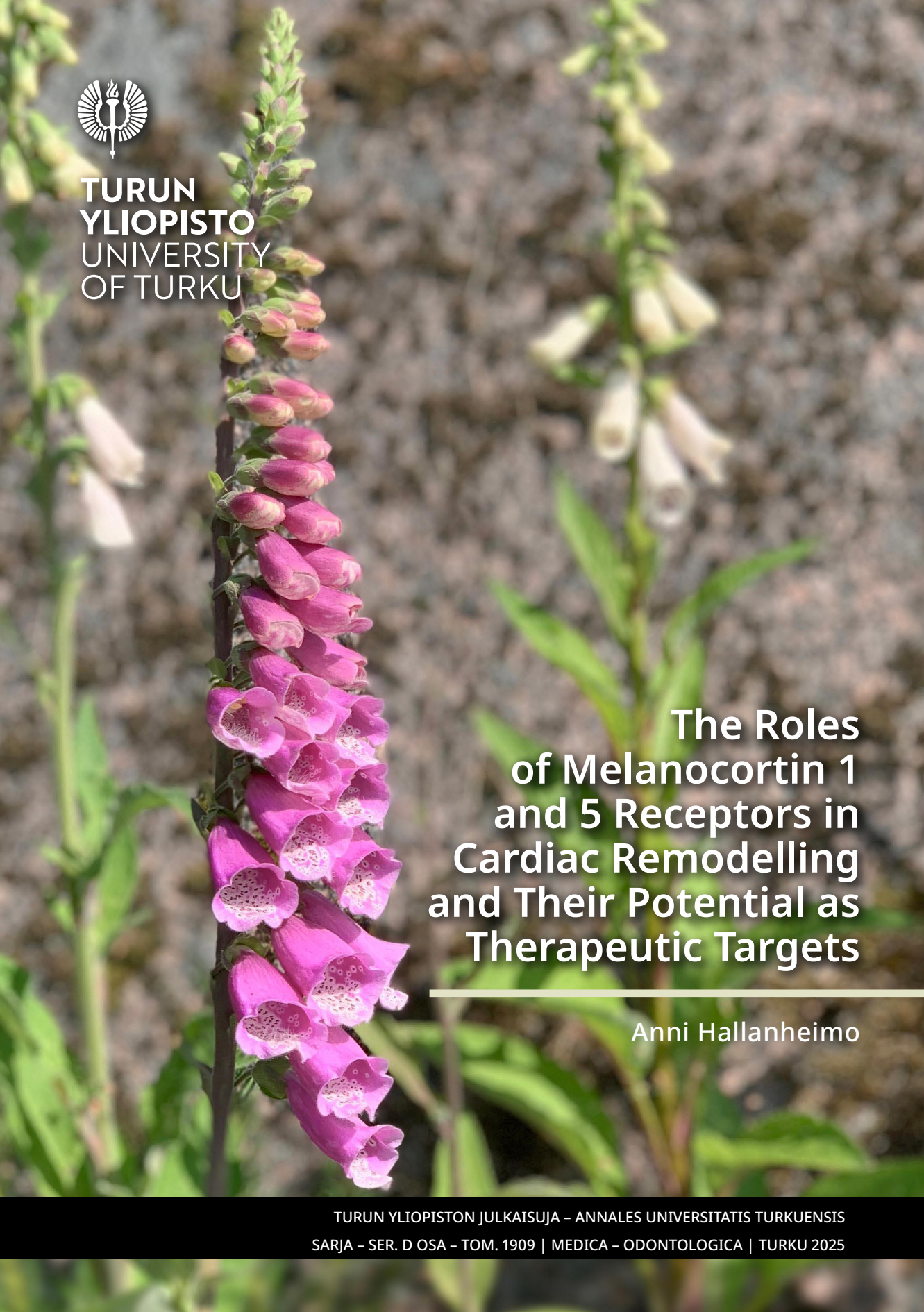

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University of Turku

Faculty of Medicine
Institute of Biomedicine
Pharmacology, Drug Development and Therapeutics
Drug Research Doctoral Programme

Supervised by

Docent Petteri Rinne, PhD
Institute of Biomedicine
University of Turku
Turku, Finland

Professor Eriika Savontaus, MD, PhD
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Professor Jaana Rysä, PhD
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
Kuopio, Finland

Associate Professor Riikka Kivelä, PhD
Faculty of Sport and Health Sciences
University of Jyväskylä
Jvväskylä, Finland

Opponent

Professor Eero Mervaala, MD, PhD
Department of Pharmacology
Faculty of Medicine
University of Helsinki
Helsinki, Finland

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To my family and myself

Kun kaatuu, kannattaa nousta.
- Lasse Virén

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ABSTRACT

Cardiac hypertrophy and heart failure are severe and progressive conditions with a poor prognosis and limited treatment options, and their morbidity and mortality rates remain high. The melanocortin system consists of melanocortin peptides and their receptors. Melanocortin peptides, including α-, β-, and γ-melanocyte-stimulating hormones (MSH), mediate their biological actions via five melanocortin receptors named from MC1R to MC5R. They are involved in multiple physiological functions, with MC1R particularly being recognised as the key regulator of skin pigmentation and MC5R in regulating exocrine gland activity. Melanocortins and their receptors have also established roles in the control of inflammation and vascular function, and the central regulation of energy homeostasis and cardiovascular function, but their local role in the heart has remained elusive. Consequently, the main objective of this thesis was to characterise the role of the melanocortin system in cardiac remodelling and to investigate pharmacological targeting of MCRs as a potential treatment for cardiac hypertrophy and heart failure.

First, it was identified that α-MSH, MC1R, and MC5R are expressed in cultured cardiomyocytes and in the mouse heart. Experimental models of pathological cardiac hypertrophy demonstrated declining levels of α-MSH, as well as MC1R and MC5R, in the failing heart. Pharmacological treatment of mice with α-MSH protected against pathological hypertrophy and heart failure. Pharmacological activation of MC1R in cellular models promoted cardiomyocyte hypertrophy, while global and cardiomyocyte-specific MC1R deficiency reduced pathological and physiological cardiac hypertrophy in mice, but led simultaneously to left ventricular dilatation and compromised left ventricular function. Conversely, pharmacological activation of MC5R attenuated hypertrophic and fibrotic responses in experimental cell and animal models, while genetic MC5R deficiency aggravated pathological hypertrophy, fibrosis, and systolic performance in mice.

In conclusion, this thesis identifies that the local melanocortin system exists in the heart and highlights novel roles for MC1R and MC5R in cardiac remodelling. These results suggest that MC1R and MC5R are promising therapeutic targets for the treatment of pathological cardiac hypertrophy and heart failure.

KEYWORDS: melanocortin receptor, melanocyte-stimulating hormone, cardiac hypertrophy, heart failure

TURUN YLIOPISTO
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TIIVISTELMÄ

Sydämen liikakasvu ja vajaatoiminta ovat vakavia ja eteneviä oireyhtymiä, joilla on huono ennuste ja rajalliset hoitomahdollisuudet, ja joiden yleisyys ja kuolleisuus on edelleen korkea. Melanokortiinit ovat peptidejä, joihin lukeutuvat α-, β- ja γ-melanosyyttejä stimuloivat hormonit (MSH). Ne vaikuttavat moniin eri elimistön toimintoihin sitoutumalla melanokortiinireseptoreihin (MC1R - MC5R). MC1R säätelee erityisesti ihon väritystä ja MC5R umpieritysrauhasten toimintaa. Melanokortiinijärjestelmä osallistuu lisäksi merkittävällä tavalla elimistön tulehdusvasteisiin, verisuonten toimintaan sekä energiatasapainon ja sydän- ja verenkiertoelimistön keskushermostoperäiseen säätelyyn, mutta sen paikallista vaikutusta sydämessä ei tunneta. Tämän väitöskirjan keskeisimpänä tavoitteena olikin selvittää melanokortiinijärjestelmän merkitystä sydämessä ja arvioida sen mahdollisuuksia sydämen liikakasvun ja vajaatoiminnan hoidossa.

Tämän väitöskirjatyön tutkimuksissa havaittiin, että α-MSH, MC1R ja MC5R ilmentyvät viljellyissä sydänlihassoluissa ja sydänkudoksessa. Kokeelliset sydämen liikakasvun mallit osoittivat α-MSH:n, MC1R:n ja MC5R:n määrien sydämessä laskevan vajaatoiminnan seurauksena. α-MSH hoito ehkäisi sairauteen liittyvää sydämen liikakasvua ja paransi sydämen supistusvaiheen toimintaa kokeellisessa tautimallissa. MC1R:n lääkinnällinen aktivointi edisti sydänlihassolujen kasvua, kun taas reseptorin puute hillitsi hiirillä sekä sairauteen liittyvää että liikunnan seurauksena kehittyvää sydämen liikakasvua aiheuttaen kuitenkin samanaikaisesti vasemman kammion laajentumista ja toiminnan heikkenemistä. MC5R:n aktivointi sekä kokeellisilla solu- että eläinmalleilla sitä vastoin vaimensi sydämen liikakasvua ja sidekudoksen muodostumista, ja reseptorin puute pahensi liikakasvua, lisäsi sidekudoksen muodostumista ja heikensi sydämen supistusvaiheen toimintaa hiirillä.

Väitöskirjan löydökset osoittavat aktiivisen melanokortiinijärjestelmän löytyvän sydämestä ja todistavat sen merkityksen sydämen kasvun ja toiminnan säätelyssä, laajentaen ymmärrystä melanokortiinijärjestelmän vaikutuksista elimistössä. Lisäksi tulokset tukevat melanokortiineja vastaavien lääkeaineiden kehitystä sekä MC1R:n ja MC5R:n mahdollisuuksia toimia uusina lääkekehityskohteina sydän- ja verisuonisairauksien hoitoon.

AVAINSANAT: melanokortiinireseptori, melanosyyttejä stimuloiva hormoni, sydämen liikakasvu, sydämen vajaatoiminta

Table of Contents

Abbreviations

α-CA	α-cardiac actin
α-SKA	α-skeletal actin
α-SMA	α-smooth muscle actin
AC	Adenylyl cyclase
ACE	Angiotensin-converting enzyme
ACE-I	Angiotensin-converting enzyme inhibitor
ACTH	Adrenocorticotrophic hormone
AgRP	Agouti-related protein
AKT	Serine/threonine-protein kinase, also known as protein kinase B, PKB
Ang II	Anigotensin II
ANP	Atrial natriuretic peptide
AR	Adrenergic receptor
ARB	AT_1 receptor blocker
ARNI	Angiotensin receptor-neprilysin inhibitor
AT_XR	Angiotensin receptor, X=type 1 or 2
BNP	B-type natriuretic peptide
cAMP	Cyclic adenosine monophosphate
CAD	Coronary artery disease
CaMKII	Calcium-calmodulin dependent protein kinase II
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CREB	cAMP response element-binding protein
CV	Cardiovascular
CVD	Cardiovascular disease
ECM	Extracellular matrix
EF	Ejection fraction
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
ET_X	Endothelin receptor, X=type A or B
GPCR	G protein-coupled receptor

HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFmrEF	Heart failure with mildly reduced ejection fraction
HFrEF	Heart failure with reduced ejection fraction
hiPSC-CM	Human induced pluripotent stem cell-derived cardiomyocyte
IGF-1	Insulin-like growth factor 1
IL	Interleukin
i.p.	Intraperitoneal
IP_3	Inositol trisphosphate
JNK	c-Jun N-terminal kinase
LV	Left ventricle
LVEDD	Left ventricle end-diastolic dimension
LVPW	Left ventricle posterior wall
MAPK	Mitogen-activated protein kinase
MCR	Melanocortin receptor
$Mc1r^{e/e}$	Melanocortin 1 receptor recessive yellow (mouse)
Mc1r-cKO	Cardiomyocyte-specific melanocortin 1 receptor knockout (mouse)
Mc5r-cKO	Cardiomyocyte-specific melanocortin 5 receptor knockout (mouse)
MHC-α	Myosin heavy chain alpha
MHC-β	Myosin heavy chain beta
MRA	Mineralocorticoid (aldosterone) receptor antagonist
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MT-II	Melanotan-II
NMCM	Neonatal mouse ventricular cardiomyocyte
p38	p38 mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
RT-qPCR	Quantitative real-time polymerase chain reaction, also known as qPCR
RV	Right ventricle
SGLT2	Sodium-glucose co-transporter 2
SERCA2a	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2a
s.c.	Subcutaneous
T2DM	Type 2 diabetes mellitus
TAC	Transverse aortic constriction
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
WT	Wildtype

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Suominen, A., Saldo Rubio, G., Ruohonen, S., Szabó, Z., Pohjolainen, L., Ghimire, B., Ruohonen, S. T., Saukkonen, K., Ijas, J., Skarp, S., Kaikkonen, L., Cai, M., Wardlaw, S. L., Ruskoaho, H., Talman, V., Savontaus, E., Kerkelä, R. & Rinne, P. α-Melanocyte-stimulating hormone alleviates pathological cardiac remodeling via melanocortin 5 receptor. *EMBO Reports,* 2024; 25(4): 1987-2014. https://doi.org/10.1038/s44319-024-00109-6

II Suominen, A., Suni, A., Ruohonen, S., Szabó, Z., Pohjolainen, L., Cai, M., Savontaus, E., Talman, V., Kerkelä, R. & Rinne, P. Melanocortin 1 receptor regulates pathological and physiological cardiac remodeling. *Journal of the American Heart Association,* 2025; 14(4):e037961. https://doi.org/10.1161/JAHA.124.037961

1 Introduction

The heart is the hardest-working muscle in the human body. Together with blood and blood vessels, it forms the cardiovascular (CV) system, whose key task is to deliver oxygen and nutrients to all tissues and organs throughout our body, and to carry away metabolic wastes. When the function of the heart is compromised, it fails to pump enough blood around the body, which can eventually lead to heart failure (HF). HF is often a consequence of cardiac hypertrophy arising from various cardiovascular diseases (CVDs). Pathological cardiac hypertrophy is a serious disease in itself, but also a major risk factor for other CVDs. The current standard-of-care to treat pathological cardiac hypertrophy and consequent HF is often targeted to the underlying cause, and despite the advancements in the diagnosis and clinical management of this disease and its associated complications during the past decades, many challenges still remain, as many patients are refractory to the currently available drug therapies and the disease tends to progress even in responsive patients. Consequently, HF remains to be the leading cause of hospitalisation worldwide. (McDonagh et al., 2021, 2023) The increasing incidence of obesity and type 2 diabetes mellitus (T2DM), together with poor lifestyle choices like a high salt diet resulting in high blood pressure, tobacco smoking, alcohol or drug misuse, and low physical activity, contribute to the high and constantly increasing global prevalence of HF. Together with the high prevalence, the nature of HF as a disease affecting patients' quality of life and survival in everyday life tasks has a huge social and economic impact, and it forms an important public health care challenge. (Ferreira et al., 2019; Martin et al., 2024; Roth et al., 2020; WHO, 2013)

The current consensus of HF as a complex clinical syndrome with various underlying reasons and related comorbidities without any disease-modifying drugs available highlights the unmet medical need for further research and development of novel therapies. As a contribution to these challenges, this thesis aimed to identify and characterise new treatment targets for cardiac hypertrophy and HF by investigating the CV actions of melanocortins. The melanocortin system is composed of melanocyte-stimulating hormones (α-, β-, and γ-MSH) derived from their precursor protein pro-opiomelanocortin (POMC), and they mediate their biological actions through a family of five closely related melanocortin receptors

(MC1R-MC5R). The melanocortin system is known to regulate diverse physiological functions throughout the body, but its role in the heart has remained unclear. The role of melanocortins in inflammation, energy homeostasis, blood pressure regulation, endothelial function, and atherosclerotic plaque formation gave further rationale for investigating their role in the heart. This thesis investigated the expression of melanocortins and their receptors in the heart and their role in regulating cardiac remodelling, and evaluated their potential as a target for developing novel drug therapies in experimental cardiac hypertrophy and HF models.

2 Review of the Literature

2.1 Cardiac hypertrophy

In principle, cardiac hypertrophy is defined as the enlargement of the heart and individual cardiomyocytes and an increase in heart mass, and it can be a consequence of various underlying reasons. Cardiac hypertrophy can be classified as physiological and pathological by the underlying initiatory stimulus, eccentric and concentric by the changes in cardiac structure, and right and left ventricular by the affected part of the heart. Furthermore, the different types of cardiac hypertrophy are driven by a variety of different molecular mechanisms, some of which are shared and some that differ between the hypertrophy types.

2.1.1 Structure and function of the heart

The human heart is a four-chambered muscular organ located slightly to the left of the centre of the chest and the apex tilted to the left, with the average size of its owner's fist and a weight of around 200 to 350 grams. The heart lies between the right and left lungs and is protected underneath the sternum and inside the thoracic cage. Furthermore, the heart sits inside a protective sac, the pericardium, which produces fluid that lubricates the heart and prevents it from rubbing against other organs. One layer of the pericardium is the epicardium, which forms the protective outer layer of the heart walls. Altogether, heart walls have three layers; under the epicardium is the myocardium, the muscular middle layer, and the inner part of the heart walls is called the endocardium, which covers the inside of the cardiac chambers. The thickness of the walls of the four heart chambers varies depending on their functions. The walls of the atria are thinner than the ventricles, since they need to pump the blood only to the adjacent ventricles. Also, the walls of the left ventricle (LV) are thicker than the right ventricle (RV) because it needs to pump blood against a higher pressure and to greater distances. (Buetow & Laflamme, 2018; Iaizzo, 2015; Weinhaus, 2015)

The human heart is divided into two halves, left and right, and the halves are separated by the muscular wall, the septum. Each half is yet separated into two chambers, the atrium and the ventricle. (Weinhaus, 2015) Interestingly, the mouse heart is divided into two ventricles by the septum, while the atria lie on top of the

heart as separate lobes, which form the most noticeable difference between the human and mouse cardiac structures. However, both species have four-chambered hearts with comparable functionality. Although there are some minor anatomical variations between the hearts of mice and humans, they are remarkably similar anatomically, and cardiac development is also comparable between the two species, making the mouse a good model for human hearts. (Buetow & Laflamme, 2018; Krishnan et al., 2014; Wessels & Sedmera, 2003)

The heart is the main organ in the CV system and its function is vital for life, since it produces the force necessary to circulate the blood. All cells need a continuous supply of oxygen and nutrients, and removal of metabolic wastes. In this circulation, blood is the transport medium, but the heart is the organ that keeps blood moving in the vessels. In humans, blood circulation is theoretically divided into pulmonary circulation and systemic circulation, which are regulated by the right and left sides of the heart, respectively. However, even though it is convenient to describe blood flow separately through the right and left sides, it is essential to remember that the human heart functions as a two-stroke engine where both atria contract simultaneously, followed by the contraction of both ventricles at the same time. (Weinhaus, 2015; Zhong et al., 2019)

Furthermore, the human heart has two types of valves, the other called atrioventricular valves, located between the atria and ventricles, and the other one called semilunar valves, located at the base of the large vessels leaving from the ventricles. The primary function of the cardiac valves is to prevent blood from flowing backwards. For this reason, atrioventricular valves and semilunar valves are never open at the same time. While the atria contract, oxygen-deprived blood from systemic veins (known as the superior and inferior vena cava) flows through the atrioventricular valve, named the tricuspid valve, into the RV, and oxygen-rich blood from pulmonary veins flows through the other atrioventricular valve, the mitral valve, into the LV. When the ventricles are full, they contract, subsequently causing semilunar valves to open and in the right side of the heart deoxygenated blood to flow through the pulmonary valve into the pulmonary trunk and eventually to the lungs, and in the left side of the heart oxygenated blood to flow into the aorta through the aortic valve and then all over the body via a complex network of arteries. (Bateman et al., 2015; Weinhaus, 2015; Zhong et al., 2019) Since the heart is the hardest-working muscle in the body, it needs itself a lot of energy and oxygen, and also the removal of metabolic wastes. Therefore, the heart has an extensive network of blood vessels on its surface, called coronary arteries, that branch from the ascending aorta and supply the energy and oxygen needed by the myocardium. The metabolic waste produced by the myocardium is removed via a system of cardiac coronary veins draining into the coronary sinus and opening into the right atrium. (Weinhaus, 2015)

During diastole, when the heart rests, the ventricles relax and the atria are filled passively with blood. When the pressure is higher in the atria than in the ventricles,

the atrioventricular valves open and the ventricles start to fill passively with blood. Conversely, during systole, the heart works actively and first, the atria contract, pushing the remaining blood to the ventricles, raising the ventricular pressure, and following immediate contraction of the ventricles, which push the blood into the arteries. Together, these two phases constitute one cardiac cycle, which takes about 0.8 seconds at a normal adult heart rate. (Hall, 2011; Loushin et al., 2015) Heart rate depicts the number of heartbeats during one minute, and in a normal situation, it is regulated by the conduction system of the heart, whose key component is the sinoatrial node, which is the pacemaker of the heart. In humans, it produces action potentials 60 to 100 times per minute on average at rest (Buetow & Laflamme, 2018; Olshansky et al., 2023). However, the heart rate is also affected by external factors, like the autonomic nervous system and hormones regulated by the endocrine system (Tiwari et al., 2021). The increase or decrease in heart rate further adjusts cardiac output, which is the volume of blood pumped by the heart in a minute, to meet the changing needs of the body. Concomitantly, due to the vital role of the heart, maladaptive changes in its structure, often followed by compromised function, can affect all over the body and even jeopardise an individual's life. (Hall, 2011)

2.1.2 Right ventricular and left ventricular hypertrophy

When cardiac workload increases, the heart tries to compensate for it by increasing its pumping efficacy. For this reason, cardiomyocytes grow in size, leading to thickening of the ventricular walls and an increase in heart weight. Cardiac hypertrophy can occur in both the right and left sides of the heart. Since the right side of the heart is responsible for pulmonary circulation, RV hypertrophy is often caused by arterial hypertension. Increased pressure in pulmonary arteries induces RV hypertrophy by thickening RV walls as a compensatory mechanism to maintain contractility in the right heart (Vonk-Noordegraaf et al., 2013). However, a long-term increase in RV afterload, meaning the pressure formed by systemic vascular resistance that the ventricle needs to work against to eject blood during contraction, leads to right HF and induces RV dilatation, tricuspid valve regurgitation and negative ventricular-ventricular interaction due to leftward translocation of the interventricular septum (IVS) and impairment of LV diastolic function as well (Urashima et al., 2008). In addition to impaired function of the pulmonary vasculature, RV hypertrophy and consequent dysfunction can occur due to congenital heart diseases or pulmonary heart diseases, also known as cor pulmonale, referring to lung diseases such as chronic obstructive pulmonary disease (COPD) that impairs RV function (Arrigo et al., 2019). Although pulmonary arterial hypertension is the main underlying factor for RV hypertrophy and it can eventually induce dysfunction of both ventricles, the majority of patients with RV HF have prior LV systolic or diastolic dysfunction, which supports

the concept that RV HF can often occur secondary to left-sided cardiac or pulmonary vascular diseases (Arrigo et al., 2019).

Since the left heart pumps blood all over the body via systemic circulation, the LV has a crucial and central role in maintaining normal cardiac function as well as the function of the systemic circulation. Therefore, cardiac hypertrophy most often refers to hypertrophy of the LV and is defined as an increase in LV mass arising from changes in LV wall thickness and chamber size. Furthermore, hypertrophy in the LV forms a leading risk factor for CV morbidity and mortality. Although cardiac hypertrophy is divided into the RV and LV, and there are some unique predisposing factors for both, they are induced by similar stimuli and share the same pathways that are up- or downregulated during the development of hypertrophy. For example, virtually all myocardial diseases involving the left heart may affect the RV as well. (Arrigo et al., 2019) Molecular pathways regulating the changes in the heart during hypertrophy differ more due to the type of hypertrophy than the side of the heart affected.

2.1.3 Physiological and pathological hypertrophy

The main function of the heart is to maintain peripheral organ perfusion in all conditions. To successfully fulfil this demand, the heart exhibits plasticity to be able to adapt to changing environmental conditions, such as increased physical stress. When cardiac workload is increased, the heart compensates for it by improving pumping efficacy by thickening cardiac muscle in the ventricular walls and thus preserving cardiac output. Based on the initiatory stimulus, cardiac hypertrophy can be divided into physiological and pathological. Although both of these hypertrophy types are initially adaptive, their phenotypes and prognoses greatly differ. When progressing, these two types also differ in their molecular fingerprints.

Physiological cardiac hypertrophy occurs due to natural postnatal growth, pregnancy, or extensive physical training, a condition also known as the athlete's heart. Except for postnatal growth, a physiologically hypertrophied heart is characterised by a mild (10-20%) increase in cardiac mass with preserved or increased contractile function and no fibrosis or cell death (Nakamura & Sadoshima, 2018). (**Table 1**) More specifically, postnatal growth can be classified as developmental, since it is a unique form of hypertrophy associated with the normal growth of the heart after birth until adulthood (Heineke & Molkentin, 2006). Furthermore, during mid-to-late pregnancy, the heart becomes hypertrophied to meet the increased need for a higher circulating blood volume and cardiac output, which is, however, reversed after the delivery of the baby (Schannwell et al., 2002). Likewise, in response to regular and intensive exercise training, especially endurance sports like long-distance running, cycling, or swimming, the heart undergoes cardiac remodelling, resulting in changes in geometry and cardiac performance to meet the body’s need for enhanced pumping efficacy.

However, these responses are fully and quickly regressed when training is discontinued. (Lovic et al., 2017; Weeks & McMullen, 2011) In physiological hypertrophy, the myocardium typically grows more uniformly than in pathological hypertrophy, with an increase in ventricular wall and septal thickness that matches the increase in chamber dimension, and even growth of myocyte length and width. (**Figure 1**) Furthermore, physiological hypertrophy is seldom associated with an elevated risk of cardiac diseases, with the exception of cases of atrial fibrillation among younger athletes (Newman et al., 2021). Nonetheless, physical activity and physiological hypertrophy overall provide cardioprotective benefits (Qiu et al., 2022).

Table 1. Basic characteristics of physiological and pathological hypertrophy. Table adapted from (Bazgir et al., 2023; Bernardo et al., 2012; Nakamura & Sadoshima, 2018).

Characteristics	**Physiological hypertrophy**	**Pathological hypertrophy**
Stimuli	Normal postnatal growth Pregnancy Exercise	Disease, i.e.: Pressure overload; hypertension, aortic stenosis, CAD Volume overload; valvular regurgitation, MI Comorbidities; obesity, T2DM, COPD Congenital; HCM, DCM
Geometry	Eccentric > concentric	Concentric or eccentric
Cardiomyocyte size	Increased	Increased
Adaptive	Yes	Initially yes, maladaptive when advanced
Reversible	Yes	No
Cardiac contractility	Preserved or increased	Preserved or decreased
Cardiac function	Preserved or enhanced	Depressed (systolic, diastolic or both)
Association with heart failure	No	Yes
Fibrosis	No	Yes
Cardiomyocyte death	No	Yes
Capillary network	Increased and sufficient for oxygenation and nourishment	Insufficient for oxygenation and nourishment, rarefaction
Inflammation	No	Yes
Fetal genes expression	Unchanged or decreased	Increased
Contractility-associated genes expression	Unchanged or increased	Decreased

CAD; coronary artery disease, COPD; chronic obstructive pulmonary disease, DCM; dilated cardiomyopathy, HCM; hypertrophic cardiomyopathy, MI; myocardial infarction, T2DM; type 2 diabetes mellitus

Cardiac hypertrophy is classified as pathological when it is associated with cardiac dysfunction, and it often develops due to unfavourable cardiac-related physiological stresses like hypertension, valvular regurgitation, loss of contractile mass due to myocardial infarction, prolonged and abnormal hemodynamic stress, and increased cardiac fibrosis (Shimizu & Minamino, 2016). It can also be developed due to other predisposing factors, such as genetic mutations (Jacoby & McKenna, 2011) or altered lipid metabolism, insulin resistance, and hyperglycaemia due to obesity and T2DM (Goff et al., 2007). Even though cardiac hypertrophy is initially a compensatory response to these stressors, if the stimulus is not removed, the response becomes pathological with time and results in unfavourable structural and functional changes in the heart. Although pathological cardiac hypertrophy generally occurs in the presence of a CVD, it is also a major player in maladaptive cardiac remodelling and serves as an independent predictor for more adverse CV outcomes, including arrhythmia, myocardial infarction, and HF (Schiattarella & Hill, 2015). (**Table 1**)

Pathological hypertrophy affects mainly the LV. Structural changes in the LV are determined by the type of cardiac insult, broadly categorised as pressure or volume overload, and underlying stressors, including diseases such as hypertension, aortic stenosis, atherosclerosis or coronary artery disease (CAD) in pressure overload, and valvular regurgitation, myocardial infarction or dilated cardiomyopathy in volume overload (Lorell & Carabello, 2000). Both types of stress increase the wall thickness, but with pressure overload, it is more marked, together with a relatively small change in ventricular volume, while, on the contrary, in volume overload, the change in chamber diameter is more evident, with a simultaneous, but comparably milder, increase in wall thickness (Schiattarella & Hill, 2015). Based on the law of Laplace, ventricular wall stress is proportional to both ventricular pressure and chamber diameter and inversely proportional to ventricular wall thickness (Burch et al., 1952). Accordingly, an increase in LV wall thickness tends to lessen the wall stress and thereby oxygen demand and is found to be a cardiac stress-adaptation mechanism (Grossman et al., 1975). Conversely, after the adaptive phase, if the stressor is not removed, pathological hypertrophy progresses to ventricular chamber dilatation with wall thinning (Nakamura & Sadoshima, 2018). (**Figure 1**) Furthermore, beyond the changes in ventricular wall and chamber sizes, pathological cardiac hypertrophy is characterised by an increase in cardiac mass, changes in individual cardiomyocyte morphology, scar formation due to increased fibrosis, changes in the matrix between the individual cells, a phenomenon known as remodelling of extracellular matrix (ECM), and even cell death (Gerdes, 2002; Li et al., 2018; Schiattarella & Hill, 2015). (**Table 1**) Together, these maladaptive changes in the heart inevitably lead to LV dysfunction and eventually HF.

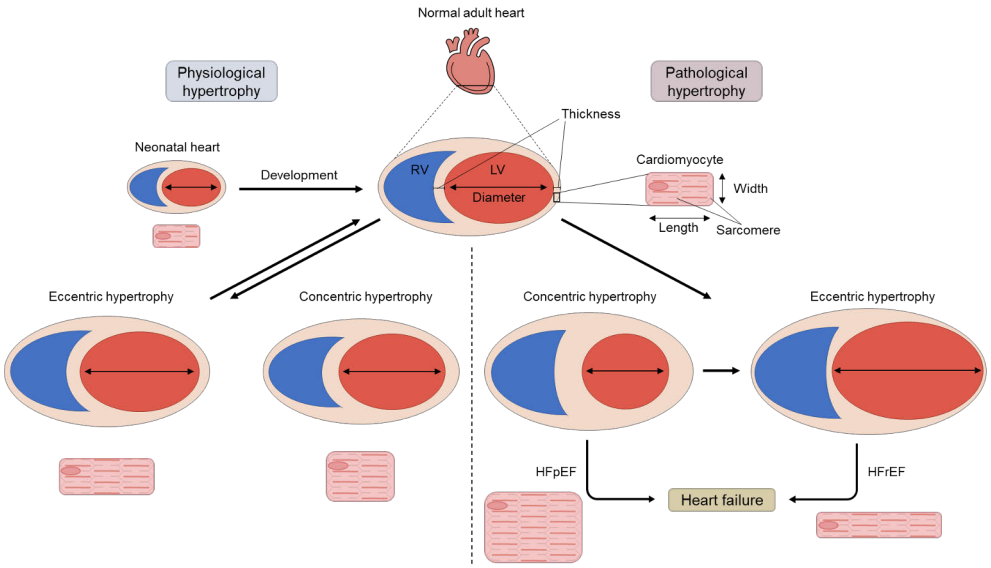


Figure 1. Overview of cardiac hypertrophy geometries. Physiological hypertrophy is identified by a mild increase in cardiac volume with a coordinated increase in wall and septal thicknesses, where individual cardiomyocytes grow both in length and width. After relief of the stimulus, physiological hypertrophy is reversed and the heart returns to its original dimensions. Increased pressure causes concentric hypertrophy, where LV walls thicken, resulting in a smaller LV chamber size. At the cellular level, sarcomeres are added in parallel, increasing myocyte width more than length. Volume overload induces pathological eccentric hypertrophy with dilated chambers, cardiomyocyte elongation and a subsequent decrease in wall thickness. Pathological hypertrophy often leads to heart failure with either preserved (HFpEF) or impaired (HFrEF) contractile function. If a stimulus is sustained, concentric hypertrophy progresses to maladaptive remodelling, leading to chamber dilatation and cardiac dysfunction. HFpEF; heart failure with preserved ejection fraction, HFrEF; heart failure with reduced ejection fraction, LV; left ventricle, RV; right ventricle. Figure adapted from (Maillet et al., 2013; Nakamura & Sadoshima, 2018).

