H&E staining the measurement of left ventricular mean cross-sectional area showed significantly enlarged ERK2<sup>fl</sup> cardiomyocytes compared to the knockout group. D. FITC-WGA staining showed similar results as to H&E staining. Data are presented as means ± SEM (n = 5 to 10). # statistical significance to respective sham group.
Figure 3.12: Expression of hypertrophic gene markers was less upregulated in ERK2\textsuperscript{cko} mice after 1 week of TAC.

Quantitative real-time PCR analyses of the hypertrophic gene markers \textit{ANP} and \textit{BNP} showed significantly less increased re-activation levels in ERK2\textsuperscript{cko} ventricles compared to ERK2\textsuperscript{ff} mice following 1 week of TAC. The data were derived from 5 independent experiments performed in triplicate and were normalised to the \textit{GAPDH} content. Data are presented as means ± SEM (n = 5). # statistical significance to respective sham group.
Figure 3.13: Less interstitial fibrosis was detected after 1 week of TAC in ERK2<sup>cko</sup> mice.

A. Picro-Sirius red staining and Masson’s trichrome staining of cardiac-ventricular cross-sections demonstrated a reduction in interstitial ventricular fibrosis in ERK2<sup>cko</sup> ventricles, whereas ERK2<sup>f/f</sup> mice showed a significantly increased response (arrows indicate areas of fibrosis, scale bar = 50µm). B. Following Picro-Sirius red staining the quantification of the relative area of interstitial fibrosis was expressed as percentage of the interstitial fibrosis area in the microscope views. C. Masson’s trichome staining showed similar values of interstitial fibrosis compared to Picro-Sirius red staining. Data are presented as means ± SEM (n = 5 to 6).
Figure 3.14: Less increased expression of fibrotic gene markers was found in ERK2<sup>cko</sup> mice after 1 week of TAC. Quantitative real-time PCR analyses revealed significantly blunted levels of procollagen type 1α2 (*Col1a2*) and procollagen type 3α3 (*Col3a3*) in ERK2<sup>cko</sup> ventricles compared to the ERK2<sup>f/f</sup>-TAC mice. The data were derived from 5 independent experiments performed in triplicate and were normalised to the *GAPDH* content. Data are presented as means ± SEM (n = 5). # statistical significance to respective sham group.
Figure 3.15: ERK2<sup>cko</sup> mice were more prone to apoptosis after 1 week of TAC. A, ERK2<sup>cko</sup> and ERK2<sup>f/f</sup> showed no apoptosis after 1 week of sham. After 1 week of TAC significantly increased apoptosis in ERK2<sup>cko</sup> LV was detected by TUNEL assay whereas ERK2<sup>f/f</sup> mice showed a significantly reduced apoptosis rate. The graph bar summarises the number of apoptotic nuclei in ERK2<sup>cko</sup> ventricles compared to ERK2<sup>f/f</sup> mice. B, Triple staining was performed: TUNEL: green; DAPI: blue; α-actinin: red (arrows indicate TUNEL positive nuclei; scale bar = 50µm). Data are means ± SEM (n = 5 to 12).
Figure 3.16: Analysis of hypertrophic regulators revealed no influence on reduced hypertrophy in ERK2\textsuperscript{cko} ventricles

Protein extracts from ERK2\textsuperscript{cko} and ERK2\textsuperscript{ff} ventricles after 1 week of TAC or sham operation were subjected to immoblot analyses for total ERK1, PKB, p38 MAPK, JNK and ERK5 expression as well as their phosphorylation levels using specific antibodies. The results suggested that the blunted hypertrophy in ERK2\textsuperscript{cko} mice is specifically reasoned by the absence of ERK2 in the ventricles.
Figure 3.17: Loss of ERK2 in left ventricle influenced hypertrophy-induced angiogenesis

A. Staining of cardiac-ventricular cross-sections: PECAM-1: red (endothelial marker); calveolin-3: green (cardiomyocyte marker); DAPI: blue (scale bar = 20µm)

B. Quantification showed less increase in blood vessel to nucleus ratio in ventricles of ERK2cko mice compared to the control group. The blood vessel to cardiomyocyte ratio, which reflects angiogenesis more precisely, displayed a significant increase in ERK2ff mice compared to the ERK2cko-TAC group. Data are presented as means ± SEM. # statistical significance to respective sham group. n.s.: no statistically significant difference found.
Table 3.4: Echocardiographic parameters of ERK2<sup>ffe</sup> and ERK2<sup>cko</sup> mice following 1 week of TAC

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ERK2<sup>ffe</sup> and ERK2<sup>cko</sup> mice were treated with 1 week of pressure overload (TAC) followed by echocardiographic assessment. Data are means ± SEM (n = 6 to 10 per group). *p<0.05, TAC-treated versus sham-operated mice within the same genotype.
3.6 Long-term hypertrophy in ERK2\textsuperscript{cko} mice sensitized to heart failure

After demonstrating the role of ERK2 in hypertrophic remodelling by 1 week of TAC, long-term pressure overload was necessary to show whether the loss of ERK2 in ventricular cardiomyocytes predisposes mice to heart failure, therefore pressure overload stimulation by TAC was extended to 5 weeks.

After 5 weeks of TAC, ERK2\textsuperscript{cko} mice showed early characteristics of heart failure. Lung weight to tibia length (LW/HW) ratio was significantly higher in ERK2\textsuperscript{cko}-TAC mice, indicating pulmonary edema due to contractile insufficiency (Figure 3.18). The HW/TL ratio in ERK2\textsuperscript{cko} mice was similar compared to the ERK2\textsuperscript{ff}-TAC mice (Figure 3.18).

The cardiomyocyte cross-sectional areas were significantly less increased in ERK2\textsuperscript{cko} mice (319.27±6.89µm\textsuperscript{2}) compared to ERK2\textsuperscript{ff} mice (364.89±7.99µm\textsuperscript{2}) (Figure 3.18).

Consistently, ERK2\textsuperscript{ff}-TAC mice revealed increased re-activation of ANP and BNP expression levels (Figure 3.19). The blunted hypertrophic growth of cardiomyocytes in LV from ERK2\textsuperscript{cko} mice was not reflected by the expression of ANP and BNP. Both expression levels of ANP and BNP were similar to the ERK2\textsuperscript{ff} group following 5 weeks of TAC (Figure 3.19).