2.2 Characteristics of cardiomyocyte hypertrophy

As previously described, cardiac hypertrophy can occur due to physiological as well as pathological stressors, which induce growth of wall thickness and chamber volume. These changes in heart geometry can be described as concentric cardiac growth, when the ventricular wall thickness is increased and eccentric growth, when the ventricular volume is increased (Calderone et al., 1995). In physiological hypertrophy due to pregnancy or extreme exercise, the eccentric form of hypertrophy is more prevalent and characterised by balanced and coordinated growth of septal and LV free walls, where individual cardiomyocytes grow evenly in length and width (Eghbali et al., 2006; Pluim et al., 2000). Although concentric hypertrophy due to physiological stimulus is rarer and controversial, it can occur in response to isometric

exercise like weight-lifting or wrestling, and induce wall thickening without enlargement of ventricles (Longhurst & Stebbins, 1997; Morganroth et al., 1975; Spence et al., 2011). (**Figure 1**)

Geometrical changes in the heart due to pathological pressure or volume overload correspond to the concentric and eccentric forms of hypertrophy, respectively (Frey et al., 2004). Pathological concentric hypertrophy induces strong wall thickening due to cellular growth in width and consequently a reduction in chamber dimension. On the contrary, pathological eccentric hypertrophy leads to excessive ventricular dilatation and wall thinning with individual cardiomyocytes preferentially growing in length (Nauta et al., 2020). (**Figure 1**) In pathological cardiac growth, the outcome, i.e. concentric or eccentric, depends greatly on the underlying disease. Concentric hypertrophy commonly occurs during the adaptive phase of pathological cardiac growth, but it can, over time, evolve into eccentric hypertrophy.

Cardiomyocytes form the predominant cell population in the heart and are responsible for the cardiac structure and contractile function (Litviňuková et al., 2020). Generally, they resemble elliptical cylinders in shape, but are highly variable in size, and their arrangement within the ventricular wall is quite complex (Gerdes, 2002). Postnatal cardiomyocytes exit the cell cycle and become terminally differentiated striated muscle cells soon after birth, with only limited proliferative capacity (Guo & Pu, 2020). Therefore, in the adult heart, individual cardiomyocytes need to grow larger to increase heart size (Karbassi et al., 2020). Furthermore, due to the spatial arrangement of cardiomyocytes within the ventricular wall, they provide the structural basis for alterations in wall thickness and chamber diameter (Gerdes, 2002). Under increased and sustained pressure, cardiomyocytes grow in thickness by parallel addition of new contractile units, sarcomeres, within the cell, which leads to concentric hypertrophy. In contrast, increased cardiac volume overload induces cardiomyocyte lengthening by serial addition of new sarcomeres, leading to eccentric hypertrophy (Martin et al., 2023). Concentric hypertrophy is characterised by an increase in cardiomyocyte cross-sectional area (CSA), while eccentric hypertrophy leads to cellular-level modifications with proportional growth of both cardiomyocyte length and CSA. When the hypertrophy progresses further, extensive lengthening of cardiomyocytes occurs without a further change of CSA, leading to a dilated chamber with thinner walls at the organ level, regardless of the underlying initial stressor (Gerdes, 2002). (**Figure 1**)

As described, cardiomyocyte thickening and lengthening occur via parallel or serial addition of sarcomeres inside the cell. Structurally, cardiomyocytes are composed of parallel bundles of myofibrils, and each myofibril consists of sarcomeres, and each sarcomere is connected in series to neighbouring

sarcomeres via the Z-disc. The basic structure of a sarcomere consists of thin actin filaments attached to the Z-disc, thick myosin filaments interconnected to the M-line in the middle, and elastic titin filaments attached to the Z-disc and running towards the M-line. (van der Velden & Stienen, 2019) (**Figure 2**) Furthermore, myosin is composed of two heavy chains (MHC) and two pairs of light chains, and in mammals, there are two MHC isoforms, MHC-α and MHC-β. Besides the changes in cardiomyocyte thickening and lengthening, sarcomere assembly induces changes in cardiomyocyte function. The interaction between myosin and actin is responsible for generating the contraction force of the heart, which leads to the ejection of blood from the ventricles to the circulation. During cardiomyocyte contraction, the actin and myosin filaments slide across each other, shortening the cell, while during cardiomyocyte relaxation, the actin and myosin filaments slide further apart, elongating the cell and enabling the relaxation of the myocardium and the filling of the heart. This interaction of actin and myosin is induced by a rise in intracellular calcium and driven by the energy from ATP hydrolysis. The MHC-α isoform exhibits higher ATPase activity and, consequently, higher contractile velocity than the MHC-β isoform. (Hamdani et al., 2008; Yin et al., 2015) However, reduced power output and contraction speed make the MHC-β isoform more economical (Holubarsch et al., 1985; Reiser et al., 2001).

This ordered assembly of sarcomeres is essential for muscle contraction and relaxation during the cardiac cycle, but it also plays a role in cardiac hypertrophy. In the stressed heart and during the development of cardiac hypertrophy and HF, the composition of sarcomeric proteins is altered, and the balance between different forms of actin, myosin, and titin proteins is disturbed, causing, for example, concentric and eccentric cardiomyocyte growth, myofilament stiffness, and impaired cardiomyocyte relaxation. Defects in sarcomere function are also vital in multiple congenital heart diseases, like hypertrophic and dilated cardiomyopathies, which result from genetic mutations in the genes encoding the thick and thin filament proteins of the sarcomere. (van der Velden & Stienen, 2019)

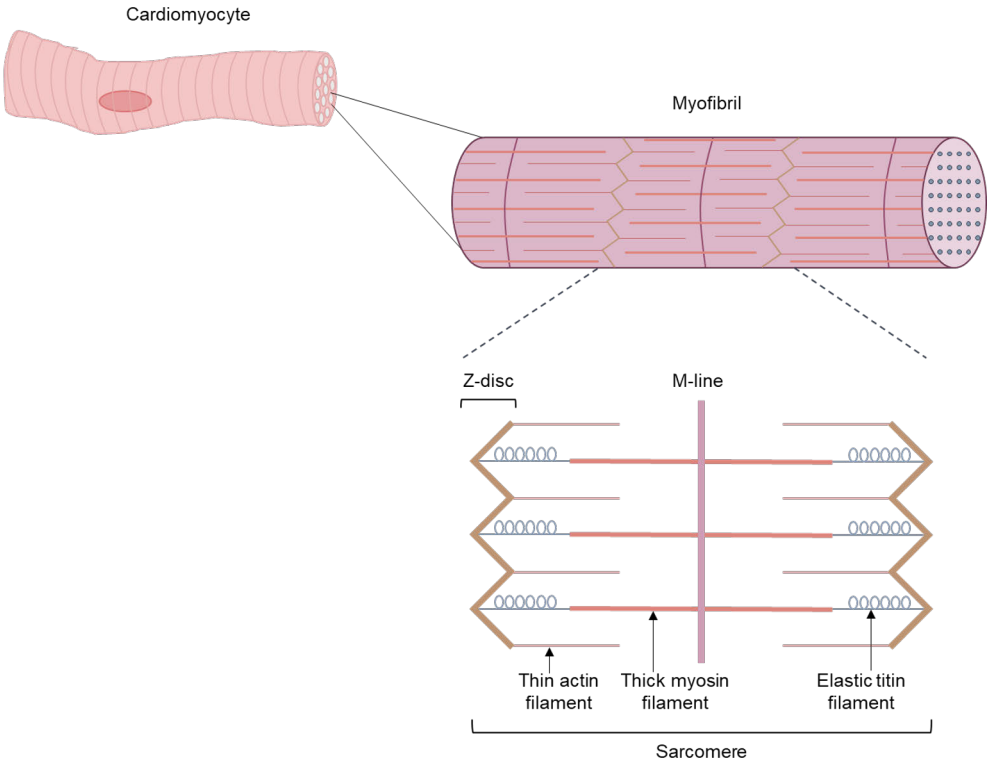


Figure 2. Basic structure of a cardiomyocyte. Cardiomyocytes are muscle cells that consist of parallel bundles of myofibrils. Each myofibril is made up of sarcomeres, which are connected in series by Z-discs. The basic structure of a sarcomere includes thin actin filaments attached to the Z-disc, thick myosin filaments connected to the M-line in the centre, and elastic titin filaments that extend from the Z-disc to the M-line and anchor to the myosin filaments. The movement of sarcomeres allows cardiomyocytes to contract. Figure adapted from (van der Velden & Stienen, 2019).

2.3 Mechanisms of physiological hypertrophy

The initial stimuli behind the hypertrophic response, along with the accompanying responses involved in cellular growth, affect the specific type of hypertrophy that develops. These concurrent responses substantially differ between physiological and pathological hypertrophy and eventually determine the nature of a hypertrophied heart. (Nakamura & Sadoshima, 2018) Physiological hypertrophy is described as fully adaptive and involves the activation of cellular signalling pathways that positively regulate cell survival, maintenance, and growth, and simultaneously antagonise pathological cardiac remodelling and dysfunction (Chen, Chen, et al., 2022; Weeks et al., 2017).

Increased physiological growth of the heart is initiated by biochemical signals, including neuroendocrine factors and hormones, and stretch-sensitive mechanisms

that sense mechanical forces. These trigger the activation of intracellular signalling pathways, which in turn lead to further gene transcription and protein translation. When compared to pathological hypertrophy, these cascades are much more limited and do not lead to fetal, fibrotic, or apoptotic gene expression, nor do they disturb sarcomeric protein assembly. Descriptive of physiological hypertrophy is also the intermittent nature of signalling pathway activation. However, some of the pathways initiating physiological hypertrophy can become maladaptive and induce pathological hypertrophy if constantly activated. (Maillet et al., 2013)

2.3.1 Hormones and growth factors

The most prevalent signalling pathways in physiological cardiac hypertrophy start from ligand binding to the network of trans-membrane tyrosine kinase receptors (RTKs), including insulin receptor (IR), type 1 insulin-like growth factor receptor (IGF1R), and other growth factor receptors, such as vascular endothelial growth factor receptor 1 (VEGFR1), and nuclear receptors, like thyroid hormone receptor (TR), and involve different growth factors and hormones, such as insulin, insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and thyroid hormone triiodothyronine (T3), as ligands (Maillet et al., 2013). (**Figure 3**) Insulin and IGF-1 signalling pathways are perhaps the most significant in regulating physiological hypertrophy, and their actions closely resemble each other in the heart (Nakamura & Sadoshima, 2018). They both regulate a broad range of cellular processes in the heart (Aroor et al., 2012), and increased serum and cardiac IGF-1 levels are related to exercise-induced hypertrophy (Neri Serneri et al., 2001). IGF-1 is mainly synthesised and secreted by the liver in response to the systemic growth hormone and delivered to the target tissues. However, IGF-1 is also produced in the heart, where it acts in an autocrine or paracrine manner. (McMullen, 2008) Insulin and IGF-1 bind to their cell surface receptors, IR and IGF1R, respectively. Insulin further activates and phosphorylates adaptor proteins insulin receptor substrate 1 (IRS1) and IRS2, which leads to the activation of phosphoinositide 3-kinase (PI3K) and the subsequent serine/threonine-protein kinase (AKT, also known as protein kinase B, PKB) pathway. IGF-1 binding to its receptor also activates the PI3K-AKT pathway. (Maillet et al., 2013) Activation of this pathway leads to activation of different downstream targets, the most well-known in physiological hypertrophy being the mammalian target of rapamycin (mTOR) (Kemi et al., 2008) and mitogen-activated protein kinase (MAPK) cascade of the extracellular signal-regulated kinase (ERK), known as a pathway of MEK1-ERK1/2 (Bueno et al., 2000). However, the role of ERK1/2 in physiological hypertrophy is debated, since MAPK cascades, including ERK1/2, have been linked to pathological hypertrophy (Bernardo et al., 2010; McMullen et al., 2007; Muslin, 2008) while other studies have demonstrated

the importance of ERK1/2 signalling in physiological hypertrophy (Bueno et al., 2000; Kehat & Molkentin, 2010). MAPK pathways are more thoroughly described in the next chapter 2.4. Since PI3K-AKT activation is so central in physiological hypertrophy, it acts as a nodal point for multiple downstream effects (Shiojima & Walsh, 2006). Besides the above-mentioned cascades, AKT activation downregulates transcription factor CCAAT/enhancer binding protein-β (C/EBPβ) in the cell nucleus. This inhibition allows CBP/p300-interacting transactivator 4 (CITED4) mediated proliferation-related gene expression activation, thereby promoting physiological hypertrophy (Boström et al., 2010). In the cell cytoplasm, AKT also promotes protein synthesis via inhibiting glycogen synthase kinase 3β (GSK3β), a kinase whose inhibition further suppresses eukaryotic translation initiation factor 2Bε (eIF2Bε) and leads to enhanced protein translation (Antos et al., 2002; Haq et al., 2000). (Figure 3) The activation of these signalling pathways further regulates the transcription of adaptive genes, protein synthesis, metabolism and energy production (Maillet et al., 2013).

The increased growth of the capillary network, proportional to cardiomyocyte growth, secures sufficient oxygen and nutrient supply for the growing myocardium and is an important controlling factor dictating the development of physiological rather than pathological cardiac hypertrophy. To maintain adequate myocardial capillary density, the levels of angiogenic growth factors, including VEGF, angiopoietin-1 and -2, platelet-derived growth factor, fibroblast growth factor, and transforming growth factor, are increased. (Oka et al., 2014) Among these, VEGF is the most crucial in myocardial angiogenesis. It regulates angiogenesis by activating the PI3K-AKT-mTOR pathway, which in turn stimulates the production of hypoxia-inducible factor 1α (HIF1α) (**Figure 3**), a major transcription factor controlling oxygen homeostasis by regulating angiogenesis, vascular remodelling, and glucose metabolism (Semenza, 2013). VEGF also regulates transcription, protein synthesis, cell growth and survival by activating other PI3K-AKT downstream pathways (Nakamura & Sadoshima, 2018).

The active form of thyroid hormone, T3, is crucial for the developing heart, and its level dramatically increases after birth (Eng & Lam, 2020; Pantos et al., 2011). During postnatal cardiac hypertrophy, T3 acts as a direct transcriptional factor when the heart matures by binding to the thyroid hormone receptors, TRα and TRβ. The binding of T3 to its receptors acts as a transcriptional switch, downregulating the expression of fetal gene *MYH7* (which encodes protein MHC-β) and upregulating the expression of *MYH6* (which encodes protein MHC-α) (Nakamura & Sadoshima, 2018; Pantos et al., 2011). In addition to the effects of T3 on contractile proteins, it contributes to improved myocardial performance when TRs interact with retinoic acid receptor (Arsanjani et al., 2011). During this receptor interaction, T3 positively regulates the expression of

sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a), while simultaneously inhibiting the Ca^{2+}-pump SERCA2a inhibitor phospholamban (PLN) expression (Arsanjani et al., 2011; MacLennan & Kranias, 2003; Nakamura & Sadoshima, 2018) and thus positively affecting cardiomyocyte electrophysiology. Furthermore, besides these main effects of cardiac contractile

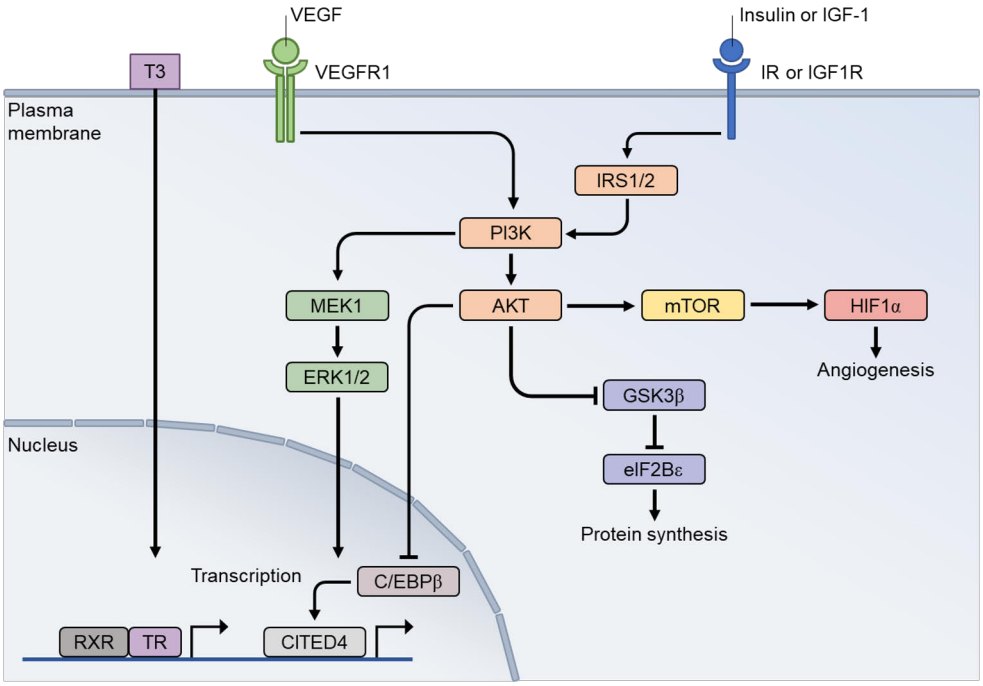


Figure 3. Signalling pathways of physiological hypertrophy. Physiological stimuli induce the activation of signalling pathways, leading to increased transcription of adaptive genes, protein synthesis, cardiomyocyte proliferation, cell survival, and angiogenesis, for example, by activating transmembrane tyrosine kinase receptors, including IR, IGF1R, and VEGFR1, as well as nuclear receptor TR. Insulin and IGF-1 promote growth and survival, whereas VEGF especially increases angiogenesis, and T3 is critical for regulating postnatal hypertrophy and contractility. These growth signals are intracellularly controlled by central molecules such as ERK1/2, PI3K, AKT, and mTOR. The figure illustrates the primary signalling pathways involved in physiological hypertrophy discussed in the text. AKT; serine/threonine-protein kinase, C/EBPβ; CCAAT/enhancer binding protein-β, CITED4; CBP/p300-interacting transactivator 4, eIF2Bε; eukaryotic translation initiation factor 2Bε, ERK1/2; extracellular signal-regulated kinase, GSK3β; glycogen synthase kinase 3β, HIF1α; hypoxia-inducible factor 1α, IGF-1; insulin-like growth factor 1, IGF1R; insluin-like growth factor receptor, IR; insulin receptor, IRS1/2; insulin receptor substrate 1 or 2, MAPK; mitogen-activated protein kinase, mTOR; mammalian target of rapamycin, PI3K; phosphoinositide 3-kinase, RXR; retinoic acid receptor, T3; thyroid hormone triiodothyronine, TR; thyroid hormone receptor, VEGF; vascular endothelial growth factor, VEGFR1; vascular endothelial growth factor receptor 1. Figure adapted from (Maillet et al., 2013; Nakamura & Sadoshima, 2018).

and calcium handling, T3 regulates the transcription of various genes in the heart (Arsanjani et al., 2011; Dillmann, 2010). (**Figure 3**)

2.4 Mechanisms of pathological hypertrophy

As stated before, the heart tries to compensate for increased workload by growing in size and increasing muscular mass. However, if the workload is extended, the heart becomes exhausted, and an initially adaptive response develops into pathological hypertrophy. Although chronic cardiac stress is vital for the development of a pathological form of hypertrophy, the more critical aspect dictating the development of pathological rather than physiological hypertrophy relies on the nature of upstream stimuli and downstream signalling mechanisms (Perrino et al., 2006). Pathological stresses promote hypertrophy mainly through neuroendocrine hormones and mechanical forces that induce distinct downstream signalling pathways and molecular features. These molecular, cellular and systemic level changes driving pathological hypertrophy are complex, and the pathophysiology of this disease is associated with fibrosis, inadequate angiogenesis, capillary rarefaction, undesirable changes in coronary arteries, disorganisation of sarcomeres, altered calcium handling, metabolic disorder, inflammation, cellular dysfunction, changes in intracellular signalling cascades, and undesirable epigenetic changes (Caturano et al., 2022; Shimizu & Minamino, 2016).

Signalling pathways underlying pathological cardiac hypertrophy have been the subject of intensive research for decades. Multiple essential pathways have been discovered, and attempts aiming at the development of therapies and clinical prevention of pathological cardiac hypertrophy have been initiated (Van Berlo et al., 2013). However, these pathways are complex, starting from the cell surface and diving into the level of DNA reading, involving cell surface receptors, intracellular activation and inhibition of signalling molecules, modulation of transcriptional elements affecting gene reading inside the nucleus and chromatin-modifying enzymes on the DNA (Carreño et al., 2006; Frey & Olson, 2003; Samak et al., 2016). Because of this complexity, research findings related to many of these signalling cascades are contradictory, highlighting the necessity for further research.

2.4.1 G protein-coupled receptors

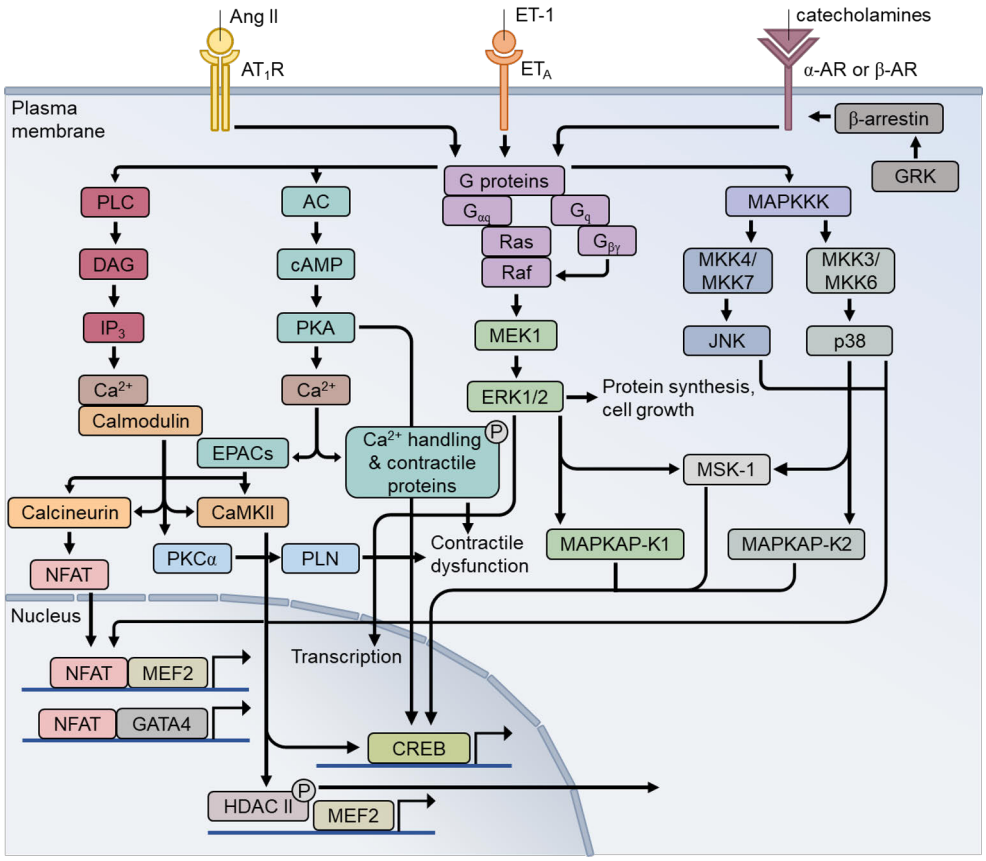
Cardiomyocytes sense the bioactive mediators released into the cardiac microenvironment by different receptors, including G protein-coupled receptors (GPCRs), growth factor receptors and cytokine receptors. The ligand binding to the cardiomyocyte cell surface receptor induces the activation of the cell signalling pathways. (Bernardo et al., 2010) In pathological hypertrophy, the most well-characterised signalling pathways start from angiotensin II (Ang II), endothelin-1 (ET-1), or catecholamines binding to their target GPCRs named as angiotensin type 1- and 2 receptors (AT_1R and AT_2R), endothelin receptors (ET_A and ET_B), and adrenergic receptors (ARs), respectively (Arai et al., 1995; Rapacciuolo et al., 2001; Yamazaki et al., 1999). Activation of these GPCRs leads to the release of various intracellular modulators and induction of signalling cascades. The most recognised of these are protein kinases (e.g. PKA and PKC) activation, calcium release alteration via calcium-dependent signalling proteins like calcineurin and calcium-calmodulin-dependent protein kinases (especially CaMKII in the heart), and MAPK cascades activation (Esposito et al., 2002; Shimizu & Minamino, 2016).

Besides the importance of the systemic renin-angiotensin-aldosterone system (RAAS) in CV function, the local RAAS in the heart is activated upon mechanical forces targeted to the heart, which increases the expression of components of this system, including angiotensinogen (AGT), renin, angiotensin I (Ang I), angiotensin-converting enzyme (ACE), Ang II, AT_1R, and AT_2R (Schunkert et al., 1990; Wollert & Drexler, 1999). Especially important in maladaptive cardiac hypertrophy is the role of the AT_1R and increased levels of Ang II throughout the cardiac microenvironment. Ang II binding to AT_1R increases cardiac contractility and heart rate, and induces vasoconstriction, thus increasing blood pressure to maintain hemodynamic homeostasis and cardiac function. (Mehta & Griendling, 2007) However, when prolonged, this Ang II/AT_1R activation leads to oxidative stress, inflammation, cell proliferation and hypertrophic responses in cardiomyocytes but also in vascular smooth muscle cells and fibroblasts via many different downstream pathways like activated MAPKs, PKC, increased intracellular calcium and further downstream signalling molecules (Bhullar & Dhalla, 2022; Forrester et al., 2018).

ET-1 is the predominant endothelin in the heart and produces a hypertrophic stimulus mainly via the ET_A receptor, which accounts for 90% of endothelin receptors in cardiomyocytes (Fareh et al., 1996; Sakai et al., 1996). ET-1 and Ang II are closely related in pathological hypertrophy and mediate similar downstream signalling pathways. AT_1R and ET_A receptor activations lead to further intracellular G protein activation, leading to phospholipase C (PLC) activation and formation of diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 promotes

intracellular Ca^{2+} release from the endoplasmic and sarcoplasmic reticulum, leading to Ca^{2+}-calmodulin complex and further protein kinase Cα (PKCα), CaMKII and calcineurin activation. Downstream pathway of PKC results in dephosphorylation of PLN, inhibition of SERCA2a activity and contractile dysfunction. (Nakamura & Sadoshima, 2018) Calcineurin is a Ca^{2+}-calmodulin-activated protein phosphatase that dephosphorylates nuclear factor of activated T cells (NFAT) and thus promotes NFAT localisation into the nucleus and interaction with transcriptional cofactors such as GATA4 and myocyte-specific enhancer factor 2 (MEF2), which stimulate hypertrophy-related gene reading (Cornwell & McDermott, 2023; Molkentin et al., 1998; Wilkins et al., 2004). Furthermore, CaMKII signalling induces cardiac hypertrophy by phosphorylating the class IIa histone deacetylases (HDAC II, e.g. HDAC4) in the nucleus, which then dissociates from MEF2 and activates it and pathological gene reading, while HDAC II translocates to the cytoplasm (Backs et al., 2006; Black & Cripps, 2010; Cornwell & McDermott, 2023). (**Figure 4**)

One of the principal modulators in pathological cardiac hypertrophy is elevated levels of circulating catecholamines, including dopamine, adrenaline and noradrenaline. They activate α- and β-type of ARs (especially α_1, β_1 and β_2 in the heart) and induce downstream signalling initially leading to beneficial adrenergic drive and increased contractility. However, this can result in pathological signalling and desensitisation of ARs by GPCR kinase (GRK)-mediated β-arrestin signalling, when the activation is prolonged (Bernardo et al., 2010; Bristow, 2000; Sato et al., 2015). Ligand binding to ARs, in the heart especially β_1-type, induces intracellular G-protein and adenylyl cyclase (AC) activation and cyclic adenosine monophosphate (cAMP) upregulation (Rockman et al., 2002). cAMP increase classically induces PKA activation leading to an increase in cytosolic Ca^{2+} and phosphorylation of Ca^{2+} handling and contractile proteins, and further alteration in cardiac contractility, fibrosis and cell growth (Nakamura & Sadoshima, 2018). More recently, cAMP activation has been demonstrated to lead to exchange proteins directly activated by cAMP (EPACs) upregulation and further induction of pathological calcineurin-NFAT and CaMKII-MEF2 pathways described above (Métrich et al., 2008; Morel et al., 2005). (**Figure 4**)



2.4.2 Mitogen-activated protein kinase pathways

Furthermore, GPCR activation in the heart by catecholamines, Ang II, and ET-1, as well as increased Ca^{2+} influx through ion channels, inflammatory cytokine signalling, activation of other cell surface receptors, such as RTKs and transforming growth factor-β (TGF-β) receptor, or various stresses, leads to activation of MAPK signalling, including three important cardiac-related MAPK subfamilies defined based on their terminal kinase: ERK1/2, c-Jun N-terminal kinase (JNK) and p38 kinase (p38) (Heineke & Molkentin, 2006; Rose et al., 2010). These subfamilies are involved in both heart development and disease, and are roughly divided into the prototypic ERK1/2 pathway, as a growth factor-stimulated, and JNK and p38, called stress-activated MAPKs (Sugden & Clerk, 1997, 1998). In cardiac disease, especially in cardiac hypertrophy, MAPKs have distinct roles, since they promote cardiac hypertrophy signalling. However, they have also been demonstrated to be involved in cardioprotective pathways. (Bueno et al., 2000; Molkentin & Dorn, 2001; Rose et al., 2010; Silberbach et al., 1999) MAPKs are highly conserved and

◄ **Figure 4.** Signalling pathways of pathological hypertrophy. Pathological stimuli via the neuroendocrine hormones Ang II, ET-1, and catecholamines, and their cognate GPCRs activate signalling pathways that increase the transcription of maladaptive genes, protein synthesis, cardiomyocyte growth, cell death, fibrosis, and contractile dysfunction. Ang II and ET-1 primarily signal via pathways downstream of PLC activation, which negatively regulates cardiac contractility and promotes maladaptive gene expression and hypertrophy. Catecholamines classically increase intracellular cAMP, thus altering cardiomyocyte contractility and fibrosis, while also upregulating hypertrophic gene transcription through EPACs signalling. Activation of GPCRs induces MAPK pathways, resulting in the activation of nuclear transcription factors, increased protein synthesis, and cellular growth. The figure illustrates the primary GPCR-mediated signalling pathways involved in pathological hypertrophy discussed in the text. AC; adenylyl cyclase, Ang II; angiotensin II, AR; adrenergic receptor, AT_1R; angiotensin type 1 receptor, Ca^{2+}; calsium ion, CaMKII; calcium-calmodulin dependent protein kinase type II, cAMP; cyclic adenosine monophosphate, CREB; cAMP response element binding protein, DAG; diacylglycerol, EPACs; exchange proteins directly activated by cAMP, ERK1/2; extracellular signal-regulated kinase, ET-1; endothelin-1, ET_A; endothelin type A receptor, GATA4; transcription factor GATA4, GPCR; G protein-coupled receptor, GRK; GPCR kinase, HDAC II; histone deacetylase class IIa, IP_3; inositol trisphosphate, JNK; c-Jun N-terminal kinase, MAPK; mitogen-activated protein kinase, MAPKAP-K1 or 2; MAPK-activated protein kinase-1 or 2, MAPKKK; MAPK kinase kinase; MEF2; myocyte-specific enhancer factor 2, MEK1; MAP-ERK kinase, MKK3/MKK6; MAP-p38 kinase, MKK4/MKK7; MAP-JNK kinase, MSK-1; mitogen- and stress-activated protein kinase 1, NFAT; nuclear factor of activated T cells, p38; p38 kinase, PKA; protein kinase A, PKCα; protein kinase Cα, PLC; phospholipase C, PLN; phospholamban. Figure adapted from (Nakamura & Sadoshima, 2018; Shah & Mann, 2011).

ubiquitously expressed kinases, which, after initial activation, lead to several phosphorylation-based amplification steps before separating into terminal kinase-specific pathways (Garrington & Johnson, 1999).