In addition, ERK2\textsuperscript{cko} mice showed significant fibrosis levels after 5 weeks of TAC (3.70±1.32%) (Figure 3.20). Long-term pressure overload led to similar interstitial fibrosis in ERK2\textsuperscript{ff} mice (3.14±0.95%) (Figure 3.20).

ERK2\textsuperscript{cko} mice showed an increased apoptosis after long-term pressure overload (13.89±2.55%) whereas ERK2\textsuperscript{ff} ventricles exhibited less apoptotic left ventricular cardiomyocytes (6.52±1.39%) (Figure 3.21).
The differences in cardiac structure between both genotypes were reflected by the significant difference of echocardiographic parameters (Table 3.5). ERK2<sup>cko</sup> mice showed substantially reduced fractional shortening whereas in ERK2<sup>f/f</sup> mice FS was significantly increased (30.29±2.92% versus 43.64±3.77%, respectively). In addition, following 5 weeks of TAC, ERK2<sup>cko</sup> mice showed decreased left ventricular posterior wall thickness (diastolic: 0.9±0.04mm; systolic: 1.10±0.12mm, respectively) compared to the ERK2<sup>f/f</sup>-TAC group (diastolic: 1.57±0.20mm; systolic: 1.80±0.20mm).

In comparison to 1 week of TAC, 5 weeks of pressure overload led to hypertrophic remodelling with early signs of heart failure, shown as significantly increased LW/TL ratio, as fluid accumulation occurred in the lungs due to elevated capillary hydrostatic pressure. In addition, the levels of ANP and BNP were upregulated compared to 1 week of TAC which correlated with the heart failure state. Following 5 weeks of TAC, ERK2<sup>cko</sup> mice showed increased levels of interstitial fibrosis that could be a result of the continuous significantly increased apoptosis levels observed at 1 week and 5 week of pressure overload.

Further analyses of angiogenesis might show differences in blood vessel development in ERK2<sup>cko</sup> mice in comparison to 1 week pressure overload leading to ischemic heart tissue. These data were confirmed by the echocardiographic analyses showing decreased FS in ERK2<sup>cko</sup> mice after 5 weeks of TAC compared to their sham group and therefore showed signs of heart failure.

Thus the absence of ERK2 initially led to development of blunted hypertrophic remodelling with increased apoptotic levels following 1 week of TAC. However, ERK2 was crucial in preventing the transition from compensated hypertrophy to heart failure during 5 week of pressure overload. Data analysing TAC of a longer...
time period might show further severe heart failure signs due to the absence of ERK2.

To summarise, these data demonstrated the functional importance of ERK2 in protecting the heart during long-term pressure overload. Loss of ERK2 led to interstitial fibrosis and increased apoptosis levels. Also, ERK2^{cko} mice showed decreased cardiac function (shown as FS) and were more vulnerable with early signs of heart failure compared to the TAC-treated control group.
Figure 3.18: ERK2^cko mice were more prone to heart failure compared to the control group after prolonged pressure overload.

A. After 5 weeks of TAC ERK2^cko mice showed signs of exaggerated cardiac dysfunction represented by increased LW/TL ratios compared to the control TAC group. B. Significantly less increased hypertrophic growth of ERK2^cko hearts compared to ERK2^ff mice after 5 weeks of TAC treatment (top panel, scale bar = 5mm). H&E staining of heart cross-sections (bottom panel, scale bar = 20µm). C, 5 weeks of TAC led to similar HW/TL ratios in both genotypes D. The quantification of the LV mean cross-sectional areas of cardiomyocytes demonstrated a significant increase in hypertrophic growth in ERK2^ff mice compared to the ERK2^cko-TAC group. Data are presented as means ± SEM (n = 9 to 10). # statistical significance to respective sham group. n.s.: no statistically significant difference found.
Figure 3.19: Increased expression of hypertrophic gene markers was found in both genotypes after 5 weeks of TAC

Quantitative real-time PCR analyses of gene markers associated with hypertrophy, ANP and BNP, demonstrated similar re-activation levels in both genotypes. The data were derived from 6 independent experiments performed in triplicate and were normalised to the GAPDH content. Data are presented as means ± SEM (n = 6). # statistical significance to respective sham group. n.s.: no statistically significant difference found.
**Figure 3.20:** ERK2\(^{ff}\) and ERK2\(^{cko}\) mice revealed similar fibrosis levels following 5 weeks of pressure overload.

**A,** Picro-Sirius red staining (top panel) and Masson’s trichome staining (bottom panel) of cardiomyocyte cross-sections showed similar levels of interstitial ventricular fibrosis in ERK2\(^{cko}\) mice compared to ERK2\(^{ff}\) mice (arrows point to fibrotic areas, scale bar = 50µm). **B,** Quantification of the relative area of fibrosis was expressed as percentage of the fibrosis area in the microscope views. Data are presented as means ± SEM (n = 9 to 10). n.s.: no statistically significant difference found.
Figure 3.21: ERK2\textsuperscript{cko} mice were more vulnerable due to a high apoptosis rates.

A. No apoptosis was found in either genotype following 5 weeks of sham operation. Increased apoptosis level in ERK2\textsuperscript{cko} LV after 5 weeks of TAC was detected by TUNEL assay. In contrast, ERK2\textsuperscript{ff} mice showed a significantly enhanced apoptosis. The graph bars summarise the percentage of apoptotic nuclei in ERK2\textsuperscript{cko} hearts compared to ERK2\textsuperscript{ff} mice after 5 weeks of pressure overload. B. Triple staining was performed: TUNEL: green; DAPI: blue; α-actinin: red (arrows indicate TUNEL positive nuclei; scale bar = 50µm). Data are means ± SEM (n = 10 to 12).
Table 3.5: Echocardiographic parameters of mice following 5 weeks of TAC

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</table>

ERK<sup>f/f</sup> and ERK<sup>cko</sup> mice were treated with 5 weeks of TAC followed by echocardiographic assessment. Data are means ± SEM (n = 6 to 10 per group). *p<0.05, TAC-treated versus sham-treated mice within the same genotype; #p<0.05, ERK<sup>cko</sup> versus ERK<sup>f/f</sup> after TAC treatment.
3.7 ERK2\textsuperscript{cko} and ERK2\textsuperscript{ff} mice showed similar physiological cardiac hypertrophy in response to swimming exercise

So far the influence of ERK2 was analysed in pathological hypertrophy induced by Iso, AngII and pressure overload. Next, the role of ERK2 in physiological hypertrophy was investigated.

To induce physiological hypertrophy ERK2\textsuperscript{cko} and ERK2\textsuperscript{ff} mice were subjected to swimming exercise for 4 weeks.

Both swimming groups developed no significant difference in HW/TL ratios (ERK2\textsuperscript{cko}: 6.97±0.44mg/mm; ERK2\textsuperscript{ff}: 6.59±0.26mg/mm) compared to their respective resting mice (7.00±0.41mg/mm [ERK2\textsuperscript{cko}] and 7.19±0.28mg/mm [ERK2\textsuperscript{ff}], respectively) (Figure 3.22). Although the HW/TL ratio showed no difference, further histological analyses at the cellular level revealed hypertrophic growth with an increase of myocardial cross-sectional areas in both swimming groups (Figure 3.22). ERK2\textsuperscript{cko} mice showed a similar cardiomyocyte cell size (267.22±4.46µm\textsuperscript{2}) to the ERK2\textsuperscript{ff}-swimming group (264.78±5.01µm\textsuperscript{2}).

Consistently, in ERK2\textsuperscript{cko} mice swimming exercise did not result in increased expression levels of ANP and BNP, biomarkers indicating pathological hypertrophy (Figure 3.23). In both groups the expression levels of ANP and BNP were not different (Figure 3.23). Furthermore, the swimming exercise did not induce development of interstitial ventricular fibrosis (Figure 3.24).

Echocardiographic assessment demonstrated similar cardiac function after swimming (Table 3.6). The heart structure remained normal in both genotypes.

Swimming induced physiological hypertrophic remodelling and therefore activated different signalling pathways. In comparison to the previous hypertrophic stimuli like Iso, AngII and pressure overload; swimming resulted in preserved cardiac
function as the levels of ANP and BNP, markers of pathological hypertrophy, remained unchanged which suggested physiological hypertrophy. In contrast to 1 week and 5 weeks of TAC, no interstitial fibrosis developed following 4 weeks of swimming. Therefore these data showed the main differences between pathological and physiological hypertrophy in development of fibrosis and expression of pathological hypertrophic signalling proteins and thereby leading to a preserved cardiac function following swimming exercise. In addition, further analyses of apoptosis following swimming might reveal no elevated levels compared to the rest mice. Despite the differences, swimming induced hypertrophic growth in both genotypes shown as increased cardiomyocyte cross-sectional areas. These levels were significantly increased in comparison to their rest groups, however, minimal compared to the hypertrophic growth following 5 weeks of TAC (swimming (ERK2^{f/f}): 264.78±5.01µm^2 versus 5 weeks of TAC (ERK2^{f/f}): 364±7.99µm^2). Therefore these changes, induced by swimming, might be too small for showing hypertrophy with echocardiography as an increased FS.

Taken together, swimming exercise induced hypertrophy which was demonstrated at the cellular level. However, ERK2 was unlikely to be involved in development of physiological hypertrophy as ERK2^{cko}-swimming mice showed similar hypertrophic growth and cardiac function compared to the ERK2^{f/f}-swimming group. No fibrosis or any other signs of pathological hypertrophy, such as increased ANP and BNP expression was developed by either genotype.
Figure 3.22: ERK2 was unlikely required for swimming-induced cardiac hypertrophy.

A. After 4 weeks of swimming exercise ERK2^{cko} mice exhibited similar physiological hypertrophy compared to the control group (top panel, scale bar = 5mm). Histological analysis of ventricular cardiomyocyte cross-sections using H&E staining (bottom panel, scale bar = 20µm). B. After swimming ERK2^{cko} and ERK2^{0/0} mice showed no increase in HW/TL ratio. C. Following swimming exercise the left ventricular mean cross-sectional areas of cardiomyocytes in ERK2^{cko} and ERK2^{0/0} ventricles were enlarged in comparison to their resting groups. Data are presented as means ± SEM (n = 4 to 7). # statistical significance to respective rest group. n.s.: no statistically significant difference found.
Figure 3.23: Swimming exercise did not re-activate fetal gene expression

Quantitative real-time PCR analyses of the hypertrophy associated biomarkers ANP and BNP demonstrated no increase of its expression levels in ERK2<sup>cko</sup> and ERK2<sup>f/f</sup> mice following 4 weeks of swimming exercise in comparison to the resting mice. The data were derived from 4 independent experiments performed in triplicate and were normalised to the GAPDH content. Data are presented as means ± SEM (n = 4). n.s.: no statistically significant difference found.
Figure 3.24: Swimming did not induce interstitial fibrosis in both genotypes

A. Picro-Sirius red staining of heart ventricle cross-sections demonstrated no development of interstitial ventricular fibrosis in ERK2
c and ERK2
 mice following 4 weeks of swimming exercise (scale bar = 50µm). Representative image of 4 to 6 animals per group.
Table 3.6: Echocardiographic characteristics of mice following swimming exercise

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ERK2^{ff} and ERK2^{cko} mice were exposed to swimming exercise for 4 weeks, followed by echocardiographic assessment. Data are means ± SEM (n = 6 to 7 per group).
3.8 ERK2 induced hypertrophy in vitro

Previously in vivo experiments demonstrated the role of ERK2 in hypertrophy following treatment with different pathological hypertrophic stimuli. Next, in vitro experiments were designed to establish the direct role of ERK2 on hypertrophic growth at the cellular level.

To inactivate ERK2, NRCMs were infected with the adenovirus encoding Ad5-constitutive cytomegalovirus (CMV)-ERK2 DN (Ad-DN-ERK2) which contained a recombinant dominant negative mutant of rat ERK2 protein.

NRCMs were infected with either Ad-DN-ERK2 or a control adenovirus (Ad-LacZ) for 24 hours prior to the PE treatment. Following 48 hours PE treatment, the hypertrophic growth was analysed by checking NRCM cell sizes. Ad-DN-ERK2 infected NRCMs did not significantly increase their cell size whereas NRCMs infected with Ad-LacZ showed significantly increased hypertrophic growth (Figure 3.25).

To further investigate the hypertrophic response, re-activation levels of ANP and BNP were analysed. Following 48 hours PE stimulation, Ad-DN-ERK2 infected NRCMs revealed no increase in ANP expression compared to its vehicle group (33.10±1.41% and 27.41±2.32%, respectively) (Figure 3.25). In contrast, the ANP expression was increased in Ad-LacZ infected NRCMs (43.87±2.97%).