ERK1/2 signalling is generally thought to be pro-hypertrophic, and it is activated by two main mechanisms, both of which rely on the Ras-Raf-MEK1-ERK1/2 cascade. Growth factor binding to RTK, Ca^{2+} influx via ion channel or GPCR activation at the cell surface by Ang II, ET-1, or catecholamines leads to intracellular $G_{\alpha q}$ activation, followed by the induction of small G proteins Ras and Raf, which act as molecular switches linking receptors to downstream signalling cascades. These Ras-Raf inductions in the cytoplasm lead to activation of MEK1 and ERK1/2 and further phosphorylation of cytosolic targets, which ultimately induce protein synthesis and cell growth. (Bernardo et al., 2010; Molkentin & Dorn, 2001; Rose et al., 2010) As another mechanism, G_q-coupled receptor activation leads to $G_{\beta\gamma}$ subunit and Raf-MEK1-ERK1/2 complex interaction and autophosphorylation of ERK1/2 in the cytoplasm and ERK1/2 localisation into the nucleus, where it phosphorylates nuclear targets and induces hypertrophy-related gene transcription (Lorenz et al., 2009). (**Figure 4**) However, while there is strong evidence suggesting that activation of ERK1/2 contributes to cardiomyocyte hypertrophy, there is also data showing that reduction of ERK1/2 is not sufficient to prevent hypertrophy either, implying that ERK1/2 can be a significant but not a necessary signalling component in

cardiomyocyte hypertrophy (Choukroun et al., 1998; Purcell et al., 2007; Ramirez et al., 1997; Zechner et al., 1997). However, since ERK1/2 signalling pathways are very complex and the same cascades can lead to both cardiac hypertrophy-promoting and cardioprotective outcomes depending on particular circumstances and ERK activation mechanisms, a lot remains to be clarified (Bernardo et al., 2010; Rose et al., 2010).

If possible, the role of the stress-activated MAPKs, JNK and p38, in cardiac hypertrophy is even more ambiguous. JNK expression is rapidly upregulated following stress induction. However, this activation is transient, and the two cardiac JNK isoforms, JNK1 and JNK2, activate differently: JNK1 is significantly translocated into the nucleus, whereas JNK2 is upregulated in the cytosol (Fischer et al., 2001; Nadruz et al., 2004). While some experiments demonstrate JNK activation as important in promoting cellular hypertrophy (Choukroun et al., 1999; Wang, Su, et al., 1998), some studies report that inhibition of JNK activity contributes to cardiac hypertrophy (Liang et al., 2003; Liu et al., 2009). It seems that the experimental model and the type of hypertrophy stressor have a major influence on whether JNK acts as a prohypertrophic or antihypertrophic regulator (Rose et al., 2010). Even though there is controversy around JNK signalling, the main pathways promoting pathological hypertrophy involve a three-tiered kinase cascade, in which the first step is the activation of a MAPK kinase kinase (e.g. MEKK1-4), which then activates a MAPK kinase MKK4 or MKK7, which in turn activates JNK through serial phosphorylation (Garrington & Johnson, 1999; Molkentin & Dorn, 2001). Activated JNK leads to the downstream activation of transcription factors MEF2 and GATA4, resulting in increased expression of hypertrophy-related genes (Nakamura & Sadoshima, 2018). (**Figure 4**) Additionally, JNK activation is related to pathological cardiac remodelling, increased expression of fetal genes, apoptosis and extracellular matrix remodelling (Petrich et al., 2003, 2004; Rose et al., 2010; Xie et al., 2009). However, since cardiac remodelling and other cardiac defects often go hand in hand, it is difficult to separate whether they are specific and distinct responses to JNK activation or ensuing from the activation of other signalling cascades recruited by hypertrophy (Petrich & Wang, 2004; Rose et al., 2010). On the other hand, the antihypertrophic regulation of JNK is related to the ability of JNK to inhibit the translocation of the prohypertrophic transcription factor NFAT into the nucleus and thus prevent the hypertrophic gene reading (Liang et al., 2003; Liu et al., 2009; Molkentin, 2004). Another pathway related to JNK antihypertrophic signalling is the activation of AKT, which is regarded as a key prosurvival protein (Shao et al., 2006).

As another stress-activated MAPK, p38 highly resembles JNK in terms of activating stimuli that p38 responds to and canonical activation pathways (Rose et al., 2010). The upstream kinases activating p38 include MAPK kinase kinase (like MEKK1-4, TGF-β-activated kinase 1 (TAK1) and apoptosis signal-regulating

kinase 1 (ASK1)), leading to the activation of MAPK kinase MKK3 or MKK6 and final phosphorylation of p38, especially isoforms p38α and p38β in the heart (Garrington & Johnson, 1999; Molkentin & Dorn, 2001). Furthermore, p38 can also be activated in noncanonical ways, like autophosphorylation (Ge et al., 2002; Tanno et al., 2003). Once p38 is activated, it can function in the cytoplasm to activate cytoplasmic proteins or translocate to the nucleus to activate nuclear proteins or transcription factors, such as MEF2 and GATA4, and thereby increase hypertrophy-related gene expression (Nakamura & Sadoshima, 2018; Ono & Han, 2000). (**Figure 4**) Activation of p38 promotes cellular growth and hypertrophy (Kinugawa et al., 2005; Nemoto et al., 1998; Wang, Huang, et al., 1998; Zechner et al., 1997), and p38 is reported to be activated in hearts under experimental pathological conditions, and demonstrated to modulate ventricular hypertrophy and remodelling, for instance, in spared myocardium after MI (Matsumoto-Ida et al., 2006). Furthermore, p38 activation *in vitro* and *in vivo* promotes pathological cardiac remodelling by inducing inflammatory actions, ECM remodelling and contractile dysfunction (Li et al., 2005; Liao et al., 2002). In addition, *in vivo* studies exploiting the overexpression of p38, its cardiac-specific deletion, or pharmacological inhibition have demonstrated that p38 is also related to increased fibrosis and apoptosis (Klein et al., 2005; Liao et al., 2001; Nishida et al., 2004). Although the role of p38 in hypertrophy is far from clear, and it appears that the type of stressor and the duration of stimulus (acute versus chronic) greatly influence whether p38 activation leads to hypertrophy-promoting or -suppressing responses, its activation has adverse effects on normal gene expression and cardiac function, and therefore, it is related more closely to pathological than physiological hypertrophy (Rose et al., 2010). Conversely, the effects induced by p38 on cardiomyocyte growth suppression are believed to stem from its capacity to inhibit the transcriptional activity of NFAT, in a similar manner to JNK (Braz et al., 2003; Molkentin, 2004).

In addition to the above-mentioned signalling pathways, one well-recognised ubiquitously expressed transcription factor that is activated by various extracellular factors and regulates the expression of several important genes, including those involved in cell proliferation, differentiation and survival and is related to the development of CVDs, is the cAMP response element binding protein (CREB). CREB is a nuclear transcription factor traditionally thought to mediate gene transcription selectively in response to elevated intracellular cAMP levels. (Ichiki, 2006; Mayr & Montminy, 2001) Therefore, the initial regulator of CREB phosphorylation and activation was found to be a cAMP-dependent protein kinase, PKA, and the activated signalling cascade to be in whole: cell surface GPCR-AC-cAMP-PKA-CREB (Gonzalez & Montminy, 1989). However, after this initial CREB activator, other CREB phosphorylating protein kinases and signalling pathways leading to CREB transcriptional activation have been found (Johannessen

et al., 2004). One of these pathways is related to an elevated level of intracellular Ca^{2+} and involves PLC-Ca^{2+}-CAMK cascade activation (Sheng et al., 1991; Zhai et al., 2022). Another is related to growth factor-mediated signalling and relies on the PI3K-AKT pathway (Du & Montminy, 1998). Furthermore, many of the signalling cascades leading to the activation of CREB have been demonstrated to involve MAPKs, especially ERK1/2 and p38 (Naqvi et al., 2014). Both ERK1/2 and p38 can activate a downstream kinase named mitogen- and stress-activated protein kinase 1 (MSK-1), which acts as CREB kinase, leading to CREB phosphorylation (Deak et al., 1998). In addition, CREB can be phosphorylated by MAPK-activated protein kinase-1 (MAPKAP-K1, also known as p90RSK and RSK2), a downstream kinase of Ras-MEK1-ERK1/2 pathway, or by MAPK-activated protein kinase 2 (MAPKAP-K2), a downstream kinase of p38 (Ichiki, 2006; Tan et al., 1996; Xing et al., 1996). (**Figure 4**) Nevertheless, despite the intensive research on CREB and its MAPK-mediated phosphorylation, relatively little is known about its functional role in pathological cardiac remodelling and failing heart (Ichiki, 2006). In conclusion, although a lot of research has been done concerning all MAPKs and many signalling pathways and molecules related to MAPK cascades have been well established, many contradictions remain, especially in the context of maladaptive cardiac remodelling.

2.4.3 Transcriptional changes in cardiac hypertrophy

Intracellular signalling pathways mediating pathological hypertrophy are coupled with transcriptional factors in the cell nucleus, as stated previously. This coupling regulates long-term alterations in gene expression by inducing the re-expression of fetal genes that are typically inactive in the adult heart, alongside the corresponding downregulation of adult isoforms of these genes (Bernardo et al., 2010). This reversed gene expression pattern is the major molecular characteristic of pathological hypertrophy. The upregulation of fetal genes includes those encoding proteins of atrial natriuretic peptide (ANP, also known as A-type natriuretic peptide and encoded by gene *NPPA*), B-type natriuretic peptide (BNP, also known as brain-derived natriuretic peptide and encoded by gene *NPPB*), α-skeletal actin (α-SKA, encoded by gene *ACTA1*), and MHC-β (encoded by gene *MYH7*). Other alterations in gene expression linked to pathological cardiac hypertrophy include the downregulation of genes typically expressed at higher levels in the adult heart and encoding proteins of MHC-α (encoded by gene *MYH6*), α-cardiac actin (α-CA, encoded by gene *ACTC1*), and SERCA2a (encoded by gene *ATP2A2*). Reactivation of fetal genes and a switch in contractile protein composition are likely to be cardioprotective responses against pathological hypertrophy; however, they

eventually lead to maladaptive changes in cardiac function. (Dirkx et al., 2013; van der Pol et al., 2020)

Natriuretic peptide isoforms ANP and BNP are cardiac hormones secreted from the heart that preferentially bind to natriuretic peptide receptor-A (NPR-A) and have an important role in regulating blood volume and pressure, and vascular tone via their diuretic, natriuretic and vasodilating actions and inhibitory effects on RAAS-system, for example, in the kidney and vascular smooth muscle and endothelial cells (Nishikimi et al., 2006). Although ANP and BNP are both produced in the atrium as well as in the ventricle in a normal adult human heart, the predominant production site for ANP is the atrium, while BNP is mainly produced in the ventricle. Ventricular ANP level is high in a neonatal heart, but it rapidly declines after birth. However, in cardiac hypertrophy and HF, the ventricular ANP production is reactivated. (Nishikimi et al., 2011) Overall, the expression and synthesis of ANP and BNP in the heart are increased in response to myocardial stretch or neurohumoral stress, and their plasma concentrations are also elevated (Nakagawa et al., 2019). Thus, genes encoding ANP and BNP (*Nppa* and *Nppb* in rodents) are classical hypertrophy-marker genes in experimental research, and ANP and BNP, especially the molecular form dominant in the human ventricle, N-terminal prohormone of B-type natriuretic peptide (NT-proBNP), are diagnostic biomarkers in clinical use (Nakagawa et al., 2019; Nishikimi et al., 2011; Nishikimi & Nakagawa, 2022). Furthermore, ANP and BNP are demonstrated to act in an autocrine and paracrine manner and have antihypertrophic actions in the heart. ANP and BNP binding to NPR-A are linked to intracellular activation of guanylyl cyclase, which further induces an increase in the formation of the intracellular signalling mediator cyclic guanosine monophosphate (cGMP) and thereby activation of cGMP-dependent protein kinase (PKG). PKG antagonises the calcineurin-NFAT signalling pathway and thus negatively regulates cardiomyocyte hypertrophy. (Nishikimi et al., 2006)

The contractile units of cardiomyocytes, sarcomeres, are composed of many structural and accessory proteins, the most abundant being contractile proteins myosin and actin, which form the thick and thin filaments, respectively, and are responsible for generating force when cardiomyocytes contract (**Figure 2**). The MHC isoform composition is central for myosin function and cardiomyocyte contraction force and velocity. Expression of MHC-α in ventricles is highly species dependent, being highest in small rodents and lowest in humans, leading to possible overinterpretation of results gained from murine experiments (Hamdani et al., 2008). The expression change between MHC isoforms from the fetal developmental stage to adulthood is more pronounced in animal models, where MHC-β dominates before birth and changes dramatically to MHC-α dominance after birth (Gupta, 2007). Although MHC-α forms the majority of adult murine ventricular MHCs (around 90%), in humans, it is quite the opposite (around 90 to 95% of ventricular MHCs are

composed of MHC-β) (Miyata et al., 2000; Yin et al., 2015). However, in humans, the ratio between these two MHC isoforms is essential, and even a small shift from minor MHC-α expression towards a barely detectable or undetectable level could negatively impact ventricular cardiomyocyte contractility (Miyata et al., 2000; Narolska et al., 2005). One of the major isoform changes observed in hypertrophied and failing hearts is indeed the shift from the fast MHC-α to the slow MHC-β, a shift that is thought to be beneficial under pathological conditions due to energy-conserving function (Lompre et al., 1979; Miyata et al., 2000). On the other hand, it is demonstrated that depressed contractile function can promote disease progression; therefore, in the long term, increased MHC-β expression may have a detrimental effect (Krenz & Robbins, 2004). Nonetheless, the functional significance of this MHC isoform change in diseased human ventricular myocardium is still contradictory and a matter of debate (Mercadier et al., 1983; Miyata et al., 2000; Narolska et al., 2005). In contrast to human ventricular dysfunction, human adult atria contain around 80% of MHC-α in normal situations, and MHC isoform shift has a more pronounced role in atrial dysfunctions, like atrial fibrillation, where the isoform expression is strongly switched towards β-isoform (Narolska, Eiras, et al., 2005; Reiser et al., 2001).

Changes in sarcomere thin filament proteins, actins, also occur during fetal to adult heart development and cardiac disease (Yin et al., 2015). The three main actin isoforms found in muscle cells are α-SKA, α-CA and α-smooth muscle actin (α-SMA), which are named according to their predominant expression sites: striated skeletal muscle, cardiac muscle and smooth muscle (e.g. in the vasculature), respectively (Tondeleir et al., 2009; Vandekerckhove et al., 1986). In developing cardiomyocytes, these three actin isoforms are sequentially expressed in a coordinated fashion (Driesen et al., 2009). In murine cardiomyocytes, α-SMA is expressed early in embryogenesis and in the initial stage of cardiac development, and has an important role in supporting the first contractile functions of the developing heart. However, the expression of α-SMA in the heart is transient, decreases during the final steps of cardiac development, and disappears completely in mature cardiomyocytes. During those final steps of cardiac development, first α-SKA and later α-CA replace α-SMA, and eventually, the adult murine heart is solely composed of α-CA. (Tondeleir et al., 2009) Thus, the re-expression of α-SKA in experimental murine models is a sign of cardiac hypertrophy and dysfunction (Stilli et al., 2006). However, the human cardiac development differs from the murine one, although all three α-actin isoforms are also expressed spatiotemporally in the developing human heart. α-SMA is also transiently expressed in human cardiomyocytes during the early stages of fetal life in a similar manner to murine hearts, and the α-SKA and α-CA replace the expression, but the isoform proportions significantly differ afterwards. Already in the developing human fetal heart (around

week 20 of gestation), α-SMA expression abates, and α-CA is the major and uniformly expressed isoform. At this time, α-SKA has a minor role and is only expressed in the region of developing papillary muscles. (Suurmeijer et al., 2003) However, during postnatal development, the amount of α-SKA increases and in a mature adult human heart, it forms around 20% of the total actin amount in a localised manner in both ventricles and atria, the α-CA being the predominant isoform, accounting for approximately 80% throughout the myocardium (Copeland et al., 2010; Vandekerckhove et al., 1986). Hence, in the human myocardium, α-SKA cannot be defined as an early or fetal isoform (Suurmeijer et al., 2003). Nevertheless, it can be used as a predictor of cardiac hypertrophy and dysfunction in a similar manner to murine models, since the relative amount of α-SKA increases dramatically and its distribution changes to homogenous in a disease state, especially due to pressure overload (Copeland et al., 2010; Suurmeijer et al., 2003). However, data on the increase in α-SKA in human cardiac hypertrophy and HF is still scarce and controversial (Adachi et al., 1998; Boheler et al., 1991). This isoform change is demonstrated in mice to be important for generating higher cardiac contractility and thus, it is considered to be an adaptive response to increased cardiac load (Hewett et al., 1994), although functional benefits have not been proven in humans (Copeland et al., 2010). On the contrary, re-expression of α-SMA is not present in human cardiomyocytes due to pathological conditions, even though it can occur in murine models (Black et al., 1991; Eppenberger-Eberhardt et al., 1990; Suurmeijer et al., 2003). Furthermore, mutations in the gene encoding α-CA produce discordance in the protein formation and lead to dilated or hypertrophic cardiomyopathy, proving the vitality of proper α-CA for normal heart function (Mogensen et al., 2004; Olson et al., 1998, 2000).

In addition to cardiac contractile function, proper relaxation is equally important for normal cardiac homeostasis. Ca^{2+} ion has an important second messenger role in the contraction-relaxation cycle of cardiomyocytes when converting electrical signals into cardiac contractility. (Eisner et al., 2017) Extracellular Ca^{2+} inflow via L-type Ca^{2+} channels triggers intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptor 2, and the elevated cytosolic Ca^{2+} concentration promotes actin and myosin interaction, resulting in cardiomyocyte contraction. In the relaxation phase, Ca^{2+} is excreted from the cardiomyocyte via Na/Ca^{2+} exchanger in the cell membrane or returned to the SR by SERCA2a, the major SERCA2 isoform in the heart. (Marks, 2013; Zhihao et al., 2020) SERCA2a-mediated Ca^{2+} uptake into SR forms the dominant way of Ca^{2+} removal from the cytoplasm in mammals, being more than 75%, and thus makes SERCA2a a key protein in the calcium cycle of cardiomyocytes (Milani-Nejad & Janssen, 2013; Piacentino et al., 2003). The regulation of the contraction-relaxation cycle of the heart, and especially the rate of myocardial relaxation, is dependent on SERCA2a

expression and activity, which are demonstrated to be reduced in the failing heart (Arai et al., 1993; Del Monte et al., 2001; Kho et al., 2012). Furthermore, impaired SERCA2a activity reduces Ca^{2+} content in the SR, and thereby fewer Ca^{2+} ions can be released from the SR, leading to decreased cardiomyocyte contraction and systolic dysfunction. On the other hand, loss of SERCA2a reduces the Ca^{2+} removal from the cytoplasm after the cardiomyocyte contraction, thereby impairing cardiomyocyte relaxation and contributing to diastolic dysfunction development. (Eisner et al., 2013; Samuel et al., 2018)

2.4.4 Mediators affecting both types of hypertrophy

Although the classification of hypertrophy into physiological and pathological is quite straightforward in terms of initiating stimuli and functional outcome, and even though there are several clearly distinct cellular responses, slightly paradoxically, some signalling cascades can turn out to be adaptive or maladaptive depending on very fine distinctions in activating molecules and downstream mediators. It is not that surprising after all, since all signalling mechanisms mediating cardiac hypertrophy are initially activated as an adaptive response. Essential determinants of whether the adaptation develops towards a physiological rather than pathological form of hypertrophy seem to be the duration and the context of the stimulus. Insulin is one of the major inducers of adaptive hypertrophic responses, but when the signalling is stimulated in the presence of existing pathological hypertrophy, it hampers the pathology and promotes HF (Shimizu et al., 2010). The short-term activation of AKT evokes the physiological growth of cardiomyocytes, whereas it leads to pathological hypertrophy and HF when sustained (Shiojima et al., 2005). In addition, activation of AKT-mTOR signalling is highly critical for physiological hypertrophy due to exercise and also for adaptive response in acute pressure overload, but it turns out to be detrimental when prolonged and chronically activated. Furthermore, complete deletion of the mTOR pathway leads to lethal consequences, while partial mTOR inhibition is shown to augment cardiac function. (Sciarretta et al., 2014, 2018) Ang II is one of the most well-known hormones to induce pathological cardiac hypertrophy via binding to AT_1R. However, besides this classical axis of RAAS, Ang II has a counterbalancing role via the non-classical axis when it binds to AT_2R and induces antihypertrophic responses (Bhullar & Dhalla, 2024). Another signalling cascade, calcineurin-NFAT, which is generally thought to regulate the pathological growth of the heart, can produce adaptive gene expression if it is downstream of IGF-1 instead of Ca^{2+} (Musarò et al., 1999). In addition, total inhibition of this pathway induces acute HF due to the lack of compensatory hypertrophy needed for sustained cardiac function under pressure overload (Nakamura & Sadoshima, 2018).

Besides neurohumoral agents, mechanical forces in the surrounding matrix of cardiomyocytes and inside the cells in the contractile unit of cardiomyocytes induce hypertrophic responses. These mechanical forces, like changes in pressure or volume, and increased cardiac tissue stiffness or stretch, are converted into biochemical signals via a process called mechanotransduction. (Maillet et al., 2013) These intrinsic mechano-sensing mechanisms of cardiomyocytes include stretch-sensitive ion channels (canonical transient receptor potential channels, TRPCs) (Patel et al., 2010) and signal-transducing proteins, such as integrins (Ross & Borg, 2001), within the plasma membrane, and intracellular mechano-sensing proteins within the sarcomeres at the Z-disc (Knöll et al., 2002). Since mechanical forces are induced in the hypertrophic process regardless of the type of hypertrophy, these mechanisms are induced in adaptive as well as pathological hypertrophy.

2.5 Extracellular matrix remodelling

Hemodynamic overload results in mechanical stretch in cardiomyocytes and induces neurohumoral activation and release of autocrine and paracrine signalling molecules such as hormones, growth factors, cytokines, and chemokines. Increased stress is sensed not only by cardiomyocytes but also by other cell types in the heart, like cardiac fibroblasts, endothelial cells, vascular smooth muscle cells, immune cells, and sympathetic neurons. These signalling molecules are sensed and secreted by all the different cell types in the heart, resulting in intercellular communication in the cardiac microenvironment, which has been underrated but whose importance has lately been understood (Tzahor & Dimmeler, 2022). Even though cardiomyocytes are the main cell type in the heart and are responsible for the weight and generating the contractile force, actually, other cell types outnumber cardiomyocytes. The homeostasis and proper interplay between different cell types in the heart are vital for proper cardiac function and health. (Bazgir et al., 2023) The space between cardiomyocytes, other cells, blood vessels, and nerves, the cardiac interstitium or ECM, is a mechanically stable and firm, fibre-containing structure that acts as a scaffold for cardiac cells (Brower et al., 2006; Hinderer & Schenke-Layland, 2019). Adult human cardiac ECM is a complex network of many substances, including the main structural components of fibrillar collagen type I and collagen type III, elastin, and fibronectin, which provide strength and plasticity, and various groups of non-fibrillar glycoproteins, proteoglycans, and glycosaminoglycans. In addition, ECM serves as a reservoir of growth factors, cytokines, chemokines, matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs). Even though ECM is stable, the structure-forming proteins in ECM are not static; instead, the balance between ECM synthesis and degradation is constantly regulated by the cells in the myocardium. (Jugdutt, 2003; Rienks et al., 2014; Silva et al., 2021; Weber,

1989; Wittig & Szulcek, 2021) Furthermore, cell-ECM interactions, for example, via integrins or other transmembrane receptors, greatly influence many cellular processes, such as cell proliferation, differentiation, migration, and survival (Frantz et al., 2010; Komuro, 2001).

2.5.1 Cardiac fibrosis

Cardiac fibrosis is the expansion of cardiac interstitium due to excessive accumulation of ECM in the myocardium and consequent distortion of myocardial architecture (Kong et al., 2014) (**Figure 5**). It disturbs electrical conduction and predisposes the heart to impaired contraction and relaxation, arrhythmias, dysfunction, and even sudden death. Cardiac fibrosis contributes to morbidity and mortality in cardiac hypertrophy and is an independent risk factor predicting HF development. On the other hand, it influences the clinical course and outcome in patients with existing HF. (Li et al., 2018; Schiattarella & Hill, 2015) One major hallmark of cardiac fibrosis is the accumulation of fibrillar collagen in the cardiac interstitium, meaning the markedly increased synthesis of both collagen type I and III, in fibrotic remodelling of the heart (Kong et al., 2014; Weber, 1989) (**Figure 5**). The internal composition and ratio between these collagen types are stated to slightly vary depending on the aetiology of fibrosis, favouring type I over III in hypertensive cardiac fibrosis and myocardial infarction, while being opposite in ischemic cardiomyopathy. However, this data is still sparse and controversial, and more essential is the overall increase of fibrillar collagens. (Brower et al., 2006; Cleutjens et al., 1995; Mukherjee & Sen, 1991, 1993; Wittig & Szulcek, 2021)

Cardiac fibrosis is present in virtually all different types of cardiomyopathies and is part of the pathophysiology of myocardial infarction, and pressure and volume overload-induced cardiac hypertrophy and HF (Assomull et al., 2006; Berk et al., 2007; Biernacka & Frangogiannis, 2011; Borer et al., 2002; Cavalera et al., 2014; Frangogiannis, 2012; Ho et al., 2010; Kong et al., 2014; Krejci et al., 2016; Russo & Frangogiannis, 2016). Since the initiator stimuli behind different cardiac dysfunctions differ, the type of cardiac fibrosis also differs and can be divided into reactive and replacement fibrosis based on the type of injury. Myocardial infarction, ischemia, and inflammation result in cardiomyocyte death, induce replacement of myocardium with fibrous tissue, and initiate scar formation, thus negatively influencing the contractility at the injury site. However, the formation of a fibrous scar is a double-edged sword, because it also protects the heart from rupture by preserving the structural integrity of the ventricle and by protecting it from dilative remodelling. On the contrary, pressure or volume overload, cardiomyopathies, brief repetitive ischemia, metabolic disturbances, and ageing do not lead to substantial

cardiomyocyte loss, but fibrosis occurs in a reactive manner by excessive ECM expanding into the interstitial and perivascular spaces, leading to mechanical stiffness throughout the myocardium. (Frangogiannis, 2019; Kong et al., 2014; Li et al., 2018; Mewton et al., 2011) In this type of interstitial fibrosis, the normal ventricular systolic and diastolic function is jeopardised due to an imbalance between the synthesis and degradation of myocardial interstitial ECM proteins. Increased interstitial collagen deposition in fibrotic regions throughout the myocardium triggers proteinase-dependent degradation of fibrillar collagens by activating MMPs, proteins known to regulate the ECM synthesis-degradation homeostasis. Contrarily, this phenomenon may not be benign in hypertrophic hearts, leading to disturbed structural integrity of the ventricle. (Berk et al., 2007; Frangogiannis, 2019; Iwanaga et al., 2002; Spinale et al., 2013) Additionally, in the case of perivascular fibrosis, the accumulation of connective tissue around the vessels endangers cardiomyocyte perfusion and thus cardiac function by causing microvascular dysfunction (Ytrehus et al., 2018). In addition, a phenotype of combined interstitial and replacement fibrosis is found in patients with hypertrophic cardiomyopathy, advanced ischemic cardiomyopathy, and idiopathic dilated cardiomyopathy (Beltrami et al., 1994; Moravsky et al., 2013; Schalla et al., 2010). Furthermore, in genetic cardiomyopathies, cardiac fibrosis may not only be an associated phenomenon but can directly promote hypertrophy and pathogenesis of myocardial dysfunction (Ho et al., 2010). Although myocardial fibrosis is an important part of the pathophysiology of maladaptive cardiac remodelling, and the extent of fibrosis is closely associated with adverse outcomes, it is still difficult to state whether it is a cause or a consequence (Frangogiannis, 2019).

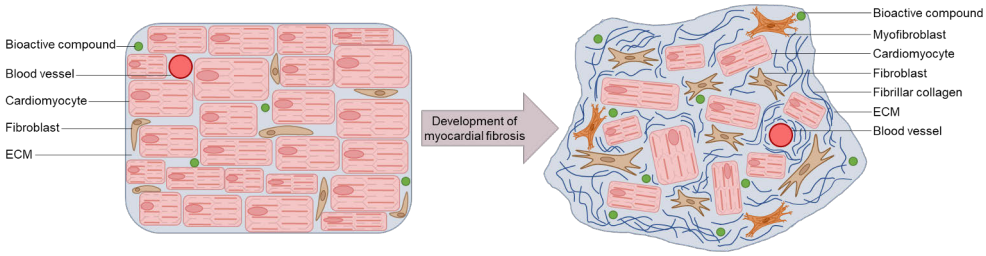


Figure 5. Overview of cardiac fibrosis. Cardiac fibrosis is characterised by an excessive accumulation of extracellular matrix in the interstitial and perivascular spaces of the myocardium. The amount of fibrillar collagens increases, cardiomyocytes are often replaced with fibrous tissue, and fibroblasts become overactivated and differentiate into myofibroblasts. The secretion of bioactive mediators promotes interaction between cardiomyocytes and fibroblasts, driving the fibrogenic process. Myocardial architecture, cardiomyocyte perfusion, and electrical conduction are compromised. ECM; extracellular matrix. Figure adapted from (Mewton et al., 2011; Kong et al., 2014).