To analyse the BNP expression level its promoter activity was detected using BNP luciferase reporter assay following PE stimulation for 48 hours. Ad-DN-ERK2 infection of NRCMs led to a similar BNP promoter activity compared to the vehicle control group, whereas NRCMs infected with Ad-LacZ showed a significant increase in BNP promoter activity in response to PE stimulation (Figure 3.25).
Taken together, *in vitro* experiments showed that ERK2 plays a crucial role in hypertrophic growth. Ad-DN-ERK2 infected NRCMs displayed no significant hypertrophic growth as well as no re-activation of ANP and *BNP*. In contrast, NRCMs infected with the control virus showed an increase in cell size following hypertrophic stimulation with PE. That hypertrophic growth was accompanied by increased ANP and *BNP* re-activation levels.
Figure 3.25: Dominant negative ERK2 adenovirus led to blunted hypertrophic growth in NRCMs

A, Cardiomyocyte cell growth was not significantly increased in NRCMs infected with Ad-DN-ERK2 following PE administration for 48 hours. In contrast, NRCMs infected with control virus showed significant hypertrophic growth B, The activity of the BNP promoter in response to PE stimulation was examined using a BNP-luciferase reporter assay. Ad-DN-ERK2 infected NRCMs displayed blunted BNP promoter activity compared to the significantly increased promoter activity in the NRCMs infected with Ad-LacZ after PE treatment. C, ANP was significantly less expressed in Ad-DN-ERK2 infected cells following PE stimulation compared to the NRCMs infected with Ad-LacZ. NRCMs were stained using triple staining (ANP: red, α-actinin: green, DAPI: blue; arrows indicate ANP; scale bar = 50µm). D, Quantification of ANP expression in NRCMs revealed significantly lower ANP levels in Ad-DN-ERK2 infected NRCMs compared to NRCMs infected with LacZ following PE treatment. Data is presented by the graph. Data are means ± SEM (n = 3 to 4). # statistical significance to the vehicle group with the same adenovirus treatment. n.s.: no statistically significant difference found.
3.9 ERK2 protected ventricular cardiomyocytes from apoptosis in vitro

In addition to the analysis of hypertrophy, the role of ERK2 in apoptosis was also investigated in vitro.

NRCMs were infected with either Ad-DN-ERK2 or Ad-LacZ for 24 hours followed by exposure to the pro-apoptotic agent hydrogen peroxide (100µM H₂O₂) for 4 or 24 hours.

Following 4 hours H₂O₂ treatment, NRCMs infected with Ad-DN-ERK2 showed significantly increased TUNEL-positive apoptotic cell nuclei compared to the Ad-LacZ-infected NRCMs (17.06±2.17% versus 5.79±2.90%) (Figure 3.26). In addition, the NRCMs infected with the control virus revealed statistically not significant apoptosis levels compared to the vehicle group with the same adenovirus treatment (Figure 3.26).

Next, the pro-apoptotic stimulation was increased to 24 hours. Consistent with the results following 4 hours H₂O₂ treatment, Ad-DN-ERK2 infected NRCMs demonstrated significantly increased apoptosis compared to infected Ad-LacZ cells (28.41±2.04% versus 17.51±3.16%, respectively) (Figure 3.26).

Seeking further confirmation, immunoblot analysis of caspase 3, which is activated in apoptotic ventricular cardiomyocytes, was performed. Cardiomyocytes were infected with Ad-DN-ERK2 or control virus and were subsequently treated with 100µM H₂O₂ for 24 hours. Consistent with the previous results, cardiomyocytes infected with Ad-DN-ERK2 showed elevated cleaved caspase 3 protein levels in response to pro-apoptotic stimulation compared to the Ad-LacZ-infected control group (Figure 3.26).

Taken together, increased apoptosis levels were detected in Ad-DN-ERK2 infected NRCMs suggesting a crucial protective role of ERK2 in cardiomyocytes.
**Figure 3.26: NRCMs containing dominant negative ERK2 were more prone to apoptosis**

A. Increased apoptosis levels were detected by TUNEL assay in treated NRCMs (infected with Ad-DN-ERK2) following 4 hours H$_2$O$_2$ (100µM) and 24 hours H$_2$O$_2$ (100µM). The graph bars summarise the number of apoptotic nuclei. B, In cardiomyocytes caspase 3 was activated following treatment with 100µM H$_2$O$_2$ for 24 hours (left panel). Tubulin expression was used as loading control. Immunoblot analyses revealed increased caspase 3 protein levels in NRCMs infected with Ad-DN-ERK2. The ratio of the caspase 3 to tubulin is represented by the graphs (right panel). Data are means ± SEM (n = 3 to 4). # statistical significance to the vehicle group with the same adenovirus treatment. n.s.: no statistically significant difference found.
3.10 Summery: role of ERK2 in vitro

The usage of the dominant negative ERK2 adenovirus enabled a simulation of the ERK2\textsuperscript{cko} mouse model at cellular level. Following PE stimulation, the dominant negative expression of ERK2 led to a similar cell size of NRCM as the vehicle group; whereas in vivo the hypertrophic growth was blunted following 1 week of TAC. Therefore hypertrophic stimulation in vitro resulted in a more distinct response in cardiomyocytes in comparison to pressure overload-induced hypertrophy in vivo. At cellular level experiments enabled a more direct approach of inducing hypertrophy as cardiomyocytes were specifically isolated and directly stimulated. In addition, the cardiomyocytes were cultivated within a controlled environment using specific cell culture conditions. 

In vitro apoptosis was induced by exposure of cardiomyocytes to hydrogen peroxide. In contrast to the in vivo mouse model, the conditions for the cardiomyocytes in cell culture were controlled by using a specific concentration of the pro-apoptotic agent and by regulating the exposure time. In vitro cardiomyocytes were more prone to apoptosis compared to the treated control NRCMs. These data confirmed the in vivo results following 1 week and 5 weeks of pressure overload. In addition, these data directly demonstrated the pro-survival role of ERK2 in apoptosis. Hydrogen peroxide treatment could be used as a positive control in experiments further analysing the role of ERK2 in apoptosis.
3.11 Overview of results

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ERK2<sup>fl</sup>: control mice; ERK2<sup>cko</sup>: mice with ventricular cardiomyocyte-specific knockout of ERK2; Ad: Adenovirus; NRCM: neonatal rat cardiomyocytes; HW/TL: heart weight/tibia length; LW/TL: lung weight/tibia length; FS: Fraction Shortening; ↑/↓: significant increase compared to respective control group; ↑↑/↓↓: statistical significance to respective control group and statistical significance between treated genotypes; ←: no significant different to respective control group; -: no data
CHAPTER 4
Chapter 4 Discussion

The mammalian heart is a dynamic organ and adapts to increased workload by undergoing hypertrophy\textsuperscript{6, 87, 215, 216}. Cardiac hypertrophy is regulated by different signalling complexes, including the MAPK pathways\textsuperscript{5, 6, 215}. These signalling complexes regulate transcription factors, such as NFAT, c-Myc and MEF2, which are collectively responsible for hypertrophic gene expression\textsuperscript{5, 6, 76, 215}.

In the present study, the biological role of ERK2, a member of the classical MAPKs, has been investigated using the cardiomyocyte-specific knockout mouse model. ERK2 is crucial for hypertrophic remodelling and cardiomyocyte survival; however it is unlikely to be involved in the regulation of physiological hypertrophy. My data also demonstrate that ERK2 protects against the transition from compensatory hypertrophy to maladaptive hypertrophic remodelling.

4.1 \textit{In vitro} and \textit{in vivo} models for ERK2 function analysis

The cultivation of cells allows important analysis with substrates incubated in a precisely timed manner. For \textit{in vitro} studies analysing the role of ERK2, short-term primary culture of neonatal rat cardiomyocytes (NRCM) is widely used\textsuperscript{125–127, 135}. However, it is difficult to extrapolate the conclusions from neonatal cardiomyocytes to the heart of a living organism. Cardiac hypertrophy is a whole organ phenomenon associated with complex remodelling which is characterised by cell growth, angiogenesis and changes of the extracellular matrix\textsuperscript{219}. In contrast, \textit{in vitro} experiments in cultured cardiomyocytes are performed under highly controlled settings that are not exactly the same as the \textit{in vivo} environment. For example cardiomyocytes in cell culture are deficient of the complex 3-dimensional extracellular matrix as well as the functional load which influences cardiac growth.
Although cardiomyocytes in cell culture may allow the analysis of a specific signalling pathway within an isolated environment, their application is limited for understanding whole organ events. Therefore in vivo studies using animals are required to understand the biological functions of signalling cascades during the development of hypertrophy.

There have been a number of genetically-modified mouse models used for studying the biological function of ERK1/2 signalling pathway during hypertrophy, including transgenic mice with overexpression of a specific gene and the gene-targeting knockout mice. Transgenic mice are generated through the process of transgenesis which involves the injection of an exogenous gene (the transgene) into the pronucleus of fertilised oocytes. After a limited number of cell divisions, the embryos are implanted into a pseudo-pregnant female and the resulting offspring are screened for the presence of the transgene. In the transgenic offspring, the gene of interest is inserted into the genome and expressed ubiquitously. For example transgenic mice overexpressing MEK1, a direct activator of ERK1/2, demonstrated hypertrophy, enhanced cardiac function and blunted apoptosis whereas ERK2 heterozygous gene-targeted (ERK2+/−) mice did not show reduced cardiac hypertrophy in response to pressure overload.

In particular, the transgenic mice under the cardiac-specific promoter α-MHC have been successfully used to achieve cardiac-specific expression of the transgene and thereby pathways that regulate hypertrophy have been identified. However, the high level of these transgenes (beyond the physiological level) may cause toxic effects, which may confound the observed phenotype in these transgenic mice. In addition, the line-to-line expression of the transgene leads to variation in copy
number and the genomic site of the transgene insertion which can cause phenotype difference. For example several transgenic mice with overexpression of active MEK1 have been generated showing 2 to 40 copies of transgene under the control of cardiac-specific α-MHC\textsuperscript{130}. The heart weight to body weight (HW/BW) ratios of the different mouse models increased with the copy-number\textsuperscript{130}.

Another model used for studying gene functions is the gene knockout mouse model generated by gene targeting. In gene targeting, embryonic stem (ES) cells are modified by insertion of an exogenous sequence which contains the desired mutation. The ES cells containing the targeting construct are injected into early blastocyst and the resulting embryo is subsequently implanted into a pseudo-pregnant female mouse to generate the knockout mouse in which the endogenous DNA is disrupted or replaced by an exogenous DNA sequence\textsuperscript{220}.

This conventional knockout is not as random and uncontrolled as transgenesis but it affects the target genes within all tissues. Therefore, the knockout of the target gene in non-cardiomyocytes may contribute to changes in the observed phenotype\textsuperscript{218, 222}.

For example, conventional knockout of ERK2 results in embryonic lethality due to disruption of ERK2 in non-cardiomyocytes during embryonic development\textsuperscript{97}.

In order to analyse the role of ERK2 specifically in the cardiomyocytes, the Cre-loxP system was used in this study to generate mice with cardiomyocyte-specific knockout (ERK2\textsuperscript{cko}). The characterisation of these ERK2\textsuperscript{cko} mice showed that the deletion appeared specifically in cardiomyocytes without affecting the expression of ERK2 in other tissues. Furthermore, the disruption of ERK2 did not affect the expression of other kinases within the MAPK pathway, including MEK1, MEK2, ERK1, ERK5, JNK and p38 MAPK. Therefore my study on this cardiomyocyte-
specific knockout mouse-line is more selective for the description of the role of ERK2 in cardiac hypertrophic remodelling.

In this study, MLC2v-Cre mice were used to generate ERK2\(^{cko}\) mice. The MLC2v-Cre mouse line was generated in Dr. KR Chien’s laboratory, where these mice survived an uneventful life time with normal reproduction and cardiac function\(^{207}\). In response to the pressure overload induced by TAC, MLC2v-Cre mice demonstrated cardiac function and hypertrophic response identical to wild type mice\(^{207}\). Therefore, this characterisation of MLC2v-Cre mice clearly suggests that the phenotype in ERK2\(^{cko}\) mice is due to the deletion of ERK2.

4.2 ERK2 versus ERK1

Various stimuli, such as growth factors and neurotransmitters interact with their respective membrane receptors. Under these circumstances, the ERK1/2 signalling pathway bridges the gap between the receptors and their intracellular targets in distinct cellular compartments\(^{225}\).