2.5.2 The role of fibroblasts in cardiac fibrosis

Although the initiating factors leading to cardiac fibrosis, mechanisms mediating and maintaining it, and even the type of cardiac fibrosis differ in different cardiac diseases and dysfunctions, the major fibrogenic mediators and fibrotic processes are shared. Many different cell types are involved in the fibrotic remodelling of the heart, either directly by producing ECM proteins or indirectly by secreting fibrogenic mediators. The relative contribution of the various cell types depends on the underlying cause of fibrosis. (Kong et al., 2014) However, fibroblasts are necessary for fibrosis, since they directly produce ECM proteins. Cardiac fibroblasts form the highest cell population in the normal myocardium and regulate the physiological turnover of ECM proteins and tissue repair. However, in cardiac injury, their number increases, and they become hyperactive, leading to ECM protein accumulation and deposition, resulting in fibrosis. In addition to ECM protein production, fibroblasts produce cytokines, peptides, and enzymes that affect ECM homeostasis. (Fan et al., 2012)

Fibroblasts in cardiac fibrosis can originate from several different sources, such as proliferation of resident cardiac fibroblasts, reactivation of epithelial and endothelial cell transformation in processes called epithelial- or endothelial-mesenchymal transition, differentiation of perivascular cells, or recruitment of bone marrow-derived hematopoietic cells into the injury site and their transformation. (Krenning et al., 2010; Travers et al., 2016) In addition to the accumulation of cardiac fibroblasts in the heart, the fibroblast transdifferentiation into secretory and contractile cells, termed myofibroblasts, is regarded as another critical contributor and the key cellular event for cardiac fibrosis in diverse pathological conditions (Fan et al., 2012; Kong et al., 2014; Li et al., 2018) (**Figure 5**). Fibroblasts accumulate at the injury site and undergo phenotypical differentiation and conversion to myofibroblasts. The well-established criterion for myofibroblasts is their structural and functional features of smooth muscles; they can produce actin/myosin-containing stress fibres that allow them to contract. They also express extensive endoplasmic reticulum, can produce a splice variant of fibronectin (fibronectin ED-A) and frequently express α-SMA. These re-expressions are considered typical molecular features of the differentiated myofibroblast, although not necessary for their unique structure and function. (Frangogiannis, 2019; Hinz, 2007, 2010) In addition to contractility, myofibroblast differentiation has an important role in cardiac injury since they have an increased rate of proliferation and greater ability to produce ECM proteins than normal cardiac fibroblasts as well as produce and secrete different cytokines, chemokines, growth factors and other bioactive mediators including Ang II, ET-1, TGF-β, tumor necrosis factor-α (TNF-α) and interleukin (IL) family members, like IL-6, which maintain inflammatory response to injury and further promote the fibroblast transdifferentiation to myofibroblast (Fan et al., 2012).

Although in most tissues myofibroblasts undergo apoptosis and disappear after completing the tissue repair process, in the injured heart, they persist, especially in infarct scars, highlighting their role in the fibrotic process of the heart (Sun & Weber, 2000).

2.5.3 Crosstalk between cardiomyocytes and fibroblasts in cardiac fibrosis

Although cardiac fibroblasts and myofibroblasts in the heart are central to initiating and maintaining cardiac fibrosis due to injury, they do not operate alone. In addition, cardiomyocytes are critical contributors to the myocardial fibrotic response. Cardiomyocytes and cardiac fibroblasts interact in multiple ways in the injured heart, including paracrine signalling, mechanical stimuli, electrotonic coupling, and exosome-mediated crosstalk (Kakkar & Lee, 2010; Nicin et al., 2022). The increased production and deposition of ECM proteins by cardiac fibroblasts rigidify the cardiac microenvironment and thus alter mechanosensing in the neighbouring cardiomyocytes. Increased mechanical stimuli induce cardiomyocyte-ECM adhesion, for example, via increased production of cardiomyocyte surface proteins, such as integrins, and further promote hypertrophic responses in cardiomyocytes. (Bildyug, 2021; Chen et al., 2023) Additionally, in the cardiac microenvironment, both cardiomyocytes and fibroblasts can produce, release, and receive miRNA and protein-containing exosomes that can either alleviate or exacerbate the fibrotic responses (Nguyen et al., 2021).

In response to stress, cardiomyocytes can produce and secrete a wide range of paracrine factors in a similar fashion to cardiac fibroblasts and myofibroblasts. Since both cell types, cardiomyocytes and fibroblasts, produce and secrete the same bioactive mediators, particularly TGF-β, TNF-α, IL family members, Ang II, and ET-1, these paracrine signalling systems critically participate in the crosstalk between cardiomyocytes and fibroblasts and establish fibrogenic signalling (**Figure 5**). (Cartledge et al., 2015; Nicin et al., 2022) First of all, cardiomyocyte loss strongly induces fibroblast activation, which serves as the initial stimulus triggering fibrosis through inflammation-driven fibrogenic signalling. In other pathological conditions where cardiomyocyte loss is not the major contributor, fibrogenic programmes and consequent fibroblast activation are still induced in cardiomyocytes. (Frangogiannis, 2019; Kong et al., 2014)

Besides the induction of fibrotic remodelling of the heart, bidirectional crosstalk between cardiomyocytes and cardiac fibroblasts is important for cardiac mechanical and electrical function in both normal and diseased hearts (Zhang et al., 2012). Traditionally, cardiac fibroblasts have been thought to affect cardiac electrophysiology indirectly by creating conduction barriers when accumulating in

the heart and physically isolating cardiomyocytes from each other. However, more recently, it has been shown that functional coupling between cardiomyocytes and cardiac fibroblasts via cell-cell interactions through connexin and gap junctions, which allow intercellular movement of signalling molecules and ions, is important. Cardiomyocyte-cardiac fibroblast interactions induce electrophysiological remodelling, leading to changes in fibroblast membrane currents and arrhythmogenic responses in cardiomyocytes, contributing to the development and worsening of cardiac arrhythmias. (Rog-Zielinska et al., 2016; Vasquez et al., 2010, 2011) Although fibroblasts are regarded as the initiators of unwanted fibrosis and contributors to cardiac dysfunction in many ways, a better understanding of how they interact with cardiomyocytes in the heart may provide ways to novel treatment approaches for HF (Kakkar & Lee, 2010).

2.6 Other processes contributing to pathological cardiac remodelling

In addition to cardiomyocytes and fibroblasts, other cells play a role in pathological remodelling. Cardiomyocytes initially attempt to adapt to changing conditions but eventually eliminate malfunctioning cells. Inflammation is an integral response to pathological cardiac remodelling, with both beneficial and detrimental effects. It attempts to alleviate and protect the cardiac tissue, but often worsens the outcome.

2.6.1 Cellular interactions and cardiomyocyte adaptation mechanisms

Cellular communication between different cell types in the heart is essential for tissue homeostasis, adaptation, repair, and regeneration, but the complex interplay can also lead to pathophysiological remodelling. Changes in the communication between cells in response to cardiac injury can aggravate tissue damage, result in remodelling, and eventually lead to HF. (Tzahor & Dimmeler, 2022) In addition to direct cellular communication, cardiac stress leads to intracellular changes that reflect altered microenvironment homeostasis. For instance, endothelial cells are needed not only for fibrosis as a source of myofibroblast formation but also to secure sufficient angiogenesis and myocardial capillary density and preclude capillary rarefaction, thus ensuring proper nutrient and oxygen delivery to cardiomyocytes. Consequently, the expression and release of autocrine and paracrine factors by endothelial cells, and changes in endothelial function and endothelial-cardiomyocyte communication, can influence, for example, cardiomyocyte contractility, energy metabolism, and oxidation as well. (Brutsaert, 2003; Esper et al., 2006; Jabs et al., 2018)

Although cardiomyocyte hypertrophy is a well-recognised cellular adaptation in cardiac remodelling, cardiomyocytes can also shrink in size and undergo atrophy in response to cardiac stress, leading to worsened cardiac function (Weber et al., 2013). Furthermore, cellular senescence, described by an irreversible cell cycle arrest, is a cellular adaptation traditionally considered protective, since it limits the proliferation of nonfunctional, aged cells. However, in the heart, senescent cardiomyocytes have a metabolically active role since they release multiple autocrine and paracrine factors, including inflammatory cytokines, TNF-α, and TGF-β, and induce inflammation, fibrosis, and senescence phenotype in other cardiac cell types. In addition, senescent cardiomyocytes have impaired contractility and abnormal conduction patterns, which contribute to cardiac dysfunction. (Chen et al., 2022; Gude et al., 2018)

2.6.2 Cell death

Pathological cardiac remodelling is accompanied by increased cardiomyocyte death, which often occurs in parallel with fibrosis. Prior to cell death, cardiac stress or injury activates autophagy, where cells degrade and recycle aged proteins and damaged organelles to prevent their accumulation in the cytoplasm. As such, autophagy is essential for maintaining tissue homeostasis, but a prolonged or exaggerated increase in autophagy is detrimental. (Chiong et al., 2011) Accelerated autophagy has been demonstrated in pathological cardiac hypertrophy and other cardiac diseases in all cell types in the heart, leading to cardiomyocyte remodelling, death, and disease progression (Fidziańska et al., 2010; Lavandero et al., 2015).

The two most well-known organised cell death programmes in cardiac dysfunctions are apoptosis and necrosis. Both are mediated by two pathways in cardiomyocytes: the extrinsic and the intrinsic pathways. (Chiong et al., 2011; Del Re et al., 2019) The extrinsic pathway, also known as death receptor-mediated apoptosis, is initiated by death ligands, such as TNF-α or Fas-ligand (FasL), binding to cognate cell surface death receptors, like TNF receptor 1 or Fas, which results in the formation of sequential protein complexes I and II and leads to activation of the specific proteases, called caspases. A cascade of cleavage and activation of different caspases eventually produces cell death. Apoptosis can also be triggered by GPCRs and activation of kinases such as apoptosis signal-regulating kinase 1 (ASK1), p38, JNK and CaMKII, which stimulate apoptosis through transcriptional upregulation and post-translational modification of pro-apoptotic genes, like members of B cell lymphoma-2 (BCL-2) family (Dorn, 2009; Shah & Mann, 2011). The intrinsic apoptotic pathway is dependent on mitochondrial outer membrane permeabilisation by BCL-2-associated X protein (BAX) and BCL-2 antagonist/killer 1 (BAK). Activation of pro-apoptotic BAX/BAK allows the release of cytochrome c from

mitochondria into the cytosol, where it functions as a cofactor in the assembly of the apoptosome and serial caspase activation, leading to cell death. (Chiong et al., 2011; Del Re et al., 2019; Konstantinidis et al., 2012)

Unlike the caspases in apoptosis, there is no single mechanism defined in necrotic signalling that would be common in both types of necrosis. In death receptor-mediated necrosis, termed necroptosis, the same ligand-receptor interactions occur as in apoptosis, but the protein complexes I and II formations lead to the activation of serine/threonine protein kinases named receptor-interacting protein kinase 1 (RIPK1) and RIPK3. This activation then results in phosphorylation and activation of a pseudokinase called mixed lineage kinase-like domain (MLKL), which oligomerizes and permeabilizes the plasma membrane to induce necroptosis. (Del Re et al., 2019; Vandenabeele et al., 2010) On the contrary, the mitochondrial-mediated necrosis pathway is related to Ca^{2+} regulation, and disturbed ATP synthesis and mitochondrial membrane potential (Weber et al., 2013). Especially, sustained Ca^{2+} stress triggers the opening of the mitochondrial permeability transition pore in the inner mitochondrial membrane, leading to loss of proton gradient, disruption of ATP synthesis, energetic deficits and necrosis with the loss of plasma membrane integrity, with a precise downstream mechanism that today remains unclear (Chiong et al., 2011; Del Re et al., 2019).

Programmed cell death of apoptosis is morphologically characterised by shrinkage, formation of apoptotic bodies, and phagocytosis of apoptotic bodies by macrophages or neighbouring cells. When apoptosis is efficient, the apoptotic bodies are removed without leakage of intracellular contents into the extracellular space, which prevents the induction of an inflammatory response. On the contrary, in necrosis, myocytes or their inner organelles become swollen and lose their defined cellular architecture, leading to organelle and plasma membrane rupture, cellular content outflow, and induction of inflammation. Furthermore, apoptosis can occur in defined cells, whereas necrosis typically involves multiple adjacent cells. Regardless of the underlying mechanism, cardiomyocyte loss is a critical component in pathological cardiac remodelling. (Chiong et al., 2011; Del Re et al., 2019; Konstantinidis et al., 2012; Vandenabeele et al., 2010)

2.6.3 Inflammation

Inflammation is closely related to cardiac dysfunction for diverse reasons, and it is a vital part of the fibrotic process, cardiomyocyte death and endothelial dysfunction, where fibroblasts and myofibroblasts, cardiomyocytes and endothelial cells can secrete inflammatory cytokines and chemokines into the cardiac microenvironment (Nicin et al., 2022; Tzahor & Dimmeler, 2022). Nevertheless, resident immune cells, such as mast cells, monocytes and macrophages, are activated, which leads to further

recruitment of immune cells to the injury site and increased production and release of cytokines, chemokines and growth factors. This can, for instance, enhance myofibroblast formation or amplify cell death. (Bazgir et al., 2023; Frangogiannis, 2019; Liu et al., 2021)

Inflammation forms a vicious cycle, where cardiac injury first stimulates the activation of inflammatory cells and the release of inflammatory mediators, which, in turn, promote the pathogenesis of cardiac hypertrophy and HF (Murphy et al., 2020). Elevated inflammatory biomarkers have even been stated to represent a hallmark feature in HF, since, according to the so-called cytokine hypothesis, HF progresses, at least partly, as a result of the deleterious effects of the endogenous cytokine cascades on the myocardium and the peripheral circulation (Mann, 2015; Seta et al., 1996). For instance, HF patients with preserved ejection fraction (EF) exhibit elevated amounts of total leukocytes and their subgroups, such as T-cells, monocytes, and macrophages in the myocardium, as well as increased plasma levels of inflammatory biomarkers, such as IL1-β, IL-6, TNF-α and MMPs (Chirinos et al., 2020; Hahn et al., 2020; Westermann et al., 2011). Furthermore, similar findings have been observed in cardiomyopathy patients, implicating an important role for inflammation in the pathogenesis of cardiac diseases (Fang et al., 2017; Monda et al., 2020).

Both innate and adaptive immune responses are activated in the heart in response to tissue injury and include multiple different inflammatory cell types (Mann, 2015). Cardiac mast cells are demonstrated to be one of the critical cell types that participate in the inflammatory response in cardiac hypertrophy and remodelling. They are a source of various groups of mediators influencing the cardiac microenvironment, including TGF-β, fibroblast growth factor, TNF-α, IL-1, IL-6, renin, histamine, and chemotactic factors that stimulate the movement of other immune cells into the inflammation site. (Mekori & Metcalfe, 2000) Besides their role in inflammation, mast cells are in close contact with other cardiac cells; for example, they induce cardiomyocyte hypertrophy by releasing histamine, interact with fibroblasts in fibrotic regions by releasing growth factors and provoking collagen production and myofibroblast turnover, and regulate endothelial cell function in perivascular sites by synthesising several endothelial cell activators. (Bazgir et al., 2023; Flier et al., 1993; Levick, 2022; Shiota et al., 2003). Furthermore, mast cell degranulation induces an alternative way to release renin by mast cell-specific protease chymase and increases Ang II in an ACE-independent way in the cardiac microenvironment. Degranulation of mast cells also induces constitutive activation of TNF-α expression, leading to further activation of nuclear factor kappa B (NF-κB) and downstream pathways of IL-6 cascade or p38 MAPK, collectively causing inflammation, and cardiac hypertrophy and dysfunction. (Bazgir et al., 2023; Frangogiannis et al., 1998; Shiota et al., 2003; Sugden & Clerk, 1998)

Additionally, monocytes and macrophages form another important group of immune cells in the heart. A heterogeneous population of macrophages lie in the healthy heart tissue, but in response to cardiac injury, the activation of macrophages and recruitment of blood monocytes and their differentiation to macrophages are increased (Epelman et al., 2014; Heidt et al., 2014). Thus, a diverse group of resident and recruited macrophages exhibit functional and phenotypic versatility, enabling them to participate in a wide range of processes, including regulation of inflammation, fibrosis, matrix remodelling, angiogenesis and regeneration. Macrophages actively take part in the early responses to stress and mediate hypertrophic cardiac remodelling. (Chen & Frangogiannis, 2017; Frangogiannis, 2015; Honold & Nahrendorf, 2018) Macrophages have been experimentally demonstrated to have a particular role in the perivascular space, where they co-localise with fibroblasts and produce inflammatory cytokines, growth factors and collagen during the cardiac hypertrophy process (Bazgir et al., 2023; Nicoletti et al., 1996). Furthermore, macrophages in the perivascular space regulate vessel contractility and function. The presence of macrophages in close contact with blood vessels and their influence on vascular endothelial cells may also lead to the up-regulation of adhesion molecules within the vasculature, affecting interactions between cells and ECM. (Azzawi et al., 2005) Macrophage recruitment to the vessels and perivascular site is primarily due to the expression of monocyte chemoattractant protein-1 by vascular cells and monocytes (Bazgir et al., 2023; Capers et al., 1997). In addition, monocytes and macrophages are a rich source of all the important growth factors and cytokines for cardiomyocyte hypertrophy and fibroblast activation, by which they can produce proteases for ECM degradation, promote cardiomyocyte cell death, and execute the clearance of dead cells and debris, thus further promoting fibrosis. (Liu et al., 2021)

In addition to mast cells, monocytes and macrophages, the cardiac microenvironment consists of a vast group of other immune cells, including neutrophils, dendritic cells, eosinophils and natural killer T cells, that have specific roles in immune responses as well as in cardiac physiology, some of them being more involved in cardioprotection and some in the regulation of maladaptive cardiac remodelling. Overall, immune cells have an essential role in initiating and advancing inflammatory, hypertrophic and fibrotic responses in the heart, which is supported by the clinical evidence on the critical role of inflammation in CVDs. (Liu et al., 2021) Furthermore, besides local inflammation in the heart, systemic inflammation due to cardiac comorbidities may lead to myocardial structural and functional alterations and predispose to the development of more severe outcomes like HF (Murphy et al., 2020; Paulus & Tschöpe, 2013).

2.7 Heart failure

When pathological hypertrophy progresses, it eventually causes maladaptive cardiac remodelling, ventricular dilatation and impaired contractile function, and the development of HF (Nakamura & Sadoshima, 2018). HF is not a single pathological diagnosis but rather a clinical syndrome with shared fundamental symptoms despite the aetiology. Most often, HF is a consequence of elevated intracardiac pressure and disturbed cardiac output, first during exercise and later also at rest. These predispose the heart to structural and functional abnormalities and eventually to either systolic or diastolic dysfunction or a combination of both forms. However, pathology of the valves, pericardium and endocardium, and abnormalities of heart rhythm and conduction can also cause or contribute to HF. (McDonagh et al., 2021)

With an estimated prevalence of more than 56 million individuals worldwide and accounting for around 1 to 3% of the general adult population, HF is a major global health concern (Khan et al., 2024; Martin et al., 2024; Savarese et al., 2022). Furthermore, since studies usually only include recognised or diagnosed HF cases and data from developing countries is often limited or missing, the true prevalence is likely to be even higher (Martin et al., 2024; Savarese et al., 2022; Van Riet et al., 2014). Consequently, HF was already designated as an emerging epidemic in 1997 (Braunwald, 1997), and its prevalence continues to increase (Bozkurt et al., 2025; Khan et al., 2024; Savarese et al., 2022). The prevalence increases with age, being around 1% for those below 55 years to over 10% for those aged 70 years or over (Martin et al., 2024; McDonagh et al., 2021). Alarmingly, however, the morbidity of younger individuals to HF is increasing, together with age-adjusted mortality in young adults (aged 15-44 years) compared to older patients (Bozkurt et al., 2025; Khan et al., 2024). The epidemiology of HF overall is highly heterogeneous and differs, for instance, due to geographical region and an individual's sex, age, and ethnicity. For example, in the United States (US), the lifetime risk of developing HF is 1 in 4 people today, meaning that every fourth person will develop HF in their lifetime. (Bozkurt et al., 2025; Khan et al., 2024) Although the age-adjusted incidence of HF has stabilised, possibly due to better management of CVDs and risk factors, the overall incidence and prevalence of HF are increasing due to ageing populations and improved survival rates, likely reflecting better treatment outcomes. Furthermore, even with enhancements in risk factor management, their increased prevalence negatively impacts HF prevalence. (Khan et al., 2024; McDonagh et al., 2021) Moreover, HF causes a considerable global economic burden, for example, due to hospitalisation, long-term care, medication, rehabilitation, loss of working competence, early retirement, and premature mortality. (Cook et al., 2014; Hessel, 2021; Lesyuk et al., 2018).

The most common causes of HF include CAD, hypertension, valvular diseases such as aortic stenosis, arrhythmias, cardiomyopathies, congenital heart diseases,

infections, and drug-induced adverse effects, for instance, due to anticancer therapy, among many others (James et al., 2018; McDonagh et al., 2021). The increased prevalence of risk factors or comorbidities, especially those related to lifestyle in the Western world, dramatically affects the occurrence of HF as well. These risk factors include, for instance, overweight, obesity, T2DM, sedentary lifestyle, smoking, excessive alcohol consumption, hypertension, hyperlipidemia, COPD, and chronic kidney disease (Dunlay et al., 2009; Khan et al., 2024). Moreover, HF often has a negative impact on patients' quality of life, and the first and most prevalent symptoms of HF or predicting its development include breathlessness when exercising and later at rest or even lying, reduced exercise tolerance, increased time to recover after exercise, fatigue, and ankle swelling. Typically, these symptoms are conjoined with diverse clinical signs prognosticating or diagnosing HF, such as elevated jugular vein pressure, cardiac murmur, changes in pulse, atypical lung sounds, fluid in the lungs, and abnormal respiratory patterns. (McDonagh et al., 2021) If the symptoms and/or signs of HF are not accurate enough to make a diagnosis, an electrocardiogram (ECG) is performed next. If the ECG is abnormal, the HF diagnostic algorithm continues with the plasma concentration measurement of NT-proBNP and echocardiography imaging. (Galderisi et al., 2017; Gardner et al., 2003; McDonagh et al., 2021; Roberts et al., 2015)

HF can be broadly divided into two categories: chronic HF (CHF) and acute HF (AHF). In CHF, patients have an established diagnosis with a gradual onset of symptoms, while AHF refers to the new onset of rapid symptoms or signs of HF, occurring secondary to an insult such as acute myocardial infarction, severe myocarditis, or acute valvular regurgitation, and is severe enough to necessitate urgent medical help. Although current guidelines also recognise sudden deterioration of CHF, termed acutely decompensated CHF (ADCHF), as the most common form of AHF, alongside de novo AHF, there is controversy surrounding this nomenclature, with an increasing recognition of ADCHF as an integral part of the natural progression of CHF rather than a subdivision of AHF. (McDonagh et al., 2021; Xanthopoulos et al., 2020)

Besides this categorisation, when considering CHF, it is also important to classify the severity of HF as a part of the diagnosis. One well-established classification system, also described by the European Society of Cardiology (ESC) guidelines, is the New York Heart Association (NYHA) classification, which categorises HF based on the severity of symptoms. The NYHA classification is divided into four classes. In class I, no limitations in physical activity and no symptoms are observed with normal physical activity. In class II, slight limitations of physical activity and mild symptoms are evident during usual physical activity, but none are observed at rest. In class III, there are marked limitations of physical activity and moderate symptoms with less than normal physical activity, but patients

are comfortable at rest. In class IV, patients are unable to perform any physical activity and might have severe shortness of breath, even at rest. (Hunt et al., 2001; McDonagh et al., 2021) The American Heart Association/American College of Cardiology (AHA/ACC) guidelines propose another widely recognised classification system, which outlines four stages of HF based on the evolution and progression of the disease. The HF is divided into preclinical stages A and B, where A represents the stage at which the patient is at high risk of HF but has no structural heart disease or HF symptoms, and stage B defines structural heart disease without signs or symptoms of HF. Stages C and D are characterised by the presence of overt clinical HF of increasing severity from C to D. In stage C, the patient has structural heart disease with prior or current HF symptoms, while in stage D, HF has evolved into the refractory stage, requiring specialised interventions. (Hunt et al., 2001; Roger, 2021) Furthermore, advanced HF is a difficult but clinically important designation since it can guide the consideration of advanced therapies, including transplant and mechanical ventricular assistance for critical patients (Crespo-Leiro et al., 2018; Truby & Rogers, 2020). However, although classifications are important, it is vital to remember that even patients with mild symptoms may have a high risk of hospitalisation and death (Caraballo et al., 2019; Solomon, Claggett, Packer, et al., 2016). In conclusion, the identification of the aetiology of the underlying cardiac dysfunction is mandatory in the diagnosis of HF, as the specific pathology can prognosticate the severity of HF and determine subsequent treatment (McDonagh et al., 2021).

2.7.1 Heart failure classification based on ejection fraction

Since the heart is a pump that serially fills and empties, it can fail in two ways. The heart with sufficient filling but inadequate emptying leads to systolic HF, while inadequate filling during diastole despite proper ejection during systole leads to diastolic HF. These syndromes are counterparts of those defined in terms of EF: systolic HF is subsequently called HF with reduced EF (HFrEF), and diastolic HF is called HF with preserved EF (HFpEF). The underlying pathophysiology in HFrEF is characterised by dilated LV, which results in impaired ejection. On the contrary, in the pathophysiology of HFpEF, end-diastolic volume (EDV) stays normal or decreases due to LV wall thickening, inducing impaired filling. In both cases, the cardiac form fails to follow function. (**Figure 1**) (Katz & Rolett, 2016)

EF is a measurement that expresses in percentage how much blood the LV of the heart can pump out per heartbeat (termed as stroke volume, SV) from the total amount of blood present in the LV during the end-diastolic phase (EDV), and is calculated by dividing SV by EDV (Katz & Rolett, 2016). Currently, the guidelines of ESC and AHA/ACC classify patients with HF into three categories based on the

LV function determined by echocardiographic measurement of LVEF. Patients with a systolic LVEF of <40% are identified to have HFrEF, patients with a systolic LVEF between 41 and 49% have HF with mildly reduced EF (HFmrEF), and patients with a LVEF ≥50% have HFpEF. Since the EF in HFpEF remains quite normal, the diagnosis is based on symptoms and signs of HF, objective evidence of cardiac structural and/or functional abnormalities, elevated levels of natriuretic peptides, and the presence of LV diastolic dysfunction or at least elevated LV filling pressure. (Heidenreich et al., 2022; McDonagh et al., 2021)

Although the definition of HF based on LVEF is the primary way of measuring, evaluating, and categorising HF in the clinic in Europe and North America, and it is consistent with a recent report on the Universal Definition of Heart Failure, some limitations need to be taken into account (Bozkurt et al., 2021; Khan et al., 2024; Roger, 2021). First of all, echocardiography is a highly subjective method, including inter-observer and intraobserver variability, and as a result, especially the definition of HFmrEF can be questionable (Greenberg et al., 2021; McGowan & Cleland, 2003). The main rationale behind using the HF definition based on LVEF relates to treatment trials that have demonstrated substantially improved outcomes in HFrEF patients but no beneficial effects in patients with LVEF ≥50%. Furthermore, patients with HFmrEF have shown possible benefits from therapies similar to those with HFrEF. (Abdul-Rahim et al., 2018; Cleland et al., 2018; Lund et al., 2018; McDonagh et al., 2021; Solomon et al., 2016) Based on clinical trial evidence, the nomenclature of patients with HF and mid-range LVEF became established in mildly reduced EF (Lam et al., 2020). Despite the similarities in symptoms and signs, clinical trials substantiate these forms of HF to be distinct disorders that are probably induced by different mechanisms, which reflect the different treatment responses and emphasise the need for a better understanding of the underlying mechanisms, especially regarding HFpEF (Abbate et al., 2015; Del Buono et al., 2020).

Although different HF types share many risk factors, some underlying factors and characteristics are clearly distinct between HFrEF and HFpEF, leading to different patient populations in these HF types. Patients of HFrEF are more often males and younger, and have CAD as a comorbidity. On the contrary, female sex, high age, arterial hypertension, previous atrial fibrillation, chronic kidney disease, COPD, obesity, and T2DM are risk factors and comorbidities for HFpEF, and are more prevalent in HFpEF than HFrEF. Overall, HFpEF is a highly complex entity involving many other defects beyond LV diastolic dysfunction. Therefore, it is also more challenging to detect and clinical echocardiography assessment includes beyond EF, detection of variables, such as left atrial dimension, blood flow velocity through the mitral valve during LV early filling (mitral E peak velocity), and concurrent septal tissue movement velocity (e' velocity). (Abbate et al., 2015; Del Buono et al., 2020; Pieske et al., 2019)

2.7.2 Current pharmacological treatment modalities of heart failure

The current standard of care for HF patients aims to enhance their clinical status, functional capacity and quality of life, prevent hospitalisation and readmission, and reduce mortality. The modulation of RAAS and the sympathetic nervous system with ACE inhibitors (ACE-I, e.g. captopril, enalapril, ramipril) or an angiotensin receptor-neprilysin inhibitor (ARNI) or AT_1 receptor blocker (ARB, e.g. candesartan, valsartan), β-blockers (e.g. bisoprolol), and mineralocorticoid receptor antagonists (MRA, spironolactone or eplerenone) form the cornerstone of pharmacotherapy recommended for all patients with HFrEF. The triad of drugs comprising an ACE-I/ARNI/ARB, β-blocker, and MRA has been shown to reduce symptoms and the risk of HF hospitalisation and improve survival in patients with HFrEF. (Heidenreich et al., 2022; McDonagh et al., 2021)

ACE-Is were the first class of drugs shown to reduce mortality and morbidity and improve symptoms. They are still recommended for all patients with HFrEF unless contraindicated or intolerant, and they should be uptitrated to the maximum tolerated recommended doses. However, according to the newest guidelines, ARNI may be considered as a first-line treatment instead of ACE-I and at least a replacement therapy for patients who remain symptomatic despite optimal treatment outlined with ACE-I, β-blocker, and MRA. (Heidenreich et al., 2022; McDonagh et al., 2021; McMurray et al., 2014; Velazquez et al., 2019) ARNI is a dual blocker treatment combining ARB (valsartan) and a neprilysin inhibitor (sacubitril) in a single compound. Neprilysin inhibitor decreases the degradation of vasoactive peptides ANP and BNP, which act antagonistically to RAAS, having favourable effects on HF. However, neprilysin also degrades Ang II, meaning its inhibition results in an accumulation of Ang II. Therefore, combining a neprilysin inhibitor with an ARB that blocks the effects of excess Ang II is essential. (Bozkurt et al., 2023) ARBs alone have a role for patients who are contraindicated or intolerant to ACE-I or ARNI (McDonagh et al., 2021). In addition to the above-mentioned optimal medical therapy, sodium-glucose co-transporter 2 inhibitors (SGLT2-I, dapagliflozin and empagliflozin) are demonstrated to reduce hospitalisation, CV death, as well as all-cause mortality, and improve the quality of life in long-term clinical trials with HFrEF patients regardless of their diabetes status, and are therefore recommended as additional therapy (Butler et al., 2021; Kosiborod et al., 2020; McMurray et al., 2019; Packer et al., 2020). Furthermore, diuretics are recommended for symptomatic patients to alleviate symptoms, improve exercise capacity, and reduce HF hospitalisation (McDonagh et al., 2021). More recently, intravenous iron supplementation has also been recommended for symptomatic HFrEF patients with iron deficiency to alleviate symptoms, improve the quality of life, and reduce HF hospitalisation (Graham et

al., 2023; Kalra et al., 2022; McDonagh et al., 2023; Vukadinović et al., 2023). In addition, there are some inotropic agents, such as ivabradine and digoxin, that can be considered in conjunction with optimal recommended therapy in selective patients with HFrEF and specific symptoms (Digitalis Investigation Group, 1997; McDonagh et al., 2021; Swedberg et al., 2010; Van Veldhuisen & Bauersachs, 2023).

Pharmacological treatment consideration for patients with HFmrEF is more challenging since, although they share similarities with patients of HFrEF, no substantial randomised clinical trial has been performed exclusively in patients with HFmrEF. For symptomatic patients, however, like in all forms of HF, diuretics are recommended for fluid retention and to alleviate symptoms. (Heidenreich et al., 2022; McDonagh et al., 2021) Furthermore, recent clinical trials have shown that the benefits of SGLT2-Is dapagliflozin and empagliflozin are not tied to EF, and thus, they are recommended for first-class treatment with diuretics for HFmrEF (Anker et al., 2021; McDonagh et al., 2023; Solomon et al., 2022). Furthermore, new trials and meta-analyses regarding iron deficiency have changed the recommendation of intravenous iron supplementation to be recommended for symptomatic patients with HFmrEF and iron deficiency to alleviate HF symptoms, improve quality of life, and possibly reduce the risk of HF hospitalisation in a similar way to HFrEF (Graham et al., 2023; Kalra et al., 2022; McDonagh et al., 2023; Vukadinović et al., 2023). Additionally, retrospective subgroup analysis of clinical trials that initially targeted patients with HFrEF or HFpEF but included patients with LVEF between 41 to 49% has also revealed that treatment with ACE-I, ARNI, ARB, β-blocker, and MRA probably has beneficial effects and may be considered in patients with HFmrEF as well to reduce the risk of HF hospitalisation and death (Cleland et al., 2018; Lund et al., 2018; Solomon, Claggett, Lewis, et al., 2016; Solomon et al., 2019, 2020). Furthermore, HFmrEF patients often have other CV indications, such as CAD, hypertension and LV systolic dysfunction due to myocardial infarction or atrial fibrillation, due to which they are already treated with the same pharmacological agents (McDonagh et al., 2021).

While HFrEF is a well-established clinical entity with several proven therapeutic strategies, and HFmrEF also responds quite effectively to medical therapy, HFpEF is a more heterogeneous syndrome with increased prevalence and a lack of effective therapeutic options. Although diastolic dysfunction has traditionally been the fundamental factor in HFpEF, the pathophysiology has been recognised to be more complex and versatile, involving other cardiac impairments such as abnormalities in LV systolic function, RV function and left atrial function, and often co-existing with multi-organ impairments, including vascular, pulmonary, renal, and peripheral skeletal muscle dysfunctions. Together with the risk factors of ageing, physical inactivity, hypertension, adiposity, and systemic metabolic disorders, with low-grade

inflammation as a result, it is essential to investigate the underlying aetiology to be able to determine the most appropriate treatment. (Del Buono et al., 2020) Consequently, in the absence of disease-modifying drugs, guideline recommendations for pharmacological treatment of HFpEF include mainly diuretics for fluid removal and symptom relief, and treatments to manage associated CV and non-CV comorbidities. Furthermore, when proper pharmacological treatments are lacking, the counselling of lifestyle changes is emphasised. Nevertheless, regardless of the lack of evidence of a reduction in clinical events, including CV and all-cause mortality, the vast majority of HFpEF patients are treated with ACE-I, ARNI, ARB, β-blocker or MRA due to concomitant CVD, e.g. hypertension or CAD. (Heidenreich et al., 2022; McDonagh et al., 2021)

However, recent clinical trials showed beneficial effects of SGLT2-Is dapagliflozin and empagliflozin consistently in patients with preserved LVEF by reducing HF hospitalisation, and as a result, they are now recommended for the first-class pharmacological treatment of HFpEF (Anker et al., 2021; McDonagh et al., 2023; Solomon et al., 2022). In addition to SGLT2-Is, another compound group that has previous recommendations for add-on therapy in patients with T2DM and atherosclerotic cardiovascular disease is glucagon-like peptide-1 receptor agonists (GLP-1 RA), which has generated considerable interest in the last few years (Khan et al., 2020). Especially one of these drugs, semaglutide, which was initially used in the management of T2DM and overweight or obesity, was proven to be safe and efficacious first for reducing adverse CV events in patients with pre-existing CVD (e.g. previous myocardial infarction, stroke or symptomatic peripheral arterial disease) and T2DM or pre-existing CVD and overweight or obesity without T2DM (Lincoff et al., 2023). Subsequently, semaglutide has proven to reduce symptoms and physical limitations, improve exercise function, and lead to weight loss in HFpEF patients with obesity and with or without T2DM (Butler et al., 2024; Kosiborod et al., 2023). Since the majority of HFpEF patients are overweight or obese and have excess adiposity, they form an important HFpEF subgroup with more adverse hemodynamic and clinical features and a greater symptom burden, worse functional capacity, and more severely impaired quality of life than those with HFpEF but no obesity (Obokata et al., 2017; Reddy et al., 2019, 2020; Sorimachi et al., 2021). Furthermore, growing evidence suggests that overweight, obesity, and excess adiposity are not just co-existing conditions but may play a role in the development and progression of HFpEF (Borlaug et al., 2023; Haass et al., 2011; Haykowsky et al., 2018; Kitzman & Nicklas, 2018). Thus, although GLP-1 RA semaglutide is not a disease-modifying drug for HFpEF or recommended by the current guidelines, it could provide a greatly needed addition to the pharmacological treatment options of HFpEF.

Although there have been significant advances in pharmacological as well as other therapeutic interventions for the clinical management of HF patients over the past decades, the short- and long-term survival rates after a diagnosis of HF still remain poor. For example, the 5-year survival rate in chronic HF after a diagnosis remains close to 50% and is even lower in acute HF. (Jones et al., 2017, 2019; Laribi et al., 2012) It draws a similar picture of mortality with many types of cancers (Mamas et al., 2017; Stewart et al., 2010). Notably, a lot remains to be done to invert HF into a curable disease. A better understanding of different types of HF and the molecular mechanisms behind these diseases could allow for better treatment options in the future.

2.8 Melanocortin system

2.8.1 Pro-opiomelanocortin and melanocortins

Melanocortins are small peptide hormones cleaved and post-translationally modified from the same precursor molecule called pro-opiomelanocortin (POMC) and include melanocyte-stimulating hormones (MSH) and adrenocorticotropic hormone (ACTH). Melanocortins were initially recognised to arise from the pituitary and to induce skin pigmentation (Donald, 1980; Harno et al., 2018). The successful gene and protein sequencing of the POMC in 1979 led to the confirmation that one larger precursor protein exists and that the sequential processing of this precursor leads to the biosynthesis of the different biologically active peptides, including MSHs (Cawley et al., 2016; Nakamura et al., 1979; Nakanishi et al., 1979). To reflect the known roles of the peptides, the precursor was named POMC in the same year (Chrétien et al., 1979).

POMC is synthesised predominantly in a few key tissues in the central nervous system (CNS) and the skin. In the CNS, these tissues include the pituitary gland, precisely corticotrophs in the anterior lobe and melanotrophs in the intermediate lobe, the arcuate nucleus of the hypothalamus, and the nucleus of the solitary tract. The POMC gene is expressed and translated in these CNS tissues and skin, and the peptides cleaved from the POMC protein are released. (Harno et al., 2018) Furthermore, since the processing of POMC in these tissues is not perfect, the precursor protein POMC is also released into the circulation (Bicknell, 2008). In addition, POMC is found to be expressed in several other peripheral tissues besides the skin, such as the heart, liver, kidney, adrenal gland, spleen, gastrointestinal tract, and immune cells (DeBold et al., 1988; Millington et al., 1993, 1999; Wang et al., 2019). However, it is unclear whether translation to the protein or processing it further to peptides occurs in these peripheral locations, and thus, it is unknown what the functional significance of POMC expression is in these sites (Harno et

al., 2018). Furthermore, according to the current understanding, the vast majority of POMC-derived peptides found in the circulation are of pituitary origin. In contrast, peptides in other tissues function in an autocrine or paracrine manner. (Bicknell, 2008)

POMC is processed in a tissue-specific manner, resulting in different sets of peptides in different tissues (Bicknell, 2008). Structurally, the POMC gene includes three exons separated by intermediate introns: exon 1 lacks the translation product, exon 2 contains the translation initiation site, and exon 3 includes most of the coding region. After the transcription of POMC mRNA and its translation to an amino acid sequence, the prohormone convertases (PCs), most importantly PC1/3 and PC2, drive the post-translational processing of POMC into biologically active components. These endoproteases cleave POMC at predefined sites of adjacent pairs of arginine and lysine amino acids (Arg-Arg, Arg-Lys, Lys-Lys or Lys-Arg), which flank all bioactive peptides within the POMC sequence. (Harno et al., 2018; Humphreys, 2004) In the anterior lobe of the pituitary, PC1/3 cleaves the POMC into larger peptides of pro-ACTH and β-lipotropic hormone (β-LPH). Pro-ACTH is further cleaved, giving rise to an N-terminal fragment of POMC, joining peptide and ACTH. In humans, POMC is not further processed in the anterior pituitary, but it is more extensively processed in the intermediate lobe of the pituitary and hypothalamus by PC2. The N-terminal POMC is further cleaved into γ3-MSH, which is subsequently cleaved to form γ-MSH. ACTH is cleaved into a smaller fraction of ACTH and corticotropin-like intermediate peptide (CLIP), and β-LPH is cleaved into β-endorphin and γ-LPH, leading further to β-MSH. The formation of α-MSH from ACTH necessitates the activity of two additional enzymes: carboxypeptidase E (CPE), which removes the COOH-terminal basic amino acid residues from ACTH, and peptidyl α-amidating monooxygenase (PAM), which then amidates the COOH-terminal and generates desacetyl α-MSH (DA-α-MSH). Desacetyl α-MSH can then be acetylated in the N-terminal region by the N-acetyltransferase (N-AT) to form α-MSH. (Cawley et al., 2016; Harno et al., 2018; Toda et al., 2017; Wardlaw, 2011) This acetylation step stabilises α-MSH against proteolytic degradation and prolongs its biological activity. However, due to rapid inactivation, the half-life of both forms, desacetyl α-MSH and α-MSH, is still short. Although the enzymatic inactivation of desacetyl α-MSH and α-MSH is incompletely understood, one enzyme responsible for it is recognised to be prolylcarboxypeptidase (PRCP), which removes one COOH-terminal amino acid, truncating and thus inactivating them. (Diano, 2011; Wallingford et al., 2009) (**Figure 6**)

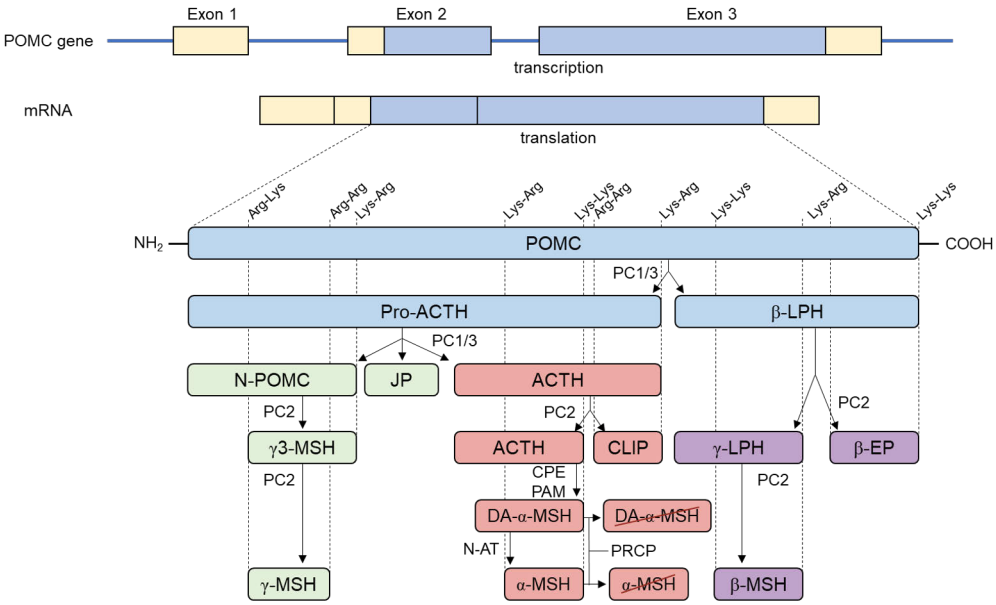


Figure 6. Melanocortin peptide processing from pro-opiomelanocortin. The POMC gene contains three exons that produce POMC mRNA during transcription and POMC precursor protein during translation. POMC is post-translationally modified by PCs at dibasic amino acid sites to produce melanocyte-stimulating hormones and other bioactive peptides. The formation of α-MSH requires additional modifications by CPE, PAM, and N-AT. PRCP can enzymatically inactivate α-MSH and DA-α-MSH (illustrated by boxes with terms crossed out). β-EP; β-endorphin, β-LPH; β-lipotropic hormone, ACTH; adrenocorticotropic hormone, Arg; arginine, CLIP; corticotropin-like intermediate peptide, CPE; carboxypeptidase E, DA-α-MSH; desacetyl α-MSH, JP; joining peptide, LPH; lipotropic hormone, Lys; lysine, MSH; melanocyte-stimulating hormone, N-AT; N-acetyltransferase, PAM; peptidyl α-amidating monooxygenase, PC; prohormone convertase, POMC; pro-opiomelanocortin, PRCP; prolylcarboxypeptidase. Figure adapted from (Harno et al., 2018).

Although all MSHs have unique structural characteristics, receptor binding properties, and biological activities, they are much alike in their amino acid sequence. Of note, α-MSH and β-MSH share the same heptapeptide sequence, and despite the slight difference in γ-MSH, all MSHs share a conserved four amino acid pharmacophore sequence (His-Phe-Arg-Trp), which is considered "the MSH signature sequence" and needed for binding and activating receptors (Gantz & Fong, 2003; Renquist et al., 2011; Wang et al., 2019). Furthermore, many POMC-derived peptides are conserved among mammalian species, particularly in the gene regions that encode the biologically active peptides of ACTH, α-MSH, and β-endorphin. However, there is some variation in the lengths of certain peptides across different species, and neither mice nor rats have the ability to produce β- and γ-MSH. (Bennett, 1986; Bumaschny et al., 2007; Donald, 1980; Harno et al., 2018)

2.8.2 Melanocortin receptors

Melanocortins mediate their physiological functions through five different but closely related G protein-coupled melanocortin receptors, named from MC1R to MC5R, corresponding to the order of their cloning (Chhajlani et al., 1993; Chhajlani & Wikberg, 1992; Gantz, Konda, et al., 1993; Gantz, Miwa, et al., 1993; Mountjoy et al., 1992). All MCRs belong to the GPCR superfamily and are therefore characterised by typical GPCR features of seven α-helical transmembrane domains, an extracellular N-terminus, and an intracellular COOH-terminus, which transduce extracellular signals to intracellular responses. However, due to the small size of MCRs within the GPCR family, they exhibit some differences as well; for example, their N- and COOH-termini are short, they have a small second extracellular loop, and they lack some amino acids typical for other GPCRs. (Yang, 2011) Most importantly, MCRs are unique GPCRs since they have two natural endogenous antagonists: agouti-related protein (AgRP) in the CNS and agouti (also known as agouti signalling peptide, ASIP) in the periphery. These antagonists act as inverse agonists by blocking agonist binding at the MCRs and inhibiting the constitutive activity of the MCRs, even when melanocortins are absent. AgRP binds to MC3R and MC4R, and agouti primarily to MC1R and MC4R, although human agouti has been shown to antagonise all melanocortins with varying affinities to MCR subtypes, suggesting a broader functional role for agouti than currently known. (Catania et al., 2004; Lu et al., 1994; Ollmann et al., 1997; Voisey & Van Daal, 2002; Yang et al., 1997)

Structurally, MCR subtypes reveal high sequence homologies varying between 38% of the identity shared by MC2R and MC4R and 60% of the identity shared by MC4R and MC5R. Despite their structural similarities, each melanocortin receptor subtype exhibits a distinct pattern of tissue expression and possesses its own profile concerning the relative potency of different melanocortin peptides. (Catania et al., 2004; Yang, 2011) This broad tissue distribution and involvement of diverse physiological functions of MCRs are summarised in **Table 2** and discussed in more detail below. Furthermore, due to the wide expression of MCRs, they are involved in many diseases as well, including obesity, diabetes mellitus, other metabolic conditions, sexual dysfunction, skin disorders, cancer, and inflammatory diseases (Hadley, 2005; Hill & Faulkner, 2016; Wikberg, 1999; Wikberg et al., 2000). From melanocortin peptides, ACTH is the only one that can bind to all MCR subtypes. Otherwise, α-, β, and γ-MSH bind with different affinities to other MCR subtypes except MC2R, α-MSH being a so-called pan-agonist with non-selective binding properties (**Table 2**). (Schiöth et al., 1997; Yang, 2011)

Table 2. Currently recognised main characteristics of melanocortin receptors. Table adapted from (Swan et al., 2024).

Receptor	Endogenous agonists binding profile	Endogenous antagonists	Primary sites of expression	Principal functions
MC1R	α-MSH = ACTH = β-MSH > γ-MSH	Agouti	Melanocytes, keratinocytes, endothelial cells, immune system	Pigmentation, anti-inflammation
MC2R	ACTH		Adrenal glands	Steroidogenesis
MC3R	γ-MSH = α-MSH = β-MSH = ACTH	AgRP	CNS, immune system	Energy homeostasis, anti-inflammation, hemodynamic regulation
MC4R	α-MSH = β-MSH = ACTH > γ-MSH	AgRP, agouti	CNS	Energy homeostasis, sexual function, hemodynamic regulation
MC5R	α-MSH > β-MSH = ACTH > γ-MSH		Exocrine glands	Production and secretion of exocrine gland products

ACTH; adrenocorticotrophic hormone, AgRP; agouti-related protein, CNS; central nervous system, MCR; melanocortin receptor, MSH; melanocyte-stimulating hormone

Ligand binding to the MCRs activates the signalling pathways, leading to cellular responses via second messengers. Two MCR accessory proteins (MRAP1 and MRAP2) regulate the transport of the MCRs towards the cell membrane and the ligand-induced signalling (Chan et al., 2009). The classical signalling pathway involves MCR coupling to the stimulatory G protein (G_s), leading to the activation of AC, an intracellular increase of cAMP and subsequent activation of PKA. All MCRs are functionally coupled to G_s and can stimulate this pathway. (Gantz & Fong, 2003) However, according to current knowledge, MCRs can couple to other G proteins ($G_{i/o}$ and G_q) and engage multiple additional downstream effectors, for example, IP_3, Ca^{2+}, and PI3K, and subsequently either activate or inhibit MAPK signalling pathways, including ERK1/2, JNK, p38, or the PKC pathway. Furthermore, it is demonstrated that MCRs can also signal in a G-protein-independent manner to some extent. (Chai et al., 2007, 2009; Konda et al., 1994; Mountjoy et al., 2001; Rodrigues et al., 2012, 2015; Roy et al., 2011; Vongs et al., 2004; Wachira et al., 2003; Watanabe et al., 1997) Due to the complex nature of MCR signalling and the significant influence of receptor-ligand pair and tissue specificity, a lot remains to be studied regarding each MCR subtype's signalling potential.

2.8.2.1 Melanocortin 1 receptor

MC1R was initially identified in melanocytes and is abundantly expressed in the skin, and has a well-known role in regulating melanin production and skin pigmentation. Human cutaneous pigmentation results from the synthesis of melanin pigments in epidermal melanocytes, followed by their transfer to keratinocytes and distribution throughout the skin. Ultraviolet (UV) radiation of sunlight induces secretion of α-MSH, which binds to MC1R, increasing the synthesis of melanin and causing a switch from the synthesis of yellow-red pheomelanin pigments to the brown-black eumelanin, which darkens skin pigmentation and provides protection against UV radiation-evoked DNA damage in the skin. Besides melanocytes and skin colour, MC1R is also found in hair follicles, and the *MC1R* gene is the best-characterised genetic determinant of human hair pigmentation and a susceptibility gene for low-penetrance skin cancers. The *MC1R* gene is highly polymorphic in humans, with several loss-of-function variants being identified and certain allelic variants being linked to red hair colour phenotype as well as increased risk of melanoma and nonmelanoma skin cancer (e.g. basal cell carcinoma and squamous cell carcinoma) and other skin disorders. (Beaumont et al., 2011; Dessinioti et al., 2011; Guida et al., 2022; Herraiz et al., 2017) Genetic studies with mice have also identified several naturally occurring dominant and recessive alleles in the mouse *Mc1r* gene. For example, the recessive $Mc1r^{e/e}$ mouse shows yellow coat colour due to a single base deletion mutation and serves as an experimental counterpart to the human red hair colour phenotype and a model to investigate suppressed MC1R function. (Robbins et al., 1993; Tamate & Takeuchi, 1984)

Besides melanocyte expression and its role in pigmentation, MC1R has been implicated in several other cell types and physiological functions. MC1R is expressed in multiple cell types of the immune system, such as monocytes, macrophages, dendritic cells, neutrophils, and CD4+ T cells, as well as in fibroblasts, endothelial cells, and cells in the intestine, indicating a special role of MC1R in immune modulation and inflammation (Andersen et al., 2005; Catania et al., 1996, 2004; Colombo et al., 2002; Hartmeyer et al., 1997; Kadiri et al., 2021; Stanisz et al., 2011). MC1R stimulation with α-MSH exerts anti-inflammatory actions by reducing pro-inflammatory and increasing anti-inflammatory cytokine production. In the intestine, MC1R plays a role in intestinal immune regulation and is demonstrated to be involved in the pathophysiology of experimental colitis (Maaser et al., 2006). In the vasculature, MC1R regulates endothelial function, arterial stiffness, and atherosclerotic plaque formation, composition, and stability (Rinne et al., 2015; Rinne, Kadiri, et al., 2018). MC1R is found in adipocytes and has proven to have a regulatory function in macrophages by promoting the clearance of excess intracellular cholesterol (Rinne et al., 2017). MC1R is also identified to have a role as a regulator of antioxidant defences, DNA-repair mechanisms, and genome

integrity (Guida et al., 2022; Li et al., 2021). In addition, although MC1R is widely distributed and has many functional roles in the periphery, it can be found in the CNS, in the periaqueductal grey matter in the midbrain, where it contributes to pain modulation (Mogil et al., 2003; Xia et al., 1995).

MC1R activity is regulated by α-MSH, ACTH, agouti, and β-defensin 3. Traditionally, MC1R has been defined as an α-MSH receptor, although α-MSH binds nonselectively to all MCRs, and in humans, ACTH has shown equal binding affinity to MC1R. Agouti is produced especially in the skin, where it competes with α-MSH for binding to MC1R and thus suppresses the production of eumelanin, resulting in the synthesis of a yellow pigment. (Abdel-Malek et al., 2000) β-defensin 3 is synthesised in the skin keratinocytes and functions in a paracrine manner by binding to the MC1R, inhibiting the receptor's interaction with both α-MSH and agouti (Swope et al., 2012; Wolf Horrell et al., 2016).

2.8.2.2 Melanocortin 2 receptor

MC2R is unique among MCRs: it has the lowest sequence homology with other MCRs and a different pharmacological profile, with ACTH being the only endogenous ligand for MC2R. Consequently, MC2R is also known as the ACTH receptor. MC2R is primarily expressed in the adrenal gland and is responsible for human steroidogenesis. (Catania et al., 2004) Several missense mutations have been identified in the coding region of the MC2R, and they are associated with familial glucocorticoid deficiency, a rare autosomal recessive disorder associated with adrenal unresponsiveness to ACTH, leading to failure to produce glucocorticoids (Clark et al., 2005; Clark & Weber, 1998; Thistlethwaite et al., 1975).

MC2R is also found in human skin (Slominski et al., 1996). Although the role of MC2R in human skin is not well-established, it is proposed to be involved in the local response to cutaneous stress in layers of skin and attached hair follicles, since human skin is shown to respond to emotional and environmental stress via the local hypothalamic-pituitary-adrenal axis. In this stress, ACTH plays a central role in stimulating cortisol production and hair growth, and consequently, the role of MC2R as an ACTH receptor mediating these effects has been under investigation. (Ito et al., 2005; Slominski et al., 2008). Stress activity of the skin is suggested to play a role in the pathogenesis of the skin disease alopecia areata, an autoimmune disease that causes hairless areas, especially on the scalp. The finding of aberrant MC2R expression and a deficit in ACTH-MC2R activity in hairless lesions of alopecia areata patients suggests the involvement of ACTH-MC2R in the disease and raises the possibility of future MC2R-targeted treatment development for this and other inflammatory skin diseases. (Hong-Wei et al., 2010).

Furthermore, MC2R is expressed in murine adipocytes in white adipose tissue, where it contributes to lipolysis when activated by ACTH (Boston, 1999; Boston & Cone, 1996; Møller et al., 2011). However, the expression and function of MC2R in human adipose tissue are controversial: some research reports that human adipocytes do not express MC2R, while some studies propose that human adipocytes express MC2R with a functional role in lipolysis regulation (Kiwaki & Levine, 2003; Møller et al., 2015; Smith et al., 2003; Xue et al., 1998; Zhang et al., 2018) Nevertheless, these findings highlight the species-specific differences in MC2R distribution and function.

2.8.2.3 Melanocortin 3 receptor

MC3R is also unique among MCRs, since it is activated by all melanocortins with similar potency. γ-MSH is considered an endogenous agonist for MC3R, since it shows significantly higher affinity and potency towards MC3R than other MCRs. Thus, MC3R is the only MCR that responds to γ-MSH stimulation at physiological concentrations. In addition, AgRP antagonises MC3R. (Cone, 2006; Renquist et al., 2011)

MC3R is predominantly expressed in the hypothalamus in the CNS, but is also found extensively in other brain areas. Expression studies investigating MC3R mRNA in the brain have mainly been conducted in rodents, and they have revealed expression in the midbrain, brainstem, and different nuclei of the hypothalamus. (Renquist et al., 2011; Roselli-Rehfuss et al., 1993; Wikberg, 1999) Furthermore, MC3R is widely expressed in multiple peripheral tissues, including the heart, gastrointestinal tract, immune cells, kidney, and skeletal muscle (Bertolini et al., 2009; Chhajlani, 1996; Gantz, Konda, et al., 1993). However, MC3R remains enigmatic regarding its physiological functions.

In the CNS, MC3R expression develops during the first postnatal weeks (Wikberg, 1999). It is suggested that MC3Rs are located presynaptically, primarily in areas of the brain that receive direct innervation from POMC immunoreactive neurons, since they act as inhibitory autoreceptors regulating MSH release from POMC neurons. However, since AgRP antagonises MC3R, it influences POMC-derived MSH signalling as well, and together, these two form a combined regulation mechanism for the control of feeding behaviour in the CNS. MC3R activation stimulates food intake by inhibiting appetite-suppressing anorexigenic POMC neurons, and AgRP antagonises this by binding to MC3R, thus suppressing appetite again. (Cone, 2006; Wikberg et al., 2000) Yet surprisingly, total deletion of MC3R in the mouse genome leads to unusual obesity syndrome with increased fat mass, stable lean mass, and mild hyperphagia, suggesting that the obesity phenotype results from increased energy efficiency and may involve behavioural aspects in feeding

regulation and energy homeostasis maintenance (Begriche et al., 2013; Butler et al., 2000; Chen et al., 2000). Furthermore, genetic mutations and polymorphism in the human MC3R gene are associated with obesity and increased risk of childhood obesity (Feng et al., 2005; Mencarelli et al., 2011).

In addition to positive and negative effects on energy balance, MC3R affects other physiological functions. MC3R and γ-MSH participate in central and peripheral hemodynamic regulation (Humphreys, 2004; Ni et al., 2003). Furthermore, the role of MC3R in inflammation has raised considerable interest. MC3R has been demonstrated to mediate anti-inflammatory effects in the periphery through leucocytes, especially macrophages, but it also regulates immunomodulatory actions in the CNS. (Lam & Getting, 2004; Muceniece et al., 2006; Wang et al., 2019) Over the years, experimental and clinical research has unveiled new physiological roles for MC3R; for example, MC3R has been shown to regulate growth, puberty, and circadian rhythm (Yanik & Durhan, 2023).

2.8.2.4 Melanocortin 4 receptor

In addition to MC3R, MC4R is the other neural and energy homeostasis-related MCR. These two receptors are often investigated together, possibly due to their partly mutual physiological functions and sites of expression, which might result from their high sequence similarity (58% overall amino acid identity and 76% similarity) (Oosterom et al., 1999) However, despite their similar features, there are distinct differences that are essential to recognise: MC4R is mainly expressed in the CNS with a wider distribution, and it is activated by ACTH, α-MSH, and β-MSH with similar affinity but binds γ-MSH with significantly lower affinity. Moreover, MC4R has two endogenous inverse agonists, agouti and AgRP.

MC4R is located abundantly and broadly in the CNS, particularly in the different areas and nuclei of the hypothalamus, but it is also found in the thalamus, cortex, brain stem, and spinal cord (Mountjoy et al., 1994). MC4R is also found in the peripheral sensory nerves (Tanabe et al., 2007). Undeniably, the most critical role for MC4R is in the control of energy homeostasis. Experimental data have shown that MC4R regulates both food intake and energy expenditure: agonist activation of MC4R decreases food intake, promotes satiety, increases energy expenditure, and contributes to weight loss, whereas inactivation of MC4R leads to the opposite phenotype. Genetic deletion of MC4R and inhibition of MC4R with agouti and AgRP resulted in obesity, hyperinsulinemia, and hyperglycemia in mice. (Adan et al., 2003; Balthasar et al., 2005; Cone, 2005; Fan et al., 1997; Huszar et al., 1997; Krashes et al., 2016; Shutter et al., 1997) Mutations in the MC4R gene in humans are also associated with obesity and account for the most common monogenic form of severe early-onset obesity (Farooqi et al., 2003; Loos, 2011; Tao, 2005; Wikberg et al., 2000).

It has also been shown that MC4R regulates glucose homeostasis and insulin sensitivity in rodents, independent of its effects on food intake and body weight. Central MC4R signalling affects fat storage and metabolism by increasing fat utilisation by receptor activation in rats and decreasing it in patients with receptor deficiency by simultaneously inducing changes in peripheral substrate utilisation and nutrient partitioning in the liver, muscle, and adipose tissue. These findings suggest a role for MC4R not only in obesity but also in obesity-associated metabolic disorders, such as T2DM, ectopic lipid deposits, and lipotoxicity. (Liu et al., 2022; Nogueiras et al., 2007; Tao, 2010) Moreover, although agouti is primarily found in the skin and was first recognised for its role in regulating pigmentation via MC1R, it is also demonstrated to be expressed in the CNS and other peripheral tissues such as the testis, ovary, heart, pancreas, and adipose tissue in humans and proposed to have a role in insulin release, lipid and energy metabolism and development of obesity. MC4R is also expressed in adipose tissue together with agouti, and consequently, the role of agouti in lipid metabolism may rely on peripheral MC4R antagonism. (Voisey & Van Daal, 2002; Wilson et al., 1995; Xue & Zemel, 2000)

MC4R also has an important role in the regulation of CV function, which is reviewed in chapter 2.10. Furthermore, MC4R has been demonstrated to regulate cellular thermogenesis in brown adipose tissue, sexual behaviour, erectile function and reproductive function, mediate central anti-inflammatory and pain responses, and even be expressed in human epidermal melanocytes that might contribute to melanogenesis (Fan et al., 2005; Martin & MacIntyre, 2004; Spencer & Schallreuter, 2009; Starowicz et al., 2009; Starowicz & Przewłocka, 2003; Tao, 2010; Wang et al., 2019; Wikberg & Mutulis, 2008).

2.8.2.5 Melanocortin 5 receptor

MC5R has a wide expression profile in peripheral tissues, and it binds all melanocortins. The distinctive feature of MC5R is its expression in secretory epithelia of exocrine and endocrine glands such as adrenal, lacrimal, and sebaceous glands, where it participates in the production and secretion of gland products (Van Der Kraan et al., 1998). This functional role of MC5R was first identified in mice lacking functional MC5R: after behavioural tests, including swimming, these mice absorbed more water in their fur, were wetter, and had impaired thermoregulation compared to wildtype (WT) mice due to impaired water repulsion and reduced production of sebaceous lipids. (Chen et al., 1997) MC5R may thus serve as a therapeutic target for the treatment of skin disorders, such as acne and dermatitis. Experimental studies have also demonstrated that MC5R deficiency affects the release of pheromones from tissues such as the preputial gland and contributes to the regulation of behaviours, including aggression and defensive behaviour, in mice

(Morgan et al., 2004; Morgan & Cone, 2006). In addition, MC5R is expressed widely, for instance, in the kidney, liver, skeletal muscle, heart, lung, skin, thymus, adipose tissue, testis, ovary, uterus, mammary gland, gastrointestinal tract, lymph nodes, and bone marrow (Catania et al., 2004; Chhajlani, 1996; Fathi et al., 1995; Wikberg et al., 2000). In addition, MC5R is expressed in immune cells and thus is involved in modulating immune responses and inflammation, especially in ocular immunity (Ng et al., 2021; Webering et al., 2019). Furthermore, in adipocytes, cytokine secretion is stated to be stimulated via MC5R (Jun et al., 2010).