ERK1 and ERK2 have a similar molecular weight (44kDa and 42kDa, respectively); are ubiquitously expressed in different tissues, such as heart and brain; and they share an amino acid similarity of 84%\(^{86, 87}\). Both kinases are activated by the classical MAPK cascade, including the MAKKK-Raf that phosphorylate MEK1 and MEK2, which subsequently activate ERK1 and ERK2 by phosphorylation within the conserved Thr-Glu-Tyr motif in their activation loops\(^{87, 106}\). Despite their similarities, ERK1 and ERK2 seem to have distinct functions which are profoundly shown by their conventional knockout mice models. Deficiency of ERK1 leads to development of fertile, viable ERK1\(^{-/-}\) mice with normal size\(^{95}\). The expression level of ERK2 is not affected in these ERK1\(^{-/-}\) mice\(^{94, 95}\). In contrast, ERK2 knockout mice die during
embryogenesis between embryonic day 6.5 to 11.5 due to secondary effects, such as a thin heart wall and growth retardation\(^97-99\). Apart from this obvious effect on viability, additional differences between ERK1 and ERK2 have also been observed. Several differences have been found in ERK1/2 regulation of cell proliferation\(^100, 224-227\). For example, silencing of ERK2 markedly reduces the cell proliferation in fibroblasts whereas ERK1 silencing does not affect this process\(^226\). However, another recent study shows that silencing of either ERK1 or ERK2 in fibroblasts reduces cell proliferation depending on their expression levels whereas their combined loss leads to complete cell arrest\(^228\).

The specific roles of ERK1 and ERK2 in cardiac hypertrophy remain an unexplored domain. Most studies have not differentiated between these two kinases during hypertrophy\(^128-130, 133, 134\). However, Lips et al. concentrated on the specific role of ERK2, as it contributes 70% to the total ERK1/2 protein content in myocardium. In the study by Lips et al., the use of ERK2\(^{+/−}\) mice has demonstrated no change in cardiac hypertrophy in response to pressure overload\(^133\). Of note, 50% expression of ERK2 still remains in these mice that would be responsible for the unchanged phenotype in hypertrophy.

In my study, a cardiomyocyte-specific ERK2 knockout mouse model was used. Although, quantitative real-time PCR and immunoblot analysis showed a remaining ventricular ERK2 expression, it can be attributed to non-cardiomyocyte population of the heart. In addition, the expression levels of ERK1 were identical to the control group (ERK2\(^{ff}\) mice). Therefore, this ERK2\(^{cko}\) mouse model uniquely portrayed the role of ERK2 in cardiac hypertrophy independent of ERK1 influence. In addition, immunostaining of cardiomyocytes prepared from the ERK2\(^{cko}\) heart using a specific ERK2 antibody can provide additional confirmation of ERK2 deletion.
4.3 Role of ERK2 in regulating cardiac hypertrophy

ERK2 is known to be important in many cellular events, such as proliferation, differentiation and cell survival in response to a wide variety of stimulation, including growth factors, cytokines and osmotic shock\textsuperscript{76,83,103}. In humans, mutations causing augmented ERK1/2 activity have been demonstrated in the protein-tyrosine-phosphates-non-receptor-type-11 (PNPN11) gene, which encodes the protein tyrosine phosphatase SHP2 that has primarily positive roles in signal transduction, particularly for ERK1/2 signalling. The increased ERK1/2 activity from this mutation causes cardiovascular defects in Noonan syndrome, where 20\% of patients develop hypertrophic cardiomyopathy\textsuperscript{228,229}. In addition, several other developmental disorders with ERK1/2 hyperactivation have been described, such as the LEOPARD syndrome and Costello syndrome that suggest aberrant ERK1/2 activation also contributes to hypertrophic disorders of any sort\textsuperscript{120,229,230}.

The role of ERK1/2 in hypertrophy has been investigated in several \textit{in vivo} studies with conflicting results\textsuperscript{130–135}. To clearly describe the role of ERK2 in hypertrophic remodelling, cardiomyocyte-specific ERK2 knockout mice were challenged in my study with pathological and physiological stresses.

4.3.1 Role of ERK2 in pathological hypertrophic remodelling

Pathological hypertrophy was induced by pressure overload (transverse aortic constriction [TAC]) and micro-osmotic pump infusion of Isoproterenol (Iso) or AngiotensinII (AngII); each of which is known to cause ERK1/2 activation\textsuperscript{208,231,232}. In this study, 1 week of TAC caused significantly reduced hypertrophy and fibrosis in ERK2\(^{cko}\) mice, in comparison to the ERK2\(^{f/f}\) mice. Moreover, the absence of ERK2 led to increased apoptosis and less microvascularisation.
A previous study assessing the role of ERK1/2 in hypertrophy have used a mouse model, in which the dual-specificity phosphatase 6 (DUSP6) is overexpressed to inhibit ERK1/2 phosphorylation. After pressure overload these mice develop hypertrophy despite the absence of phosphorylated ERK1/2. However, phosphatases like DUSP6 can influence other unknown protein kinases in addition to ERK1/2, while analysis of ERK2<sup>cko</sup> mice only demonstrates the direct effect of ERK2. The function of ERK2 on cardiac hypertrophy can be exerted through ERK1/2 activation of substrates, such as transcription factors GATA4, Elk-1 and c-Myc, critical regulators of cardiac hypertrophy.

Furthermore, the role of ERK2 in hypertrophic remodelling was analysed at the cellular level. The adenovirus-mediated inactivation of ERK2 was used for its known efficiency. This allowed precise measurement of hypertrophic effect of ERK2 in the entire population of NRCMs. NRCMs infected with Ad-DN-ERK2 showed a decrease in cell size following hypertrophic stimulation and was accompanied with reduced re-activation levels of ANP and BNP compared to the control group infected with Ad-LacZ. This further indicates the crucial role of ERK2 in hypertrophic growth. Consistently, a previous study has reported that adenovirus-mediated overexpression of constitutively active MEK1 induces ANP mRNA expression and results in an increase in NRCMs cell size, while adenovirus-mediated overexpression of dominant negative MEK1 inhibits PE- and Iso-induced ERK1/2 activation and downregulates ANP mRNA expression. Moreover, these in vitro results confirm the observations following treatment with 1 week of TAC in ERK2<sup>cko</sup> mice.

A recent study investigating the role of ERK1/2 report preferentially eccentric hypertrophy in ERK1<sup>−/−</sup> ERK2<sup>fl/fl-Cre</sup> mice. However, another study in PTPN11
mutant mice has demonstrated results suggesting otherwise\textsuperscript{240}. The ERK1\textsuperscript{-/-} ERK2\textsuperscript{f/f-Cre} mouse model used by Kehat and colleagues is different from the one used in my study, as it contains a deletion of both ERK2 and ERK1 which results in generation of spontaneous hypertrophy without external stimuli and ultimately causes premature death at 2 months of age onwards.