Although MC5R is recognised as a peripheral receptor, it was initially found in the brain, a finding that has been later supported by the Human Protein Atlas, Mouse and Human Brain Atlas, and experimental research (Chhajlani et al., 1993; Fathi et al., 1995; Hawrylycz et al., 2012; Lein et al., 2007; Uhlén et al., 2015). MC5R in the brain has experimentally been demonstrated to be involved in fetal brain development and, later in life, proposed to have a role in maintaining or restoring cognitive function in aged rodents and rodents with neurodegenerative diseases, which has led to a hypothesis that MC5R could serve as a therapeutic target for cognitive diseases (Simamura et al., 2011; Zhou et al., 2021). Furthermore, brain expression of MC5R is related to physical activity and lean phenotype in rats (Shukla et al., 2015). In adipocytes, MC5R regulates lipid mobilisation and decreases adipocyte fat mass, while in skeletal muscle, MC5R regulates fatty acid oxidation and glucose uptake (An et al., 2007; Enriori et al., 2016; Ji et al., 2022; Rodrigues et al., 2013). Together, these data indicate a regulatory role for MC5R in energy metabolism. However, the physiological functions and role of MC5R are not yet completely understood.

2.9 Synthetic melanocortin receptor ligands and drug development

The close relations of MCR subtypes due to their high sequence homology and the lack of their three-dimensional protein structures have hampered subtype-selective ligand development (Hruby, 2002). In addition, the ability of MCRs to form constitutive homodimeric and heterodimeric or oligomeric complexes affects their functions and activity, but also the progress in the development of subtype-selective compounds (Wikberg & Mutulis, 2008). Furthermore, the unique property of the melanocortin system with endogenous agonists and antagonists has further challenged the subtype-selective ligand design due to the different binding properties, partially different binding sites, and effects of agonist versus antagonist on the same receptor (Yang, 2011). Nevertheless, this also offers possibilities for developing specialised drugs with receptor activating and silencing action, less off-target effects, and better efficacy after understanding the structure, binding and

function properties between ligands and receptor subtypes. Additionally, natural ligands, such as α-MSH, have short half-lives due to rapid enzymatic degradation in a physiological environment (D'agostino & Diano, 2010; Diano, 2011). Hence, natural MCR ligands are quite ineffective therapeutic agents since disease conditions need prolonged receptor modulation. Therefore, the development of synthetic MCR ligands, with improved stability and pharmacokinetic profile, is constantly in progress.

The first synthetic ligand for MCRs was an analogue of α-MSH, [Nle^4-D-Phe^7]-α-MSH (NDP-α-MSH), also known as afamelanotide or melanotan I (MT-I). This superpotent analogue was characterised in 1980 and has modifications in two amino acid residues compared to endogenous α-MSH, which makes it more resistant to enzymatic degradation, thus prolonging its duration of action and improving the affinity and potency towards all MCR subtypes. (Sawyer et al., 1980) Thereafter, another α-MSH analogue, Ac-Nle^4-cyclo[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2, known as melanotan II (MT-II), was developed. The cyclic peptide structure of MT-II enhanced metabolic stability, with an even longer half-life and improved bioavailability with high potency for all MCRs. (Al-Obeidi et al., 1989) Additionally, the structure of MT-II has served as a basis for developing other MCR ligands with better subtype selectivity. This has led to the development of selective agonists for MC1R and MC5R, as well as antagonists for MC3R, MC4R, and MC5R. The MC1R selective agonist LD211 (compound 28 in the original publication), MC5R agonists and MC3R and MC4R antagonists PG-901 and PG-911, MC3R and MC4R antagonist SHU9119, and MC5R antagonist PG-20N are utilised as investigational compounds to study the biological role of specific MCRs in preclinical studies, but are not in clinical use (Cai et al., 2015; Doedens et al., 2010; Grieco et al., 2002, 2008; Hruby et al., 1995; Zhou & Cai, 2017) The development of MC3R selective agonists has been more challenging, and today the most prominent compound for investigational use has been the synthetic analogue of γ-MSH, [D-Trp^8]-γ-MSH, which clearly shows the highest selectivity for MC3R and has higher affinity for MC3R than γ-MSH (Grieco et al., 2000; Hruby et al., 2007). Furthermore, small synthetic receptor selective agonists for MC4R have been developed and characterised, the most well-known being synthetic non-peptide compound THIQ (N-(3R)-1 4-tetrahydroiso-quinolinium-3-ylcarbonyl-(1R)-1-(4-chlorobenzyl)-2-4-cyclohexyl-4-(1 H-1,2,4-triazol-1-ylmethyl) piperidin-1-yl-2-oxoethylamine) (Pogozheva et al., 2005; Sebhat et al., 2002; Yang et al., 2009).

Since the melanocortin system is extensively distributed and has a central role in regulating multiple physiological functions, it has aroused a great deal of interest and promise for drug discovery across multiple therapeutic areas. Extensive efforts have been made to develop peptide and peptidomimetic drugs targeting the melanocortin system. However, the lack of stability, bioavailability, and selectivity of MCR

ligands is still the most significant hurdle for their application in clinical use. One major challenge is the delivery of the ligand; it is critical to design the proper structure for the peptide drug that permits delivery to a specific site, tissue, or cell in the body, where the desired biological process is regulated. For instance, MC3R and MC4R are mostly expressed in the CNS and peptides targeting them need to penetrate the blood-brain barrier, while peptides targeting peripheral MC1R and MC5R are safer without the ability to access the brain. Subsequently, closely related to these challenges is the selectivity issue due to the ubiquitous presence of MCRs: it is challenging to design a ligand that binds only one MCR subtype and only in certain regions without affecting other receptor subtypes or the selective receptor in other regions of the body besides the desired one. Another major barrier for peptide drugs to be accepted for the market and therapeutic use is oral bioavailability: since most peptides are easily degraded in the gut, they are mostly administered by injection, which patients strongly disfavour. (Zhou & Cai, 2017) To address these issues many peptide modification approaches are utilised: cyclisation of peptide enables blood-brain barrier crossing, conformational modulation in peptide backbone with N-methylation is suggested to improve oral bioavailability and receptor subtype selectivity, design of constrained tetrapeptide with four amino acid MSH-signature pharmacophore sequence and additional side chain groups for better potency, selectivity and binding pocket fit for linear peptides, and the usage of biased signalling effects of MCRs with preferential activation of a specific signalling pathway to distinguish and selectively regulate physiological processes (Hruby, 2002; Montero-Melendez et al., 2015; Zhou & Cai, 2017). Additionally, entirely different types of therapeutics, such as miRNA drugs or pro-drug approaches, could address the peptide-mediated challenges in the future.

The very first melanocortin peptide drug approved by the FDA for use in human inflammatory and autoimmune diseases was ACTH already in 1952 (Hench et al., 1949, 1950; Montero-Melendez, 2015). Soon after the initial finding of ACTH treatment, glucocorticoid treatments were developed, and they have largely replaced ACTH. However, over 70 years after its approval, a better understanding of the physiological functions and pharmacological mechanisms of action of ACTH and MCRs has revealed new treatment properties for ACTH and led to the repositioning of this drug. ACTH-like new medicines devoid of cortisol-related side effects and having extra-adrenal actions could now be developed, and ACTH is being tested in several active clinical trials (Montero-Melendez et al., 2022). Today, the first reported oral nonpeptide MC2R or ACTH antagonist that blocks the action of ACTH at MC2R, atumelnant (CRN04894), is in clinical phase 2 development for the treatment of ACTH-dependent Cushing's disease, a disease with excess cortisol production of the adrenal glands due to adrenal tumours, pituitary tumours with elevated ACTH production, or ACTH-producing tumours outside the pituitary

gland, and for congenital adrenal hyperplasia (ClinicalTrials.gov numbers NCT05804669 and NCT05907291) (Ragnarsson et al., 2024).

Another FDA-approved melanocortin drug from 2019 is the first synthetic analogue of α-MSH, afamelanotide, which serves as the active ingredient in the drug Scenesse®. It induces photoprotection via activating MC1R and is used for treating erythropoietic protoporphyria, a disease with intolerance to sun and artificial light. (Harms et al., 2009) Today, afamelanotide is under investigation for other skin disorders as well (Montero-Melendez et al., 2022). Two other currently approved melanocortin drugs for human use are agonists for MC4R. The first is setmelanotide, which was FDA-approved in 2020 and is marketed as Imcivree®. It is used as an anti-obesity drug for patients with mutations in POMC, proprotein convertase subtilisin/kexin type 1 (PCSK1), or leptin receptor (LEPR) genes (Clément et al., 2020; Kühnen et al., 2016; Markham, 2021). The second is bremelanotide (Vyleesi®), which was approved by the FDA in 2019 for the treatment of hypoactive sexual desire disorder in premenopausal women (Kingsberg et al., 2019; Markham, 2021; Simon et al., 2019). In addition, MT-II and particularly the cyclic melanocortin analogue bremelanotide have demonstrated high potential for treating male erectile function. Bremelanotide showed beneficial results in phase 2 clinical trials, but thereafter, the phase 3 clinical trial was postponed by the FDA due to concerns about the CV safety, and no compound has reached the market in this indication so far (Diamond et al., 2004, 2005; Hadley & Dorr, 2006; Rosen et al., 2004; Wikberg & Mutulis, 2008).

All the above-mentioned approved melanocortin drugs are pan-agonists and, hence, cause unwanted side effects by affecting other MCRs besides their desired target. Consequently, current drug development aims to improve subtype selectivity. Since MC1R is preferentially expressed in the skin and crucial for regulating skin and hair pigmentation, it represents a potential therapeutic target for skin disorders. Given the protective role of MC1R against UV-induced skin cancers in melanocytes, interest has been sparked in investigating whether activating MC1R could yield therapeutic benefits against melanoma. Even though there is evidence demonstrating that MC1R activation promotes DNA repair and may have anti-carcinogenic and tumour-suppressive effects, other results indicate detrimental effects of MC1R activation (Castejón-Griñán et al., 2018, 2024; Chen et al., 2017; Cui et al., 2023; Eves et al., 2003; Kokot et al., 2009). Thus far, some promising results have emerged from studies utilising α-MSH as a homing device for drug targeting: the α-MSH-drug conjugate is specifically recognised and internalised by MC1R-expressing melanoma tumour cells, where the conjugated drug is released. However, research of this nature is still in its infancy. (Szabó et al., 2024) Another investigational approach against melanoma has been MC1R-targeted alpha-particle radiotherapy (Tafreshi et al., 2019). Today, early clinical phase 1/2a first-in-human studies are

investigating alpha-particle-emitting therapeutic agents (VMT01/02 and MTI-201) targeted to MC1R in advanced melanoma and metastatic uveal melanoma, respectively (ClinicalTrials.gov numbers NCT05655312 and NCT05496686).

Besides skin cancers, MC1R targeting could be beneficial in other types of skin disorders. A novel oral MC1R agonist, dersimelagon (MT-7117), has demonstrated therapeutic potential for erythropoietic protoporphyria and a rare form of the disease, X-linked protoporphyria, in a completed phase 3 clinical trial (Kondo et al., 2022). Moreover, recent research has demonstrated a novel hydrogel agonist, called peptide 1, that stimulates MC1R, thereby enhancing melanin production. This compound has shown resistance to enzymatic proteolysis in *in vitro* and *ex vivo* experiments as well as potential for the treatment or prevention of vitiligo, a pigmentation disorder characterised by the loss or destruction of melanocytes in the skin and hair follicles, which results in the inability to produce melanin needed for normal pigmentation. Peptide 1 has also shown potential effects for other skin pigmentation disorders and cancers. (Zhu et al., 2023)

In addition to skin diseases, inflammation and obesity have been the dominating areas of melanocortin investigation and drug discovery in recent years. Targeting MC1R in the immune system has revealed therapeutic potential for the treatment of inflammation-related disorders. In systemic sclerosis, a disease characterised by dysregulation of the immune system, inflammation, microvascular dysfunction, and widespread fibrosis in various organs without approved medication, a novel oral MC1R agonist, dersimelagon (MT-7117), has revealed disease-modifying effects with anti-inflammatory and anti-fibrotic benefits in preclinical models, and it has proceeded into clinical phase with currently completed phase 2 trial (Kondo et al., 2022). Furthermore, MC1R activation has been shown to have anti-inflammatory and neuroprotective effects in multiple neurological disorders in preclinical experiments, including reduced neuroinflammation in rats treated with the selective MC1R agonist BMS-470539 (Kang et al., 2006; Wu et al., 2019; Yu et al., 2021). BMS-470539-induced MC1R activation has also shown neuroprotection in models of Parkinson's disease (Cai et al., 2022). The selective MC1R agonist PL-8177 has brought therapeutic benefits for intestinal and ocular inflammation in preclinical studies and is currently being investigated in a phase 2 clinical trial for the treatment of ulcerative colitis (Spana et al., 2019), and the non-selective MCR agonists PL-8331 and PL-9643 have demonstrated anti-inflammatory actions in ocular diseases and are in the late clinical research phase (Evans et al., 2023; Ng & Taylor, 2023). A novel compound, resomelagon (AP1189), has been shown to decrease cytokine levels in macrophages and to alleviate inflammatory arthritis in mice, and it is currently under clinical evaluation for the treatment of rheumatoid arthritis and idiopathic membranous nephropathy (Montero-Melendez et al., 2015, 2022).

Although obesity has been one of the most studied areas related to melanocortins, and the role of MC3R and MC4R in obesity and energy homeostasis was among the earliest discoveries of the melanocortin system, drug development has not thus far been so successful in this field. The only approved obesity-related drug, setmelanotide, is effective with a limited patient population. Nevertheless, new clinical studies for widening its therapeutic applications are ongoing, and some of them have already been completed. In addition, two new investigational oral MC4R agonists, bivamelagon (LB54640) and RM-718, are in clinical phase 2 and 1 studies, respectively, and are being investigated for the treatment of hypothalamic obesity (ClinicalTrials.gov numbers NCT06046443 and NCT06239116). Besides investigating the role of the melanocortin system in obesity, an ongoing clinical trial investigates the effect of α-MSH on glucose clearance via MC5R in skeletal muscle in patients with T2DM (ClinicalTrials.gov number NCT06293664). Additionally, one MC4R-specific antagonist, PF-07258669, is in early clinical development and another, TCMCB07, is in preclinical development for the treatment of anorexia and cachexia, with the desired function of inducing appetite and reducing weight loss (Garnsey et al., 2023; Gruber et al., 2022; Montero-Melendez et al., 2022; Sweeney et al., 2023).

Clearly, there is a great deal of excitement surrounding the development of new compounds that target the melanocortin system, as well as the clinical investigations into the drug repositioning of approved medications, such as bremelanotide. Although developing synthetic melanocortin ligands has proven to be challenging in multiple ways, the few first-in-class drugs have demonstrated safety, tolerability, and therapeutic efficacy potential, which encourages further drug development in this field.

2.10 Melanocortins and cardiovascular system

The melanocortin system is crucial in CV function, especially in regulating blood pressure and heart rate via central MCRs. Particularly, MC4R is implicated in the central regulation of CV function. MC4R is expressed in the nucleus of the solitary tract in the brain, a region known to be important for regulating CV function. Activation of MC4R raises arterial pressure and heart rate, likely through adrenergic activation, whereas receptor inhibition decreases both. (Kuo et al., 2004; Tao, 2010) Although the rise in blood pressure and heart rate could be due to mutual activation of MC3R and MC4R, experimental studies have demonstrated that α-MSH treatment increases these parameters in WT mice but not in MC4R-deficient mice, indicating that the effects were mediated via MC4R. Furthermore, α-MSH induced elevation of blood pressure and heart rate only when administered into the brain, while no effect was observed when administered intravenously, indicating purely central MCR

involvement. In addition, mice lacking MC4R showed no hypertension or renal damage despite severe obesity, hyperinsulinemia, and hyperglycemia. (do Carmo et al., 2009; Hill & Dunbar, 2002; Ni, Butler, et al., 2006) Similar observations were also recognised in humans with MC4R deficiency. These individuals were obese but had significantly lower blood pressure, resting heart rate, and 24-hour urinary noradrenaline excretion than their equally obese control subjects. Additionally, MC4R agonist treatment in overweight or obese, but otherwise healthy, volunteers with normal MC4R led to an increase in both systolic and diastolic blood pressure. (Greenfield et al., 2009) Furthermore, MC4R and its integrity are associated with the development of cachexia, a severe loss of lean body mass due to different chronic diseases and inflammatory stages, and patients with HF are also at risk of being affected by it. Different preclinical models have suggested that antagonising MC4R could lead to therapeutic benefits against cachexia. (Tao, 2010) Consequently, even though MC4R agonism has raised a lot of hope and seems to be a promising therapeutic approach for obesity treatment, close attention is warranted, since increased blood pressure and heart rate are important adverse effects associated with MC4R agonism.

MC3R and γ-MSH have been demonstrated to have a role in central and peripheral hemodynamic regulation: MC3R expression is upregulated in the kidney of rats with a high-sodium diet, while excess dietary sodium upregulates the expression of pituitary γ-MSH and its secretion into the circulation (Humphreys, 2004; Ni, Bhargava, et al., 2006). The rise in γ-MSH attenuates the hypertensive effect of a high-sodium diet, possibly by reducing renal sodium reabsorption and plasma volume. Additionally, experimental natriuresis induced by γ-MSH could be reversed by MC3R antagonists in control rodents but not in mice lacking MC3R, indicating that natriuresis occurred at least partly via MC3R (Humphreys, 2004; Ni et al., 1998). Conversely, deficiency of γ-MSH or MC3R in mice markedly increased blood pressure when exposed to a high-sodium diet. Furthermore, administration of exogenous γ-MSH corrected hypertension in γ-MSH-deficient mice but not in MC3R-deficient mice, demonstrating that blood pressure normalisation requires the integrity of MC3R. (Humphreys, 2004; Humphreys et al., 2011; Ni et al., 2003)

Although low doses of γ-MSH elicit beneficial renal and CV actions through MC3R, higher doses of γ-MSH paradoxically induce hypertensive effects in rodents. It was demonstrated that inhibiting the sympathetic nervous system by blocking ARs could inhibit this hypertensive effect of γ-MSH, suggesting its dependence on stimulation of sympathetic outflow. (Callahan et al., 1984; Gruber & Callahan, 1989) The structure-function studies of γ-MSH resulted in the finding that a specific Arg-Phe amino acid sequence in γ-MSH is critical for the hypertensinogenic action. Further experiments demonstrated that the hypertensive action of γ-MSH was not mediated by MC3R but rather by an entirely different type of receptor expressed in

the CNS, an FMRFamide (Phe-Met-Arg-Phe-NH_2) gated sodium channel. The involvement and activation of this channel in the hypertensive effect of γ-MSH have been demonstrated through both pharmacological studies and studies using MC3R-deficient mice. (Humphreys, 2007; Ni, Butler, et al., 2006) It has been postulated that the balance between the protective effect against hypertension from a high sodium diet and the hypertensinogenic effect of γ-MSH depends on its concentration. Physiological levels of γ-MSH in mice consuming a high sodium diet may activate MC3R in the CNS, likely in the nucleus of the solitary tract, and in the kidneys. These interactions could reduce sympathetic activation, typically triggered by a high sodium intake, and additionally promote natriuresis. In contrast, supraphysiological concentrations of γ-MSH are required for the interaction with the FMRFamide sodium channel, resulting in hypertension due to increased sympathetic nervous system activity. (Humphreys et al., 2011)

Furthermore, ACTH is involved in blood pressure regulation. Hypertension is a crucial feature of patients with Cushing's disease and ectopic ACTH syndrome, diseases with excess cortisol production of the adrenal glands. These diseases form a link between ACTH and blood pressure, and in addition, Cushing's disease is associated with compromised cardiac function, including LV hypertrophy and impaired contractility (Muiesan et al., 2003). Furthermore, as demonstrated already in early studies, chronic administration of ACTH produced blood pressure elevation in normotensive and hypertensive humans but not in patients with adrenal insufficiency (Whitworth et al., 1983). In addition, the effect of ACTH was mimicked by cortisol (Whitworth et al., 1984). Due to these observations, the rise in blood pressure was hypothesised to be due to an ACTH-induced increase in steroid hormone cortisol production and secretion from adrenal glands. Cortisol was postulated to induce blood pressure elevation by increasing vascular tone by potentiating the vasoconstrictor action of many pressor hormones, like Ang II. Besides ACTH hormonal effects, it is suggested to have direct effects on vascular tone through an adrenally independent mechanism via binding to MC2R in vascular endothelial cells and thus downregulating the expression of enzyme 11β-Hydroxysteroid dehydrogenase, which converts cortisol to inactive cortisone. Reduced activity of this enzyme may be relevant for the pathogenesis of hypertension. (Hatakeyama et al., 2000)

The versatile role of the melanocortin system in inflammation also affects CV health. For instance, one major type of CVDs is atherosclerotic cardiovascular disease, which includes various conditions such as CAD, ischemic heart disease, peripheral artery disease, and different aneurysms (Makover et al., 2022). The underlying cause for all of these is atherosclerosis, which leads to plaque buildup in the arterial walls and chronic inflammation in the vasculature (Hansson & Libby, 2006). It has been demonstrated that the melanocortin system plays a protective role

in experimental atherosclerotic plaque formation, composition, and vulnerability. Furthermore, melaocortin system activation decreases arterial accumulation of immune system cells, improves vascular endothelial function and cholesterol handling, and overall protects against the development of atherosclerosis in mice. (Kadiri et al., 2021; Nuutinen et al., 2018; Rinne et al., 2014; Rinne, Kadiri, et al., 2018) Additionally, the expression of POMC and its processing into biologically active α-MSH has been demonstrated to occur in human atherosclerotic plaques as well (Rinne, Lyytikäinen, et al., 2018). Data regarding the involvement of the melanocortin system in atherosclerosis indicate the necessity for future investigations aimed at harnessing the system's potential to enhance inflammation resolution and produce plaque-stabilising effects. Such advancements could lead to innovative therapeutic strategies against atherosclerotic cardiovascular disease.

Melanocortin system activation reduces heart damage in experimental models of ischemia, reperfusion injury, and transplantation (Colombo et al., 2005; Getting et al., 2004). Treatment with α-MSH and γ-MSH has exerted protective effects via MC1R and MC3R during ischemia or at reperfusion time by reducing the expression of inflammatory mediators and by diminishing tissue damage (Mioni et al., 2003; Vecsernyes et al., 2003). Furthermore, MC1R activation by an α-MSH synthetic analogue has been demonstrated to induce phenotypic changes in the heart that closely resemble ischemic preconditioning, which could protect against subsequent reperfusion injury (Catania et al., 2010). In addition, endogenous circulating α-MSH has been shown to be elevated in patients with acute myocardial infarction who received thrombolytic therapy, implying that α-MSH is released during ischemia and reperfusion and might contribute to cardioprotection under these conditions (Airaghi et al., 1995). These experimental findings could be of particular importance, since the phenomenon of ischemic preconditioning is stated to be the most effective method to protect the heart tissue against irreversible ischemic damage. However, it is not exploitable clinically, since it involves the induction of repetitive sublethal ischemic events. Therefore, pharmacological treatment modalities mimicking it are of great interest. (Lochner et al., 2009)

Lastly, relatively little is known about the expression and functions of MCRs in the heart. Early studies have demonstrated that *Pomc* gene expression and its processing into α-MSH occur in the rat heart (Millington et al., 1993, 1999). In addition, as a relevant clinical observation, elevated plasma levels of α-MSH were found in patients with hypertrophic or dilated cardiomyopathy or ischemic heart disease (Yamaoka-Tojo et al., 2006). Otherwise, the role of the melanocortin system in the heart remains unknown.

3 Aims

The main aim of this thesis was to investigate the undefined role of the melanocortin system in cardiac health and disease. Particularly, focus was on characterising the role of α-MSH and its potential target receptors in pathological cardiac hypertrophy and HF.

The specific aims of this research were:

1. To uncover which MCR subtypes are expressed, functionally active, and mediate the effects of the endogenous MCR agonist α-MSH in cardiomyocytes, and resolve whether α-MSH is locally produced in the heart.
2. To investigate the effects of global and cardiomyocyte-specific MC1R knockout in experimental models of pathological and physiological cardiac hypertrophy, and examine the selective MC1R agonist in cultured cardiomyocytes.
3. To characterise the cardiac phenotype of cardiomyocyte-specific MC5R knockout mouse model in experimental models of pathological cardiac hypertrophy, and evaluate the therapeutic effects of a selective MC5R agonist in cultured cardiomyocytes and an experimental model of heart failure.

4 Materials and Methods

4.1 Experimental animals and animal models

All experiments were performed with adult male mice kept in the Central Animal Laboratory, University of Turku. Mice were group-housed, unless otherwise indicated, under standard conditions with constant temperature (22 ± 3°C), humidity (50 ± 20%), a 12-hour light/dark cycle (lights on at 7 a.m.), and free access to food and tap water.

An overview of the animal models, along with an overview of all models and methods employed in this thesis, is presented in **Table 3**.

4.1.1 Ethical consideration

All animal experiments were planned and performed according to the ethical principles of the 3Rs. The number of animals needed was optimised to detect biologically significant differences in the measured parameters. To ensure minimum pain and suffering of the animals, sufficient pre- and post-operative care and constant well-being monitoring were provided. Humane endpoints, such as considerable weight loss (>15%) and behavioural changes, were defined, and euthanasia was carried out according to general guidelines. All animal experiments were approved by the national Animal Experiment Board in Finland and the Regional State Administrative Agency for Southern Finland (Licence numbers: ESAVI-438/04.10.03/2012, ESAVI/6280/04.10.07/2016, ESAVI/1260/2020 and ESAVI/45421/2022) and conducted in accordance with the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and with the institutional and national guidelines for the care and use of laboratory animals.

4.1.2 Pharmacological intervention studies

To study the effects of non-selective chronic activation of the melanocortin system in pathological cardiac hypertrophy, 8-week-old male C57Bl/6J mice were subjected to transverse aortic constriction (TAC) as described below, and after a 2-week

Table 3. Overview and summary of experimental models and methods used in studies of this thesis and reference to the original publications where they are initially presented.

Model or method	Original publication
In vivo pharmacological intervention:	
MT-II dosing	I
PG-901 dosing	I
Genetic mouse model:	
$Mc1r^{e/e}$	II
Mc1r-cKO	II
Mc5r-cKO	I
In vivo hypertrophy model:	
TAC	I and II
Ang II infusion	I and II
Voluntary wheel running	II
Echocardiography	I and II
Cell model:	
H9c2	I and II
NMCM	I and II
hiPSC-CM	I and II
In vitro hypertrophy model:	
Pharmacological induction (Ang II/ ET-1)	I and II
Mechanical stretching	I and II
In vitro signalling mechanism assays	I and II
siRNA MCR silencing	I
Human cardiomyopathy samples	I
RT-qPCR	I and II
Western blotting	I and II
[^{3}H]-Leucine incorporation assay	I and II
cAMP assay	I and II
ELISA	I and II
Histology and immunohistochemistry	I and II

Ang II; angiotensin II, cAMP; cyclic adenosine monophosphate, ELISA; enzyme-linked immunosorbent assay, ET-1; endothelin-1, hi-PSC-CM; human induced pluripotent stem cell-derived cardiomyocyte, $Mc1r^{e/e}$; MC1R recessive yellow mouse, Mc1r-cKO; cardiomyocyte-specific melanocortin 1 receptor knockout, Mc5r-cKO; cardiomyocyte-specific melanocortin 5 receptor knockout, MCR; melanocortin receptor, MT-II; melanotan-II, NMCM; neonatal mouse ventricular cardiomyocyte, PG-901; melanocortin 5 receptor selective agonist, RT-qPCR; quantitative real-time polymerase chain reaction, TAC; transverse aortic constriction

recovery period, randomly assigned to receive once daily injection of either vehicle (phosphate-buffered saline (PBS), i.p.) or the α-MSH analogue melanotan-II (MT-II, 0.3 mg/kg/day, i.p., Tocris, #2566). As a control group, sham-operated mice were treated with vehicle or MT-II according to the same dosing scheme. Mice were sacrificed after 6 weeks of treatment.

To evaluate the therapeutic potential of selective MC5R activation in the context of HF, 8-week-old male C57Bl/6N mice were subjected to TAC and, after a 1-week recovery period, randomly assigned to receive either vehicle (0.5% dimethyl sulfoxide (DMSO) in PBS, s.c.) or the selective MC5R agonist PG-901 (0.005 or 0.5 mg/kg/day, s.c.) once daily. Sham-operated mice were dosed with the same treatment scheme and served as controls. Mice were sacrificed after 4 weeks of treatment.

4.1.3 Genetic mouse models

Recessive yellow mice, $Mc1r^{e/e}$, that lack functional MC1R due to a spontaneous single-base deletion mutation, were used as a model of global MC1R deficiency (the Jackson Laboratory, strain #000060). Their age-matched non-mutant littermates were used as controls.

Inducible cardiomyocyte-specific MC1R knockout mouse model (Mc1r-cKO) was generated by intercrossing MC1R floxed mice ($Mc1r^{fl/fl}$, the Jackson Laboratory, strain #029239) (Takeo et al., 2016) with tamoxifen-inducible Myh6-MerCreMer transgenic mice (Myh6-MCM, the Jackson Laboratory, strain #005657) (Sohal et al., 2001). To generate cardiomyocyte-specific MC5R knockout mice (Mc5r-cKO), MC5R floxed mice ($Mc5r^{fl/fl}$, GemPharmatech, strain #T005911) were intercrossed with previously mentioned tamoxifen-inducible Myh6-MerCreMer transgenic mice. Age-matched Cre-positive and MC1R/MC5R WT mice (referred to as Myh6-MCM) were used as controls. In experiments with Mc5r-cKO mice, age-matched Cre-negative and MC5R homozygous mice (referred to as $Mc5r^{fl/fl}$) were used as another control group. All mice were on C57Bl/6J background and equally treated with tamoxifen.

To induce the conditional knockout in cardiomyocytes, six-week-old mice were treated with tamoxifen (20 mg/kg, i.p., Cayman Chemicals, #13258) on 4 consecutive days. Tamoxifen was dissolved in peanut oil by heating at +37°C. Mice were allowed to recover for a minimum of 7 days from tamoxifen treatment before any experimentation in all of the studies. At the end of the experiments, genomic DNA samples from the heart and different reference tissues (skeletal muscle, liver and spleen) were genotyped to verify that the desired recombination had occurred only in the heart.

4.2 Animal models of cardiac hypertrophy

4.2.1 Transverse aortic constriction

For the surgery, mice were first anaesthetised with the ketamine-xylazine mixture (110 mg/kg and 15 mg/kg, respectively, i.p.), intubated and ventilated (MiniVent,

Harvard Apparatus). Median sternotomy was performed, the pericardium covering the aorta was opened, and the aortic arch was exposed by carefully removing the surrounding connective tissue. The transverse aorta was ligated with a 27-G needle and 7-0 silk suture. The thoracic cage and skin were closed with 6-0 surgical silk sutures. Sham-operated mice underwent the same procedure without the constriction of the aorta and served as controls. For peri- and post-operative analgesia, buprenorphine (0.05 mg/kg, s.c., 2x/day) and carprofen (5 mg/kg, s.c., 1x/day) were given on the day of surgery and for 3 consecutive days.

4.2.2 Angiotensin II infusion

As another model of cardiac hypertrophy, mice were subjected to a 4-week subcutaneous infusion of angiotensin II (Ang II) (1.4 mg/kg/day) via osmotic minipumps (Alzet, Model 1004). The minipumps were loaded with Ang II based on animal weights. To implant the minipumps into the subcutaneous space, mice were first anaesthetised with isoflurane (induction 4%, maintenance 1.5%), and the fur from a small area in the back was shaved, and the skin cleaned with 70% ethanol. A dorsal midline incision was made to create a subcutaneous pocket for the insertion of the minipump. Skin was thereafter closed with surgical sutures. Sham-operated mice served as controls and underwent the same surgical procedure, without implantation of a minipump. Buprenorphine (0.05 mg/kg, s.c., 2x/day for 3 days) and carprofen (5 mg/kg, s.c., 1x/day for 3 days) were given for pre- and post-operative analgesia.

4.2.3 Voluntary wheel running

To induce physiological cardiac hypertrophy, mice were subjected to a 5-week voluntary wheel running experiment. Mice were individually housed, and all mice had free access to a running wheel (Low Profile Wireless Running Wheel for Mice, ENV-004, Med Associates Inc.) placed into their home cages. Individual running data was recorded with the magnetic counter in each running wheel and collected and stored in wheel manager software (SOF-860, Med Associates Inc.) throughout the experiment. Sedentary mice that did not exercise served as controls and followed the same protocol, but their running wheels were rendered non-rotating using special stoppers.

4.3 Echocardiography imaging

A dedicated small animal ultrasound system (Vevo 2100, Visual Sonics Inc.) with MS-550D (frequency of 22-55 MHz) linear array transducer was used to perform transthoracic echocardiography before the induction of cardiac hypertrophy or drug

administration and at the end of the experiments. For imaging, mice were anaesthetised with isoflurane (induction 4%, maintenance 1.5%), hair from the thorax was removed with chemical hair remover, and imaging gel was applied to the thorax between the skin and transducer. Mice were kept on a heated table during imaging to maintain normal body temperature and to avoid the influence of body temperature changes on cardiac parameters.

Brightness and motion mode (B- and M-mode, respectively) images from two main directions, the parasternal long axis view (PSLAX) and the short axis view (SAX), were acquired. LV dimensions, including LV end-diastolic dimension (LVEDD) and LV posterior wall thickness (LVPW), as well as functional parameters and heart rate, were analysed from B- and M-mode images. EF was calculated using the equation: 100x((LV Vol;d-LV Vol;s)/LV Vol;d), where Vol;d refers to end-diastolic volume and Vol;s to end-systolic volume. Endocardial fractional area change (FAC) was calculated using the equation: 100x((LV EDA-LV ESA)/LV EDA), where EDA refers to end-diastolic area and ESA to end-systolic area.

All measurements and analyses were conducted in a blinded manner. All analyses were done using Vevo software (Vevo LAB 5.5.0) and all parameters were averaged from three consecutive cardiac cycles.

4.4 Cell models

4.4.1 H9c2 cell line

Rat heart myoblast H9c2(2-1) cells (ATCC®, CRL-1446™) served as a first cell model to investigate the effects of MCR modulation on intracellular signalling pathways and gene expression. H9c2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Merck Life Science Oy, #D6429) supplemented with 1% penicillin-streptomycin (Gibco, #15140-122 or Merck Life Science Oy, #P4333) and 10% heat-inactivated fetal bovine serum (FBS; Biowest, #S181B-500 or Gibco, #10270-106) at +37°C under 5% CO_2 level. Cells were subcultured with a ratio of 1:3 to 1:5 when confluency reached 60–70%.

4.4.2 Neonatal mouse ventricular cardiomyocytes

To verify effects observed in the H9c2 cell line, neonatal mouse ventricular cardiomyocytes (NMCM) were isolated from 1- to 3-day-old C57Bl/6N and C57Bl/6J mouse pups, and used as another cardiomyocyte model. Isolation, differentiation and culturing were accomplished using Pierce Primary Cardiomyocyte Isolation Kit (ThermoFisher, #88281) according to the manufacturer's protocol. Cardiomyocytes were seeded on sterile 24-well plates at an

average density of 500,000 cells/well in DMEM (ThermoFisher, #88281) supplemented with 1% penicillin-streptomycin (Gibco, #15140-122 or Merck Life Science Oy, #P4333) and 10% heat-inactivated FBS (Biowest, #S181B-500 or Gibco, #10270-106) at +37°C under 5% CO_2 level. NMCMs were used in experiments after 5 to 7 days of culturing.

4.4.3 Human induced pluripotent stem cell-derived cardiomyocytes

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) were differentiated from iPS (IMR90)-4 line (WiCell) using small molecule induction, cultured and matured for a minimum of 30 days after the initiation of differentiation before any experimentation, as previously described (Burridge et al., 2014; Karhu et al., 2018; Pohjolainen et al., 2021).

4.4.4 Induction of cardiomyocyte hypertrophy, inhibition of signalling pathways and melanocortin receptor silencing

To induce cardiomyocyte hypertrophy, cells were treated with Ang II (Abcam, #ab120183) or ET-1 (Merck Life Science Oy, #E7764) for 24 hours. In addition to pharmacological induction, mechanical stretching for 24 or 48 hours was applied as another model of cell hypertrophy to hiPSC-CMs, in a way previously described (Pohjolainen et al., 2022).

Different pharmacological agents were used to inhibit key signalling pathways in cardiomyocytes. To inhibit the JNK pathway, H9c2 cells were treated with SP 600125 (Tocris, #1496). To inhibit p38 and CREB pathways, H9c2 cells were treated with TAK-715 (CaymanChemical, No.26170) and 666-15 (MedChemExpress, No.HY-101120), respectively. To inhibit G_i and G_s protein-mediated signalling pathways, H9c2 cells were treated with pertussis toxin (PTX, Tocris, #3097), or PKA inhibitor H89 (Tocris, #2910) or cAMP analogue cAMP-Rd (Tocris, #1337).

To knock down MC5R in cardiomyocytes, siRNA-mediated silencing technique was applied in H9c2 and NMCM cells. MC5R siRNA (ThermoFisher, Silencer™ Select Mc5r siRNA, #4390771, assay ID s69671 and s69672 for NMCM or s130589 and s130590 for H9c2) or negative control siRNA (Silencer™ Select Negative Control No. 1 siRNA, #4390843) was diluted in Opti-MEM® medium (ThermoFisher, #31985062) and combined with Lipofectamine® RNAiMAX (ThermoFisher, #13778030) to form siRNA-lipid complex to be exposed to the cells. Thereafter, cells were incubated with the siRNA-lipid complexes for 24 or 48 hours to silence MC5R before collection and analyses.

4.5 Human cardiomyopathy samples

Human LV samples were obtained from dilated (n=15) and ischemic (n=8) cardiomyopathy patients undergoing cardiac transplantation in Helsinki University Hospital between 2014 and 2019. The patient characteristics have been previously reported (Lin et al., 2022). Control samples (n=13) were from victims of traffic accidents with no history or evidence of cardiovascular diseases at autopsy. The study was approved by the Ethics Committee of Helsinki and Uusimaa Hospital District and conducted according to the Declaration of Helsinki, and the study subjects gave informed consent.

4.6 Biochemical analysis

4.6.1 Gene expression analysis

H9c2, NMCMs and hiPSC-CMs were collected in QIAzol Lysis Reagent or Trizol Reagent (Invitrogen), and total RNA extraction was performed using Direct-zol RNA Miniprep and Microprep (Zymo Research) kits or by chloroform phase separation method following RNeasy™ MinElute™ Cleanup (Qiagen) kit according to the manufacturer's instructions. Cardiac tissue samples were first homogenised in QIAzol Lysis Reagent using TissueLyser LT Bead Mill and stainless-steel beads (Qiagen), and total RNA was thereafter extracted using Direct-zol RNA Miniprep. The concentration and purity of extracted total RNA were determined with a NanoDrop Microvolume Spectrophotometer (ThermoFisher Scientific) and reverse-transcribed to cDNA using PrimeScript™ RT reagent kit (Takara Clontech) according to the manufacturer's instructions. Specific mRNA expressions were analysed using the quantitative real-time polymerase chain reaction (RT-qPCR or qPCR) method with SYBR Green protocols (Kapa Biosystems) and real-time PCR detection system (Applied Biosystems 7300 Real-Time PCR system). mRNA expression levels of target genes were normalised to mRNA expression of housekeeping gene (β-actin; ACTB, glyceraldehyde-3-phosphate dehydrogenase; GAPDH or ribosomal protein S18; RPS18) and the fold induction was determined by using the comparative ΔCt method. The results are presented as relative transcript levels ($2^{-\Delta\Delta Ct}$). Primer sequences for mouse genes are given in **Table 4**, for rat genes in **Table 5** and for human genes in **Table 6**.

Table 4. Quantitative real-time polymerase chain reaction primers for mouse genes.

Gene name **Accession number**	**5'-3' primer sequence**
Acta1 NM_001272041.1	Forward: cccaaagctaaccgggagaag Reverse: ccagaatccaacacgatgcc
Acta2 NM_007392.3	Forward: agattgtgcgcgacatcaaag Reverse: gcagactccataccgataaagga
Actb NM_007393.5	Forward: tccatcatgaagtgtgacgt Reverse: gagcaatgatcttgatcttca
Col1a2 NM_007743.3	Forward: tgcagtaacttcgtgcctagc Reverse: acgtggtcctctgtctcca
Ctgf NM_010217.2	Forward: agacctgtgggatgggcat Reverse: gcttggcgattttaggtgtcc
Fn1 NM_010233.2	Forward: atgtggacccctcctgatagt Reverse: gcccagtgatttcagcaaagg
Il6 NM_031168.2	Forward: ggccttccctacttcacaag Reverse: atttccacgatttcccagag
Mc1r NM_008559.3	Forward: gtgctggttgtgatagccatc Reverse: tgctgacacttaccatcaggt
Mc3r NM_008561.3	Forward: tccgatgctgcctaacctct Reverse: ggatgttttccatcagactgacg
Mc4r NM_016977.3	Forward: cccggacggaggatgctat Reverse: tcgccacgatcactagaatgt
Mc5r NM_013596.2	Forward: caagaccagagcccggtaaac Reverse: gcgcaaaggtaagcatgattct
Mmp2 NM_008610.3	Forward: gatgtcgcccctaaaacagac Reverse: cagccatagaaagtgttcaggt
Mrps18a NM_026768.3	Forward: cagctccaagcgttcctgg Reverse: ggccttcaattacagtcgtct
Myh6 NM_010856.4	Forward: ccacttctccttggtccactatg Reverse: acaaacccaccaccgtctca
Myh7 NM_080728.3	Forward: agggtggcaaagtcactgct Reverse: catcacctggtcctccttca
Nppa NM_008725.3	Forward: gcttccaggccatattggag Reverse: gggggcatgacctcatctt
Nppb NM_008726.6	Forward: cccaaaaagagtccttcggtc Reverse: cggtctatcttgtgcccaaag
Pomc NM_008895.4	Forward: caagccggtgggcaagaaacg Reverse: ctaatggccgctcgccttccag
Tgfb1 NM_011577.2	Forward: ccgcaacaacgccatctatg Reverse: cccgaatgtctgacgtattgaag

Table 5. Quantitative real-time polymerase chain reaction primers for rat genes.

Gene name **Accession number**	**5'-3' primer sequence**
Acta2 NM_031004.2	Forward: actgggacgacatggaaaag Reverse: catctccagagtccagcaca
Ctgf NM_022266.2	Forward: gaggaaaacattaagaagggcaaa Reverse: cggcacaggtcttgatga
Fn1 NM_019143.2	Forward: gctgctgggacttccacgt Reverse: tctgttccgggaggtgca
Gapdh NM_017008.4	Forward: gacatgccgcctggagaaac Reverse: agcccaggatgcccttagt
Il6 NM_012589.2	Forward: cctggagtttgtgaagaacaact Reverse: ggaagttggggtaggaagga
Nppb NM_031545.1	Forward: acaatccacgatgcagaagct Reverse: gggccttggtcctttgaga
Rn18s NR_046237.2	Forward: cattcgaacgtctgccctat Reverse: gtttctcaggctccctctcc
Tgfb1 NM_021578.2	Forward: gcaacaacgcaatctatgac Reverse: cctgtattccgtctcctt

Table 6. Quantitative real-time polymerase chain reaction primers for human genes.

Gene name **Accession number**	**5'-3' primer sequence**
ACTB NM_001101.5	Forward: caccattggcaatgagcggttc Reverse: aggtctttgcggatgtccacgt
FN1 NM_212482.4	Forward: cggtggctgtcagtcaaag Reverse: aaacctcggcttcctccataa
GAPDH NM_002046.7	Forward: tcaaggctgagaacgggaag Reverse: cgccccacttgattttggag
MC1R NM_002386.4	Forward: acacctggaggggaagaact Reverse: aggaagcaggaaggagtcgt
MC5R NM_005913.3	Forward: ttggatctcaacctgaatgcc Reverse: gcccctatgaccaagatgttctc
NPPA NM_006172.4	Forward: acaatgccgtgtccaacgcaga Reverse: cttcattcggctcactgagcac
NPPB NM_002521.3	Forward: tctggctgctttgggaggaaga Reverse: ccttgtggaatcagaagcaggtg
RPS18 NM_022551.3	Forward: cgccgctagaggtgaaattc Reverse: ccagtcggcatcgtttatgg
TGFB1 NM_000660.7	Forward: tacctgaacccgtgttgctctc Reverse: gttgctgaggtatcgccaggaa

4.6.2 Western blotting

Cell and tissue samples were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Complete Mini, Roche and Halt™ Phosphatase Inhibitor Cocktail, ThermoFisher). Cardiac tissue samples were homogenised using TissueLyser LT Bead Mill and stainless-steel beads (Qiagen), followed by quantification of total protein concentration using copper-based bicinchoninic acid method (Pierce™ BCA Protein Assay Kit, ThermoFisher). Protein samples were mixed with Laemmli buffer or Lane marker buffer (4X or 5X Laemmli Buffer, Bio-Rad Laboratories; 5X Pierce™ Lane Marker Reducing Sample Buffer, ThermoFisher) and boiled for 5 minutes at 95°C in order to denaturate, linearise and negatively charge proteins before loading samples into 10 to 15% sodium dodecyl sulphate polyacrylamide gels for electrophoresis (SDS-PAGE). Size-fractioned protein samples were transferred onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes by electroblotting and non-specific binding was blocked with 5% skimmed milk or 3% bovine serum albumin in Tris-Buffered Saline containing 0.1% Tween-20 detergent (TBST). Membranes were probed with specific primary antibodies overnight at +4°C. Specific primary antibodies used were: anti-α-MSH (Bioss, #BS-1848R), anti-MC1R (Alomone Labs, #AMR-020), anti-MC5R (Alomone Labs, #AMR-025), anti-NT-proBNP (Abcam, #ab13115), anti-phospho-CREB (Thermo Fisher, #MA5-11192), anti-CREB (Cell Signaling Tech, #9197), anti-phospho-p38 (Cell Signaling Tech, #9215), anti-p38 (Cell Signaling Tech, #9212). After primary incubation, membranes were washed with TBST and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Cell Signaling Tech) for 1 hour at room temperature, followed by detection using an enhanced chemiluminescence system (Pierce™ ECL Western Blotting Substrate, ThermoFisher) and Sapphire Biomolecular Imager (Azure Biosystems). The results for target protein expression were normalised to β-actin (Merck Life Science Oy, #2066) or vinculin (BioRad, #MCA465GA) expression to correct for loading and protein bands were analysed with ImageJ software (Fiji, NIH).

4.6.3 Protein synthesis measurement by [^{3}H]-Leucine incorporation assay

In [^{3}H]-Leucine incorporation method (Marinovich et al., 1990, 1994), tritium-labelled ^{3}H-leucine is added to the cells, and the amount of incorporated leucine into newly synthesised proteins can be measured to estimate the rate of intracellular protein synthesis. Cells were cultured in DMEM medium containing L-[4,5-^{3}H]

leucine (1 µCi/ml, PerkinElmer) for 24 hours, after which cells were rinsed with PBS and incubated with 10% trichloroacetic acid (TCA) at +4°C for 30 minutes. Then the precipitates were washed with ice-cold MilliQ water and suspended in 0.5 M sodium hydroxide (NaOH). Sample suspensions were mixed with liquid scintillation counter cocktail (Optiphase Supermix, PerkinElmer) and the radioactivity was measured with an automatic liquid scintillation counter (Hidex 600 SL, Hidex). Immediately after adding L-[4,5-^{3}H] leucine, cells were treated with Ang II and non-selective MCR agonist α-MSH or MC1R or MC5R selective agonists, or MC1R selective agonist with or without pretreatment of p38 or CREB inhibitor, or with MC5R targeted siRNA with or without pretreatment of JNK inhibitor, to estimate their effects on protein synthesis.

4.6.4 Cyclic AMP determination

To measure intracellular cAMP concentrations, H9c2 cells and NMCMs were pretreated with 3-isobutyl-1-methylxanthine (0.1 mM, IBMX, Sigma-Aldrich) for 30 minutes and thereafter stimulated with the non-selective MCR agonist α-MSH (0.1 nM) for 5, 15, 30, and 60 minutes. Cells were thereafter lysed with 0.1 M HCl and assayed for cAMP levels with a commercial kit (Cyclic AMP Select ELISA kit, Cayman Chemical, #501040) according to the manufacturer's instructions. Results were normalised against total protein concentrations (Pierce™ BCA Protein Assay Kit, ThermoFisher) and expressed as a percentage of control samples that were left untreated. To study concentration responsiveness of MC1R and MC5R selective agonists on intracellular cAMP production, H9c2 cells were harvested, pipetted into a 96-well OptiPlate (6,000 cells/well) and stimulated with different concentrations of LD211 or PG-901 for 30 minutes in the presence of IBMX (0.1 mM). cAMP levels were determined using a commercial kit (LANCE Ultra cAMP Detection Kit, PerkinElmer, #TRF0262) according to the manufacturer's instructions.

4.6.5 Enzyme-linked immunosorbent assay (ELISA)

Tissues and plasma were harvested from sham- and TAC-operated mice and α-MSH concentrations were assayed using a commercial kit (antibodies-online GmbH, #ABIN6969629). Tissue samples were homogenised in PBS supplemented with a protease inhibitor cocktail (Complete Mini, Roche) and centrifuged for 5 minutes at 5,000 g. The resulting supernatant was diluted with assay buffer and analysed for α-MSH concentration. The results were normalised against total protein concentrations (Pierce™ BCA Protein Assay Kit, ThermoFisher). The effects of MCR activation on ERK1/2 and JNK

phosphorylation were detected by commercial kits (DuoSet IC ELISA, R&D Systems, #DYC1018B & DYC1387B) according to the manufacturer's instructions. Briefly, cells were treated with the non-selective MCR agonist α-MSH or MC5R selective agonist PG-901, or with Ang II in the absence or presence of α-MSH, or with α-MSH with or without MC5R antagonist PG-20N, or with PG-901 with pretreatment of G_i or G_s protein inhibitors, or with MC5R targeted siRNA, and thereafter lysed with Lysis Buffer #6 (R&D Systems) and assayed for the expression levels of phospho-ERK1/ERK2 and phospho-JNK. The optical density of each well was determined with an EnSight™ multimode plate reader (PerkinElmer) and results were normalised against total protein concentrations (Pierce™ BCA Protein Assay Kit, ThermoFisher).

4.6.6 Histology and immunohistochemistry

For histological analysis, mouse heart samples were cut in the midline of the base-apex-axis and fixed in 10% formalin overnight, followed by transfer to 70% ethanol, embedding in paraffin and cutting into 5 µm-thick serial sections at the level of papillary muscles. For measuring CSA and length of cardiomyocytes, cardiac cross-sections were stained with hematoxylin and eosin (H&E), and then scanned with Pannoramic P1000 digital slide scanner (3DHISTECH Kft) and analysed using Case Viewer software (3DHISTECH Kft). At least 100 individual, longitudinally oriented cardiomyocytes with well-defined cell membranes and visible cell nuclei were selected and measured for CSA quantification. Cardiac cross-sections were also stained with Picrosirius Red to quantify the extent of fibrosis using ImageJ software (Fiji, NIH).

For immunohistochemistry, cardiac cross-sections were deparaffinised, rehydrated and then incubated in a pressure cooker in 10 mM sodium citrate buffer for 20 minutes for antigen retrieval, followed by 10 minutes quenching in 1% H_2O_2 and 60 minutes blocking in 5% normal horse serum containing 1% BSA. Sections were incubated overnight with primary antibodies against α-MSH (Bioss, #BS-1848R), MC1R (Elabscience, #E-AB-15765) or MC5R (Alomone Labs, #AMR-025), followed by biotinylated horseradish peroxidase-conjugated secondary antibody incubation and detection with diaminobenzidine (ABC kit, Vector Labs) for α-MSH and MC5R or by detection with ImmPRESS® HRP Horse Anti-Rabbit IgG PLUS Polymer Kit (Vector Labs, #MP-7801-15) for MC1R. For isotype control, a consecutive heart section was treated similarly except that the primary MC1R or MC5R antibody was replaced by purified normal rabbit IgG (Novus Biologicals, #NB810-56910). Sections were counterstained with hematoxylin (CarlRoth), cover-slipped and scanned with Pannoramic 250 digital slide scanner (3DHISTECH Kft).

Images were processed with Case Viewer software (3DHISTECH Kft) and ImageJ software (Fiji, NIH).

4.7 Melanocortin receptor ligands

Non-selective agonist α-MSH (Abcam, #ab120189) and selective MCR agonists were used to study the effects of MCRs activation in cardiomyocytes *in vitro*. LD211 was used as a selective MC1R agonist (compound 28 in the original publication) (Doedens et al, 2010), [D-Trp8]-γ-MSH as a selective MC3R agonist (Grieco et al, 2000), THIQ as a selective MC4R agonist (Cayman Chemical, #312637-48-2) (Sebhat et al, 2002) and PG-901 as a selective MC5R agonist (Grieco et al, 2002). In addition, selective MC5R antagonist PG-20N (Grieco et al, 2008) was used to block the MC5R *in vitro*. α-MSH and THIQ were commercially purchased, and LD211, [D-Trp8]-γ-MSH, PG-901 and PG-20N were synthesised and provided as a generous gift by Professor Minying Cai. α-MSH, [D-Trp8]-γ-MSH and THIQ were dissolved in ultrapure water (referred to as MilliQ water based on the MilliQ® purification system), LD211 and PG-901 were dissolved in PBS, and PG-20N was dissolved in DMSO to form stock solutions and further diluted in PBS to obtain working solutions.

For *in vivo* studies, α-MSH analogue MT-II (Tocris, #2566) (Al-Obeidi et al., 1989) was dissolved in PBS and used as a non-selective MCR agonist. Control animals received only PBS as a vehicle treatment. PG-901 was used as an MC5R-specific agonist *in vivo* and dissolved in DMSO to form a stock solution and further diluted in PBS to obtain desired concentrations for administration (0.05 mg/ml & 0.0005 mg/ml in 0.5% DMSO). Control animals received PBS containing 0.5% DMSO as a vehicle treatment.

4.8 Statistical methods

Statistical analyses were performed with GraphPad Prism software (versions 8, 9 and 10). The normality of the data was evaluated using D'Agostino and Pearson omnibus normality test, and the possible outliers in the data sets were detected using the regression and outlier removal (ROUT) method at Q-level of 1%. Data are expressed as mean ± standard error of the mean (SEM) and considered statistically significant for p-value <0.05 in all experiments. Unpaired Student's *t*-test was used to evaluate statistical significance between two independent groups, one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test was used to compare three or more groups and two-way ANOVA followed by Dunnett's, Bonferroni's or Šídák's *post hoc* test was used to compare two independent factors. Data that did not pass D'Agostino and Pearson normality test or had fewer than 6

samples per group were analysed using Mann-Whitney *U* test or Kruskal-Wallis test followed by Dunn's *post hoc* test. Randomised block ANOVA (individual experiments and treatment as factors) followed by Dunnett's *post hoc* test was used to analyse MC5R-related hiPSC-CM data, using ΔCt-values with qPCR data and β-actin-adjusted values with Western blot data, as previously described (Karhu et al., 2021).

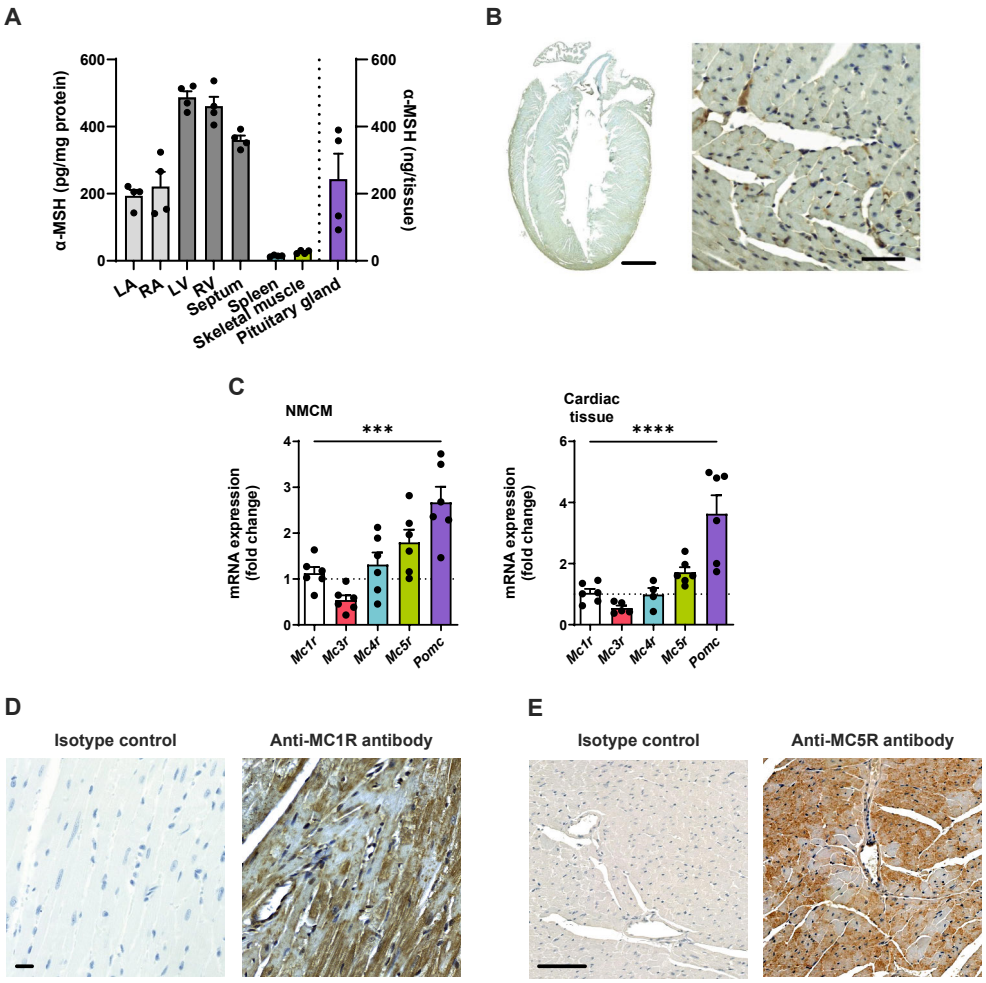
5 Results

5.1 Expression of α-MSH, MC1R and MC5R in the mouse heart

The first was aimed at investigating whether α-MSH and its target receptors are expressed in the mouse heart. Using an ELISA assay, detectable levels of α-MSH were found in different parts of the heart, the highest expression occurring in the ventricles. As expected, the overall highest expression appeared in the pituitary gland, roughly 500-fold higher than in the heart. However, the concentration of α-MSH in the other reference tissues, such as the spleen and skeletal muscle, was clearly lower compared to the heart (**Figure 7A**). Immunohistochemical analysis also confirmed that a small subset of cardiac cells in the mouse heart were positive for α-MSH (**Figure 7B**). Since α-MSH is a ligand of MCRs, the study aimed to determine which receptor subtypes are expressed in the mouse heart. Quantifying MCR subtypes in NMCMs and adult mouse hearts showed particularly high expression of MC5R, alongside POMC (**Figure 7C**). Moreover, immunohistochemical staining showed that MC1R and MC5R were uniformly present in the heart (**Figure 7D-E**).

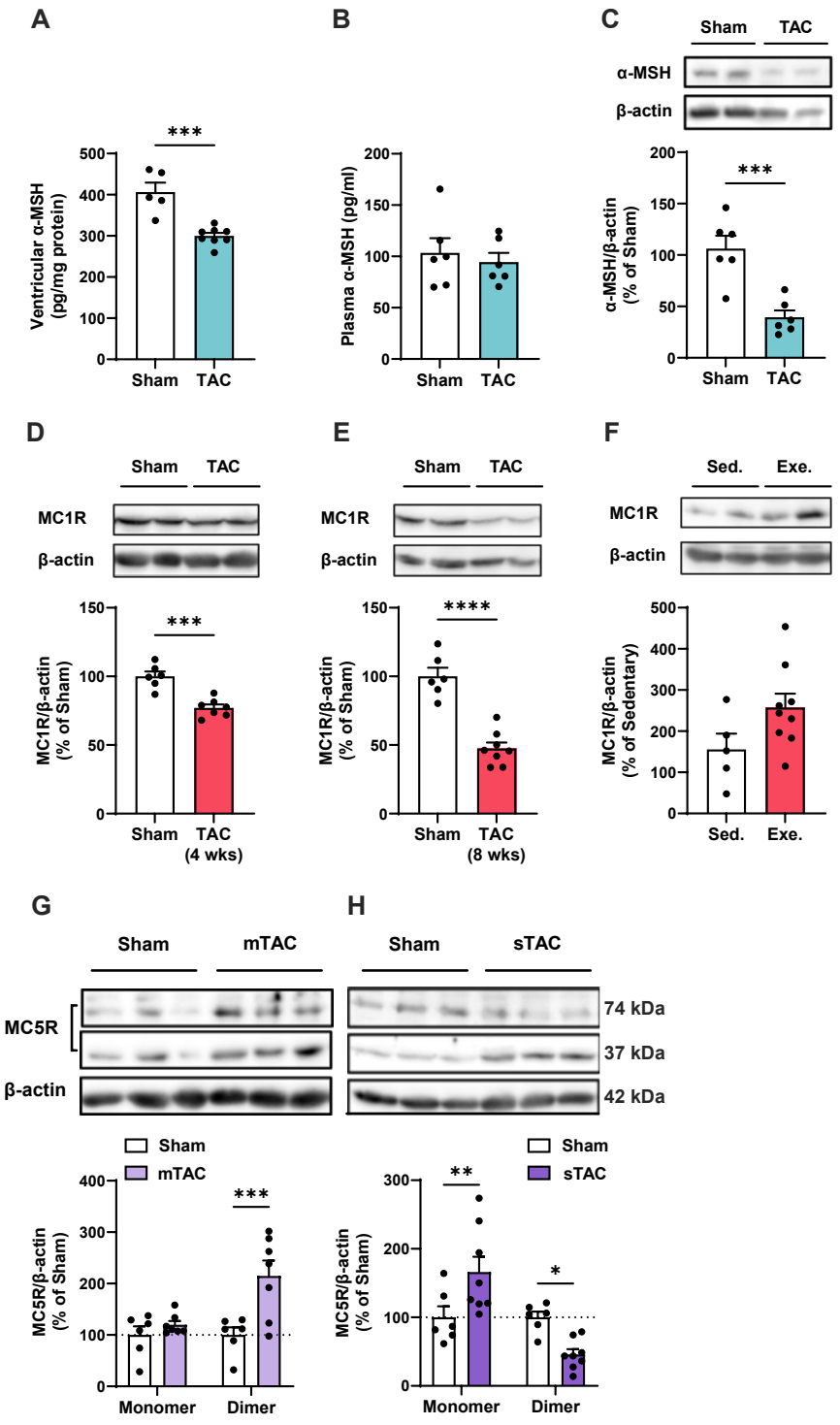
The subsequent studies focused on investigating whether pathological cardiac remodelling influences the expression of α-MSH and MCRs. In mice subjected to TAC surgery to induce pressure overload and pathological cardiac hypertrophy, a clear reduction in the cardiac α-MSH concentration was observed after 5 weeks of

Figure 7. ► α-Melanocyte-stimulating hormone and melanocortin 1 and 5 receptors are produced locally in the heart. **(A)** α-MSH concentration (pg/mg protein) in the mouse left atrium (LA), right atrium (RA), left ventricle (LV), right ventricle (RV), septum, spleen, skeletal muscle and pituitary gland (ng/ whole gland, plotted on the right Y-axis). **(B)** Immunostaining of α-MSH in a longitudinal heart section of a C57Bl/6J mouse. Scale bar, 1 mm (left) and 50 µm (right). **(C)** Quantitative real-time polymerase chain reaction (qPCR) analysis of relative *Mcr* (melanocortin receptor) subtype and *Pomc* (pro-opiomelanocortin) expression in neonatal mouse ventricular cardiomyocytes (NMCM) and mouse cardiac tissue. **(D-E)** Immunostaining of MC1R and MC5R in cardiac cross-section (D) and longitudinal heart section (E) of a C57Bl/6J mouse. In the control section, anti-MC1R or anti-MC5R antibody was replaced by purified normal rabbit IgG (isotype control). Scale bar 20 µm (D) and 100 µm (E). Data are mean ± SEM. Each dot represents an individual mouse. ***P<0.001 and ****P<0.0001 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc test. α-MSH; α-Melanocyte-stimulating hormone, MCR; melanocortin receptor.



surgery (**Figure 8A**) with no simultaneous change in the plasma level of α-MSH (**Figure 8B**). The result was also verified by Western blotting, which showed lower α-MSH protein level in the hearts of TAC-operated mice compared to sham-operated mice (**Figure 8C**).