In my study, 1 week of TAC ERK2\textsuperscript{cko} mice resulted in less fibrosis than in the control group. AngII and TGF-\(\beta\) are important factors for regulating cardiac fibrosis\textsuperscript{57, 239, 240}. The ERK1/2 pathway has been shown to negatively regulate AngII via downregulation of angiotensin converting enzyme 2 (ACE2)\textsuperscript{243}. In addition, ERK1/2 signalling regulates connective tissue growth factor (ctgf), which itself mediates the stimulatory effects of TGF-\(\beta\) on proliferation and matrix production by fibroblasts\textsuperscript{242, 243}. That evidence suggests that ERK1/2 regulates pro-fibrotic signalling. Moreover, ERK5, another member of the ERK family, has also been shown to regulate hypertrophic growth and fibrosis by activation of myocyte enhancer factor-2 D (MEF2D)\textsuperscript{162, 244}. MEF2D knockout mice show less response in heart growth after pressure overload. In addition, the induction of ctgf is compromised in MEF2D knockout\textsuperscript{246}. Consistently, the MEF2-dependent expression of ctf and foetal genes (ANP and BNP) has been demonstrated to be significantly depressed in mice with cardiac ERK5 knockout\textsuperscript{163}.

In my studies, ERK2\textsuperscript{cko} mice demonstrated increased apoptosis following 1 week of TAC. Furthermore, in cultured neonatal rat cardiomyocyte inhibition of ERK2 activity led to increased apoptosis following treatment with a pro-apoptotic agent. This is in line with \textit{in vivo} studies reporting that DUSP6 overexpression leads to higher apoptotic cell death rates whereas transgenic mice overexpressing MEK1 are resistant to apoptosis\textsuperscript{129, 133}. Therefore, my study has suggested a cardioprotective
role of ERK2 in response to pressure overload-induced stress. ERK1/2, as a pro-survival regulator, has been demonstrated to suppress the apoptotic pathway by interacting with caspase 9 and inhibiting the activation of the pro-apoptotic Bid and caspase 8142,143. Furthermore, the role of ERK2 was analysed in angiogenesis following 1 week of TAC in my project. Cardiac hypertrophy is normally accompanied by angiogenesis to supply the enlarged cardiomyocytes with nutrients247. Physiological hypertrophic growth is associated with preserved contractile heart function and enhanced angiogenesis while persistent pathological stress triggers an imbalance between cardiomyocyte growth and the capillary number245,246.

In my study, ERK2cko mice demonstrated that cardiomyocyte-expressed ERK2 has an impact on angiogenesis during TAC-induced hypertrophy. Initiation of angiogenesis with fibroblast growth factor or vascular endothelial growth factor is known to activate ERK1/2 in endothelia cells249–251. Therefore future studies using ERK2 endothelial cell knockout models may provide more insights into the role of ERK2 in angiogenesis.

Next, in this study the role of ERK2 in pathological hypertrophic remodelling caused by β-adrenergic stimulation was analysed. While the increase in the heart weight to tibia length (HW/TL) ratio was similar following Iso treatment, ventricular cross-sectional analysis revealed blunted hypertrophic growth in the knockouts compared to the ERK2f/f mice. In addition, ERK2cko mice showed less increased expression of the foetal genes ANP and BNP compared to the control group. Echocardiographic assessment demonstrated less increased contractility in the knockout mice. Taken together, these data suggested that ERK2 might be involved in β-adrenergic-stimulated hypertrophy.
Moreover, ERK2\textsuperscript{cko} mice were treated with AngII to analyse the role of ERK2 in cardiac hypertrophy induced by the renin-angiotensin pathway, as ERK1/2 activation though AngII has been previously reported\textsuperscript{250, 251}. ERK2\textsuperscript{cko} mice demonstrated a similar cardiac hypertrophy compared to ERK2/\textsuperscript{+/f} mice in our experimental setting. Further investigation using higher dosage and/or increased duration of AngII stimulation is required to analyse the role of ERK2 further.

AngII did not induce interstitial fibrosis at the dosage used whereas Iso resulted in minimal development of interstitial fibrosis. It could be explained that Picro-Sirius red staining is not sensitive enough to detect small amount of collagen deposition. Therefore analysis using the Hydroxoproline method would be an additional approach which has been proven to be highly sensitive\textsuperscript{254}.

4.3.2 ERK2 prevents the transition from hypertrophy to heart failure

The important role of ERK2 in preventing heart failure has been suggested by a study analysing left ventricular assist device (LVAD)-supported human failing hearts that has shown dramatic inactivation of ERK1/2\textsuperscript{255}. In my study, ERK2\textsuperscript{cko} mice demonstrated early signs of heart failure indicated by increased lung weight to tibia length weight (LW/TL) and impaired contractility after 5 weeks of TAC.

It is known that increased apoptosis and fibrosis can lead to cardiac dysfunction\textsuperscript{254}. In concordance, my results showed a consistently significant increase in cardiomyocyte apoptosis in ERK2\textsuperscript{cko} mice compared to the control group. The high apoptosis levels in ERK2\textsuperscript{cko} mice were accompanied by a progressive increase in interstitial fibrosis. In addition, the expression levels of ANP and BNP were progressively increased in the ERK2\textsuperscript{cko} mice from 1 week to 5 weeks of TAC; suggesting progression to heart failure\textsuperscript{255, 256}.
The transition from pathological hypertrophy to heart failure has also been suggested by some studies to be attributed by impaired angiogenesis\(^{248}\). In consistent, we observed blunted angiogenesis in the ERK2\(^{\text{cko}}\) following 1 week of TAC.

In summary, this data clearly show that ERK2 protects the heart from progress to heart failure during long term pressure overload.

### 4.3.3 ERK2 in physiological hypertrophic remodelling

For inducing physiological hypertrophy there are different methods, including treadmill running, voluntary wheel running and swimming. Treadmill running for 7 to 13 weeks resulted in a 15\% increase in cardiomyocyte dimensions\(^{68}\). Voluntary wheel running is known to induce physiological hypertrophy following 3 to 4 weeks of training; although it is largely dependent on the animals willingness, offering the researcher less control over the experiment\(^{257, 258}\).

In this study, swimming exercise was used as a physiological hypertrophic stimulation. After 4 weeks of swimming, physiological hypertrophy was demonstrated in both groups of mice by increased cardiomyocyte cell size. These results are in concordance with previous studies which showed development of physiological hypertrophy following 4-6 weeks of swimming exercise in mice and rats\(^{261-263}\). The echocardiographic assessment did not reveal any functional changes associated with this hypertrophy\(^{264}\). Several previous studies have shown the absence of ERK1/2 activation during swimming in different mouse models\(^{69, 186, 231, 263}\). Further experiments with increased exercise intensity or duration can be used to investigate the role of ERK2 on physiological hypertrophy. Such experiments might include swimming exercises performed with attached external weights, which has been demonstrated to result in greater cell hypertrophy\(^{262}\).
4.4 ERK2 as a therapeutic target

Selective ERK1 and ERK2 inhibitors have been used as an important approach for development of anti-cancer therapeutics, as in 50% of human cancers the Ras/ERK1/2 pathway is massively activated\textsuperscript{266}.

The first generation of ERK1/2 inhibitors included PD98059 and U0126, which were identified in cell based assays\textsuperscript{267–269}. Their inhibitions are non-competitive for ATP binding or ERK1/2 binding to MEK1/2 and they interact more strongly with the non-phosphorylated MEK1/2 form than the activated version. Therefore, these inhibitors seem to prevent phosphorylation of MEK1/2 by changing conformation of the MAPKKs and locking them in their inactive form\textsuperscript{267,268}. None of these compounds was moved to clinical evaluation due to their limitations, but have been widely used as academic research tools to analyse the role of ERK1/2 pathway\textsuperscript{270}.

More recently, the non-competitive MEK1/2 inhibitors PD184352 and PD0325901 have been recommended to inhibit MEK1\textsuperscript{271}. However, the inhibitors PD98059, U0126 and PD184352 have been shown to block the activation of MEK5 and ERK5 due to the structure of the binding site for these inhibitors\textsuperscript{270,271}.

In clinical trials PD184352 and PD0325901 have been used and demonstrate that MEK1/2 can be inhibited in cancer cells in humans. However, their clinical development has been discontinued due to low bioavailability or severe toxicity on the physiological proliferation regulated by ERK1/2, especially cells with a high turnover\textsuperscript{264, 268}. The extensive side effects include diarrhea, visual disturbance and fatigue\textsuperscript{266}. Several other MEK1/2 inhibitors, such as XL518 and BAY 869766 show fewer side effects due to low central nervous system penetration\textsuperscript{270}. Currently, they are mostly used in cancer patients receiving combination therapy\textsuperscript{270}.
My study analysed the role of ERK2 during hypertrophic remodelling. Considering the central role of ERK2 in precise regulation of hypertrophic remodelling and its cardioprotective effects, inhibition of ERK2 might prove to be harmful with transition to heart failure. On the contrary, pharmacological activation of ERK2 might be accompanied by excessive hypertrophic growth and interstitial fibrosis. Importantly, ERK2 is strictly regulated by multiple elements and its sub-localisation affects the activation of different pathways. Under resting conditions, ERK2 molecules are situated in the cytoplasm whereas their phosphorylation leads to re-localisation. ERK2 phosphorylates multiple targets with half of the proteins localised in the nucleus and the other half can be found in cytosol, plasma membrane and cell organelles. Therefore ERK2 signalling could be regulated by influencing ERK2 downstream regulators or by a partially confinement of ERK2 to a specific cell compartment. Compared to ERK2 its scaffold proteins seem to be better therapeutic targets as they regulate the duration and intensity of the signals. At the same time these scaffold proteins play an important role in the selectivity of ERK2 signals. In summary, ERK2 might not be suitable as a direct target for therapeutic treatment. However, treatment of hypertrophy and heart failure may be accomplished by interfering with local sub-signals, and specific regulation of ERK2 effectors that might provide therapeutic control over the pathway.

4.5 Future directions

In this study, ERK2cko mice displayed impaired hypertrophic remodelling due to the ERK2 deficiency in cardiomyocytes. The precise role of ERK2 in cardiac fibroblasts has not been studied. A recent study showed in cardiac fibroblasts that micro RNA-21 (miR-21) inhibits Sprouty-1, an antagonist of ERK1/2 activation. Silencing
of miR-21 attenuated ERK1/2 activity, reduced fibrosis and hypertrophy, as well as prevented LV chamber dilation following 3 weeks of pressure overload that suggests an important role of ERK1/2 in cardiac fibroblasts in regulation of hypertrophic remodelling 274.

The future work will therefore focus on the in vivo effects of ERK2 knockout in cardiac fibroblasts during pathological hypertrophy. Mice with cardiac fibroblast-specific deletion of ERK2 will be subjected to either TAC or Iso treatment. The hypertrophic response and cardiac function regulated by cardiac fibroblast-expressed ERK2 will be subsequently examined.

4.6 Conclusion

Cardiac hypertrophy is characterised by an increase in cardiac mass in response to increased workload. Depending on the cause of stress, either pathological or physiological hypertrophy may develop5, 215, 275.

Physiological hypertrophy is a reversible and the adaptive cardiac growth leads to normal cardiac function whereas pathological hypertrophy is associated with complex cardiac remodelling, including upregulation of fetal genes and fibrosis. Initially pathological hypertrophy may be compensatory; but sustained stress is associated with ultimate development of heart failure6, 276. Therefore therapeutic strategies for treating heart failure include prevention of hypertrophy and antagonising the hypertrophy-induced cardiac remodelling.

In this context, the understanding of the regulatory mechanisms leading to pathological and physiological hypertrophy will give therapeutically beneficial information. The MAPK pathway is crucial in regulating hypertrophy and is activated in response to various hypertrophic stimuli, such as pressure overload and
neurohormones. Influencing the MAPKs signalling might be an attractive therapeutic approach for treating heart failure, for which detailed and precise evaluation for the functions and regulations of MAPK pathway is required.

The data generated in this study will significantly contribute to the knowledge of hypertrophic signalling in the heart. ERK2, a member of the classical MAPKs, plays a key role in pathological hypertrophic remodelling. Furthermore, ERK2 is crucial for cardiomyocyte survival during hypertrophy. In contrast, ERK2 is unlikely involved in the development of physiological hypertrophy. Specific pharmacological regulation of ERK2 effectors requires future evaluation.
CHAPTER 5
Chapter 5 References


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Co-author contributions

Pak1 as a Novel Therapeutic Target for Antihypertrophic Treatment in the Heart

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