Next, ventricular MC1R protein expression after 4 or 8 weeks of TAC surgery was quantified by Western blotting. Already after 4 weeks of pressure overload, the amount of MC1R protein was clearly decreased (**Figure 8D**) and continued to decline progressively towards more severe pathological cardiac hypertrophy (**Figure 8E**). A similar but milder decline of cardiac MC1R expression was detected in mice subjected to Ang II infusion for 4 weeks (data not shown). On the contrary, physiological cardiac hypertrophy induced by 5-week voluntary wheel running tended ($P = 0.080$) to increase MC1R protein level in the mouse heart (**Figure 8F**).


A
Ventricular α-MSH (pg/mg protein)
***
Sham
TAC
B
Plasma α-MSH (pg/ml)
Sham
TAC
C
Sham
TAC
α-MSH
β-actin
α-MSH/β-actin (% of Sham)
***
Sham
TAC
D
Sham
TAC
MC1R
β-actin
MC1R/β-actin (% of Sham)
***
Sham
TAC (4 wks)
E
Sham
TAC
MC1R
β-actin
MC1R/β-actin (% of Sham)
****
Sham
TAC (8 wks)
F
Sed.
Exe.
MC1R
β-actin
MC1R/β-actin (% of Sedentary)
Sed.
Exe.
G
Sham
mTAC
MC5R
β-actin
Sham
mTAC
MC5R/β-actin (% of Sham)
***
Monomer
Dimer
H
Sham
sTAC
74 kDa
37 kDa
42 kDa
Sham
sTAC
MC5R/β-actin (% of Sham)
**
*
Monomer
Dimer

◄ **Figure 8.** Cardiac expression of α-Melanocyte-stimulating hormone and melanocortin 1 and 5 receptors is modulated by different hypertrophic stimuli. **(A-B)** α-MSH concentration in the left ventricle (LV) (A) and plasma (B) of sham- and TAC-operated mice 5 weeks after the surgery. **(C)** Representative Western blots and quantification of α-MSH in the LV of sham- and TAC-operated mice. **(D-E)** Representative Western blots and quantification of MC1R protein expression in LV of sham- and TAC-operated (4 or 8 weeks) mice. **(F)** Representative Western blots and quantification of MC1R protein expression in the LV of mice subjected to voluntary wheel running (exercise, exe.) or non-exercising period (sedentary, sed.) for 5 weeks. **(G-H)** Representative Western blots and quantification of MC5R protein monomer (37 kDa) and dimer (74 kDa) forms expression in the LV of sham- and TAC-operated mice with mild (mTAC) or severe (sTAC) hypertrophy. All analysed proteins were normalised to β-actin. n=5–6 per sham groups, n=6–8 per TAC groups, n=5 in sedentary group and n=9 in exercise group. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 for the indicated comparisons by unpaired Student's t-test (A-F) and by 2-way ANOVA and Bonferroni's post hoc test (G-H). α-MSH; α-Melanocyte-stimulating hormone, MCR; melanocortin receptor.

Similarly, the amount of MC5R protein in the heart was changed due to pressure overload. For measuring MC5R abundance, cardiac samples from TAC-challenged mice that displayed either preserved EF (EF -3% vs sham, P=0.23) and mild hypertrophy or advanced systolic dysfunction with reduced EF (EF -25% vs sham, P<0.001) and marked hypertrophy, were selected. Since the dimer form of MC5R is considered to be functionally more active (Milligan, 2004), the protein amount of MC5R monomer and dimer forms was analysed separately by Western blotting. In mildly hypertrophied hearts (referred to as mTAC), the dimer form was significantly upregulated with no change in MC5R monomer expression (**Figure 8G**). On the contrary, in a severely hypertrophied heart (referred to as sTAC), the amount of MC5R dimer was clearly reduced, which was accompanied by an increase in the MC5R monomer (**Figure 8H**). However, Ang II infusion for 2 or 4 weeks did not affect cardiac MC5R protein level (data not shown). Collectively, these results demonstrate that endogenous melanocortin receptor agonist α-MSH, as well as MC1R and MC5R, are expressed in the mouse heart, and different hypertrophic stimuli modulate their expression.

5.2 The effects of α-MSH in cultured cardiomyocytes

To investigate whether cardiomyocytes have functional MCRs, experiments with H9c2 cells, NMCMs, and hiPSC-CMs were performed, and cells were treated with the general MCR agonist α-MSH. Since MCRs are known to be coupled to G_s protein and thus expected to evoke changes in intracellular cAMP level, the cAMP concentration in α-MSH-treated cells was measured. Surprisingly, α-MSH treatment caused a quick and transient reduction in cAMP level in both H9c2 cells and NMCMs (**Figure 9A**). Screening of other possible downstream targets of MCR

signalling revealed a substantial reduction in JNK phosphorylation after α-MSH treatment (**Figure 9B**), while no change in ERK1/2 phosphorylation (**Figure 9C**) was detected. Next, to explore whether these signalling cascade changes are associated with changes in gene expressions, qPCR analysis on α-MSH-treated H9c2 cells and NMCMs was performed. Subnanomolar concentration of α-MSH downregulated hypertrophy-related gene *Nppb* (B-type natriuretic peptide), many fibrosis-related genes, including *Acta2* (alpha-smooth muscle actin) and *Tgfb1* (transforming growth factor beta 1), in both cell types, and also pro-inflammatory gene *Il6* (interleukin 6) in NMCMs but not in H9c2 cells (**Figure 9D-G**).

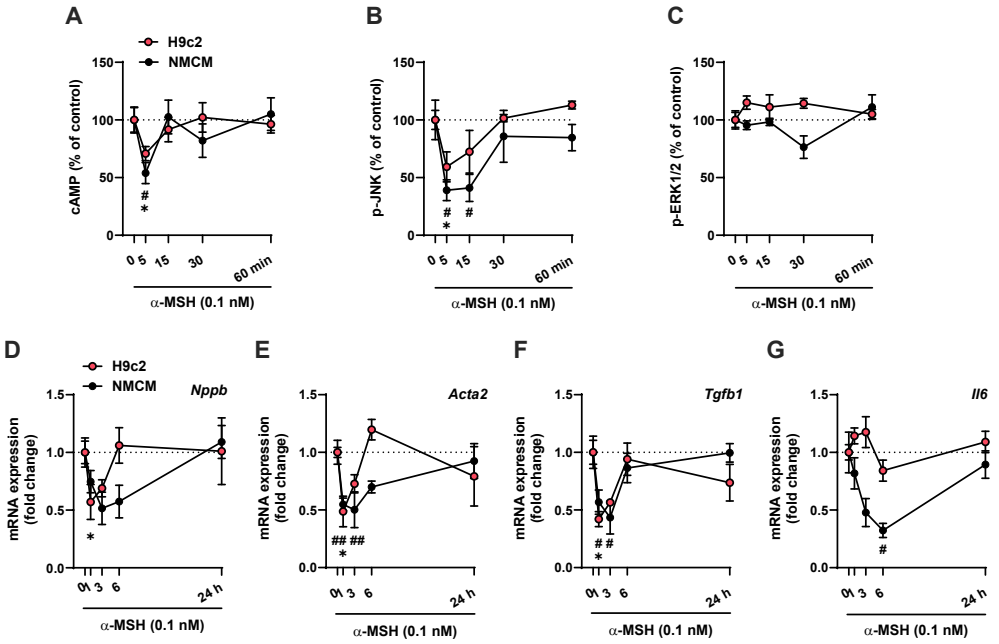


Figure 9. α-Melanocyte-stimulating hormone reduces the levels of cyclic adenosine monophosphate, phosphorylated JNK and mRNA expressions in cultured cardiomyocytes. **(A-C)** Quantification of intracellular cAMP (A), p-JNK (B) and p-ERK1/2 (C) by ELISA assays in H9c2 cells and neonatal mouse ventricular cardiomyocytes (NMCMs) treated with α-MSH (0.1 nM) for 5, 15, 30 or 60 minutes. **(D-G)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppb* (B-type natriuretic peptide), *Acta2* (alpha-smooth muscle actin), *Tgfb1* (transforming growth factor beta 1) and *Il6* (interleukin 6) in H9c2 cells and NMCMs treated with α-MSH (0.1 nM) for 1, 3, 6 or 24 hours. n=3–7 per group (technical replicates) in each graph from 2–3 independent experiments. Data are mean ± SEM. *P<0.05 versus control (0 min/ 0 h) in H9c2 cells, #P<0.05 and ##P<0.01 versus control (0 min/ 0 h) in NMCMs by 1-way ANOVA and Dunnett's post hoc test. α-MSH; α-Melanocyte-stimulating hormone, cAMP; cyclic adenosine monophosphate, ELISA; enzyme-linked immunosorbent assay, ERK; extracellular signal-regulated kinase, JNK; c-Jun N-terminal kinase.

Next, cellular hypertrophy was promoted with Ang II, and the effects of α-MSH were investigated in these hypertrophic conditions in H9c2 cells. A leucine incorporation assay was performed to measure the protein synthesis rate, and it was observed that α-MSH effectively prevented Ang II-induced increase in leucine incorporation (**Figure 10A**). Additionally, α-MSH prevented Ang II-induced increase in JNK phosphorylation (**Figure 10B**), and *Nppb* (**Figure 10C**) and *Acta2* (**Figure 10D**) gene expression.

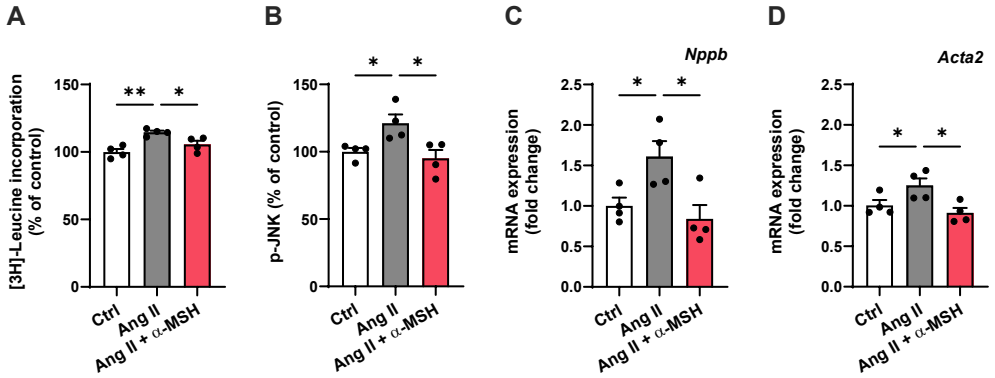


Figure 10. α-Melanocyte-stimulating hormone reverses angiotensin II-induced hypertrophy in cultured H9c2 cells. **(A)** Quantification of [^{3}H]-Leucine incorporation as a measure of protein synthesis in H9c2 cells treated with Ang II (0.1 μM) for 24 hours in the absence or presence of α-MSH (0.1 nM). **(B-D)** Quantification of p-JNK by ELISA assay (B) and quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppb* (B-type natriuretic peptide) and *Acta2* (alpha-smooth muscle actin) in H9c2 cells treated with Ang II (0.1 μM) for 3 hours in the absence or presence of α-MSH (0.1 nM). n=4 per group. Data are mean ± SEM. *P<0.05 and **P<0.01 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc test. α-MSH; α-Melanocyte-stimulating hormone, Ang II; angiotensin II, ELISA; enzyme-linked immunosorbent assay, JNK; c-Jun N-terminal kinase.

Since α-MSH is a pan-agonist of all MCRs, qPCR analysis on H9c2 cells treated with subtype-selective MCR agonists was performed to investigate which receptor subtype mediates the effects of α-MSH. The only compound mimicking the effects of α-MSH was the MC5R selective agonist PG-901 that showed downregulation of *Tgfb1* and a tendency towards reduced expression of *Nppb*, *Acta2* and *Il6* (**Figure 11A-D**). To test the dependency of the α-MSH-induced effects on MC5R subtype, H9c2 cells were simultaneously treated with α-MSH and the MC5R antagonist PG-20N. The presence of PG-20N abrogated the effect of α-MSH on JNK phosphorylation (**Figure 11E**). In addition, PG-20N seemed to reverse the effect of α-MSH on various gene expressions. Particularly, it abolished the α-MSH-induced downregulation of *Nppb* mRNA expression. (**Figure 11F-H**) Taken together, the results demonstrate that functional MCRs exist in cardiomyocytes and suggest that α-MSH acts as an anti-hypertrophic regulator in cardiomyocytes by activating MC5R subtype.

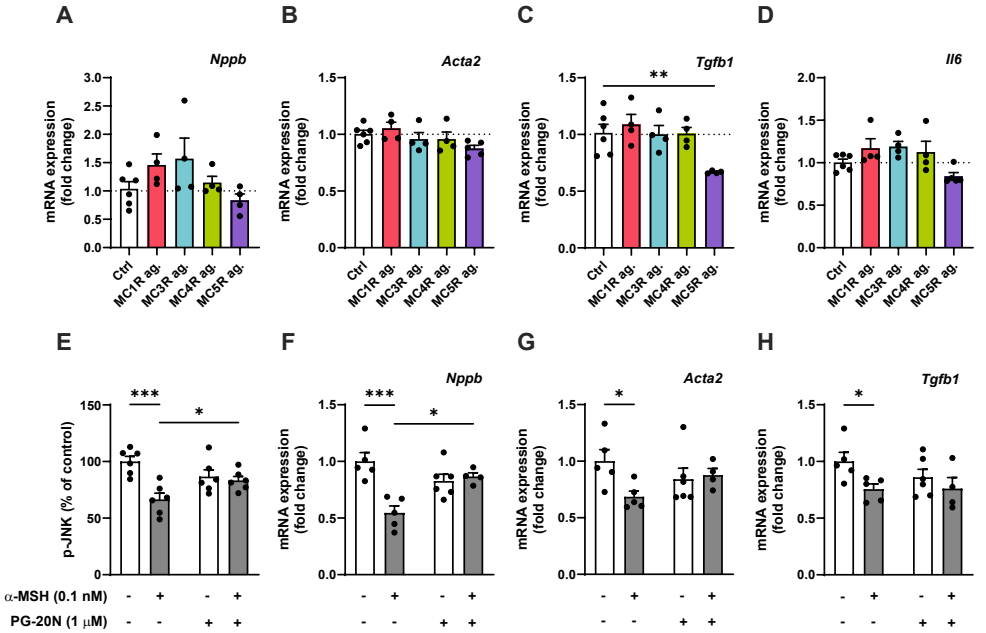


Figure 11. Melanocortin 5 receptor mediates the effects of α-Melanocyte-stimulating hormone in H9c2 cells. **(A–D)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppb* (B-type natriuretic peptide), *Acta2* (alpha-smooth muscle actin), *Tgfb1* (transforming growth factor beta 1) and *Il6* (interleukin 6) in H9c2 cells treated with subtype-selective MCR agonists for 3 hours. **(E–H)** Quantification of p-JNK by ELISA assay (E) and qPCR analysis of *Nppb*, *Acta2* and *Tgfb1* expression (F-H) in H9c2 cells treated with α-MSH (0.1 nM) for 1 hour in the absence or presence of the selective MC5R antagonist PG-20N (1 μM). n=4–6 per group in each graph from 2 independent experiments. Data are mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc test (A-D) or by 2-way ANOVA and Bonferroni's post hoc test (E–H). α-MSH; α-Melanocyte-stimulating hormone, ag.; agonist, ELISA; enzyme-linked immunosorbent assay, JNK; c-Jun N-terminal kinase, MCR; melanocortin receptor.

5.2.1 MC1R-mediated effects

Even though MC1R did not appear to be the primary receptor for mediating the effects of α-MSH in cardiomyocytes, the effects evoked by the selective MC1R agonist LD211 were explored based on the abundant expression of MC1R in the mouse heart. Thus, H9c2 cells were first treated with different concentrations of LD211 for 24 hours and then assayed for leucine incorporation as a measure of protein synthesis rate. Activation of MC1R increased leucine incorporation, and the most significant effect was observed in the subnanomolar concentration of LD211 (**Figure 12A**). Next, H9c2 cells were treated with Ang II and LD211 to investigate whether LD211 potentiates Ang II-induced hypertrophy. Both LD211 and Ang II alone increased leucine incorporation, but simultaneous treatment did not amplify the effect further (**Figure 12B**). To investigate the effects of MC1R activation on gene expression, H9c2 cells and NMCMs were treated with LD211 for 1, 3, 6 or 24

hours and the mRNA expression of hypertrophy- and fibrosis-associated genes was analysed by qPCR. The analysis revealed that the hypertrophy-related gene *Nppb* was upregulated in LD211-treated H9c2 cells (**Figure 12C**), whereas no change in *Nppa* expression was detected in LD211-treated NMCMs (**Figure 12D**). Furthermore, LD211 induced transcriptomic changes, especially in various fibrosis-related genes, including *Acta2* and *Tgfb1*, in NMCMs (**Figure 12E-F**).

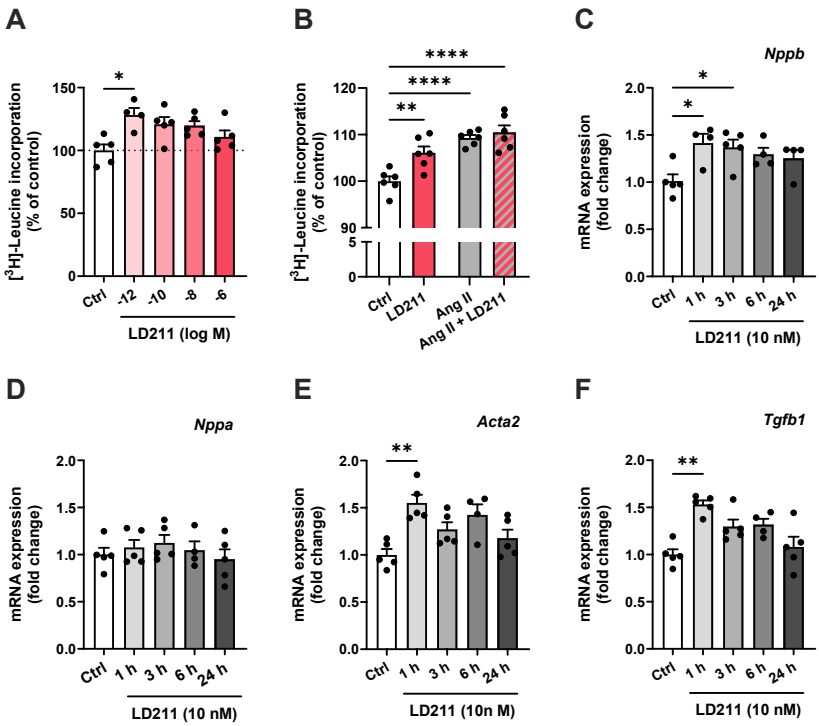


Figure 12. Pharmacological activation of melanocortin 1 receptor promotes cardiomyocyte hypertrophy. **(A)** Quantification of [^{3}H]-Leucine incorporation as a measure of protein synthesis in H9c2 cells treated with different concentrations of LD211 for 24 hours. **(B)** [3H]-Leucine incorporation assay in H9c2 cells treated with LD211 (10 nM) or control (PBS) for 24 hours in the absence or presence of Ang II (1 µM). **(C–F)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppb* (B-type natriuretic peptide) in H9c2 cells (C) and *Nppa* (atrial natriuretic peptide), *Acta2* (alpha-smooth muscle actin) and *Tgfb1* (transforming growth factor beta 1) in neonatal mouse ventricular cardiomyocytes (NMCM) (D–F) treated with LD211 (10 nM) for 1, 3, 6 and 24 hours. Data are mean ± SEM, n=4–6 per group in each graph. *P<0.05, **P<0.01 and ****P<0.0001 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc test (B) or Kruskal-Wallis and Dunn's post hoc test (A, C-F). Ang II; angiotensin II, LD211; melanocortin 1 receptor selective agonist.

Finally, the effects of MC1R activation on intracellular signalling pathways in cardiomyocytes were investigated. No change in cAMP was observed after

stimulation with LD211 in H9c2 cells (**Figure 13A**). Other possible signalling pathways were then screened, and it was observed that LD211 increased the phosphorylation of CREB and p38 after 30 or 15 minutes of treatment in H9c2 cells (**Figure 13B-C**), while no change in the phosphorylation of ERK1/2 or JNK was detected (data not shown). To investigate the dependency of the hypertrophic response on CREB and p38-mediated signalling after MC1R activation, H9c2 cells were treated with LD211 in the presence or absence of the CREB and p38 selective inhibitors 666-15 and TAK-715, respectively. The selective CREB inhibitor 666-15 blocked LD211-induced increase in leucine incorporation (**Figure 13D**). The inhibition of p38 signalling with TAK-715 reduced p38 phosphorylation and blocked

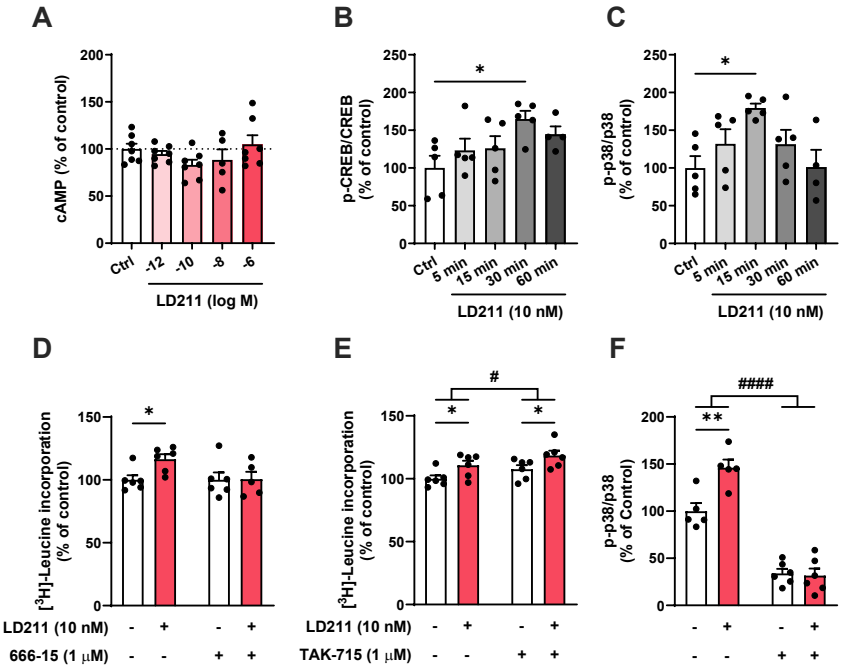


Figure 13. LD211 induced changes in intracellular signalling cascades in H9c2 cells. **(A)** Quantification of intracellular cAMP level by ELISA assay in response to different concentrations of LD211 for 30 minutes. **(B–C)** Quantification of p-CREB (B) and p-p38 (C) normalised against total protein amounts by Western blot after LD211 (10 nM) treatment for 5, 15, 30 or 60 minutes. **(D–E)** [^{3}H]-Leucine incorporation assay quantification after pre-treatment with or without the CREB inhibitor 666-15 (1 μM) for 2 hours (D) or the p38 inhibitor TAK-715 (5 μM) for 30 minutes (E), followed by treatment with LD211 (10 nM) or control (PBS) for 24 hours. **(F)** Quantification of p-p38 protein expression normalised against total protein amount by Western blot after pretreatment with or without the p38 inhibitor TAK-715 (5 μM) for 30 minutes, followed by treatment with LD211 (10 nM) for 15 minutes. n=4–7 per group in each graph. Data are mean ± SEM. *P<0.05 and **P<0.01 for the indicated comparisons by Kruskal-Wallis and Dunn's post hoc test (B-C) or 2-way ANOVA and Šídák's post hoc test (D–F). $^{\#}$P<0.05 and $^{\#\#\#\#}$P<0.0001 for the main effect of TAK-715 by 2-way ANOVA. cAMP; cyclic adenosine monophosphate, CREB; cAMP response element-binding protein, ELISA; enzyme-linked immunosorbent assay, LD211; melanocortin 1 receptor selective agonist, p38; p38 mitogen-activated protein kinase.

the LD211-induced effect in this regard (**Figure 13F**), but it did not, however, interfere with the effect of LD211 on leucine incorporation (**Figure 13E**).

In addition to mouse and rat cardiomyocytes, it was lastly investigated if MC1R is also expressed in human cardiomyocytes and whether the expression is affected by hypertrophic stimuli. For this intention, hiPSC-CM were induced to hypertrophy with prolonged stretching for 24 or 48 hours (**Figure 14A**) or 24 hours of ET-1 exposure (**Figure 14B)**. The expression of MC1R in hiPSC-CMs showed downregulation in both hypertrophy models. The decrease occurred in relation to the state of hypertrophy; the stronger the hypertrophy stimulus, the greater the decrease of *MC1R* expression. Collectively, these results show that MC1R is expressed in mouse and rat as well as in human cardiomyocytes, and different hypertrophic stimuli modulate the expression. These results demonstrate that activation of MC1R promotes cardiomyocyte hypertrophy, possibly through CREB signalling.

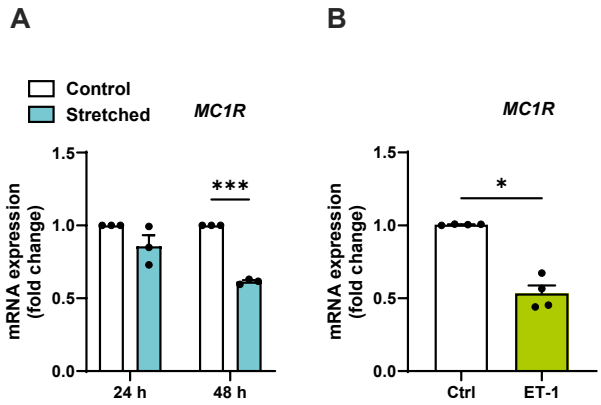


Figure 14. Melanocortin 1 receptor mRNA expression in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM). **(A–B)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *MC1R* (melanocortin 1 receptor) in hiPSC-CMs that were mechanically stretched for 24 or 48 hours (A) or treated with endothelin-1 (ET-1, 100 nM) for 24 hours (B). n=3–4 individual experiments/ batches of differentiation. Data are mean ± SEM. *P<0.05 and ***P<0.001 vs control by 2-way ANOVA and Šídák's post hoc test (A) or Mann-Whitney *U* test (B).

5.2.2 MC5R-mediated effects

Since MC5R appeared to mediate the anti-hypertrophic effects of α-MSH in cardiomyocytes, more thorough research was conducted using the MC5R-selective agonist PG-901. It was first explored whether PG-901 evokes similar changes in intracellular signalling as detected with α-MSH. For this purpose, H9c2 cells were treated with different concentrations of PG-901 and the intracellular cAMP level was measured. PG-901 reduced cAMP level (**Figure 15A**). Other possible signalling pathways were also explored, and a reduction in phosphorylated JNK (**Figure 15B**)

and no change in phosphorylated ERK1/2 (data not shown) in PG-901-treated H9c2 cells and NMCMs were observed, thus mimicking the responses evoked by α-MSH. In addition, PG-901 downregulated various fibrosis-related genes, including *Acta2*, *Tgfb1* and *Fn1* (fibronectin-1), in H9c2 cells (**Figure 15C-E**).

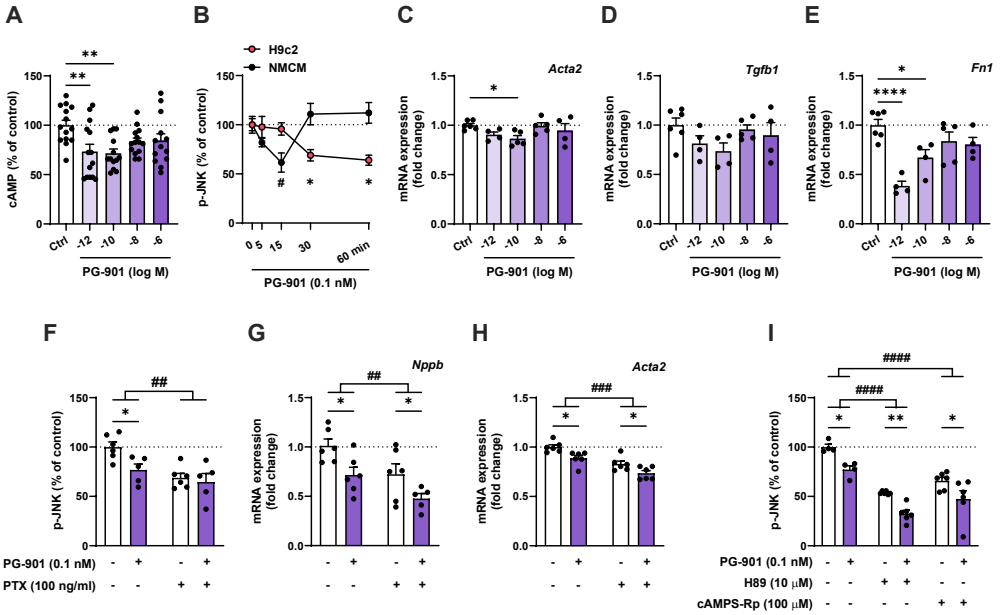


Figure 15. The effects of selective melanocortin 5 receptor agonist PG-901 on intracellular signalling cascades. **(A)** Quantification of intracellular cAMP levels in H9c2 cells treated with different concentrations of PG-901 for 30 minutes. n=13–14 per group from 3 independent experiments. **(B)** Quantification of p-JNK by ELISA assay in H9c2 cells and neonatal mouse ventricular cardiomyocytes (NMCM) treated with PG-901 (0.1 nM) for 5, 15, 30 or 60 minutes. *P<0.05 versus control (0 min) in H9c2 cells, $^{\#}$P<0.05 versus control (0 min) in NMCMs by 1-way ANOVA and Dunnett's post hoc test. n=4–6 per time point from 2 independent experiments. **(C–E)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Acta2* (alpha-smooth muscle actin), *Tgfb1* (transforming growth factor beta 1) and *Fn1* (fibronectin-1) in H9c2 cells treated with different concentrations of PG-901 for 3 hours. **(F–H)** Quantification of p-JNK by ELISA assay (F) and *Nppb* and *Acta2* mRNA expression levels by qPCR (G–H) in H9c2 cells treated with or without pertussis toxin (PTX) for 18 hours, followed by PG-901 treatment for 60 minutes (F) or 3 hours (G–H). **(I)** Quantification of p-JNK by ELISA assay in H9c2 cells treated with or without PKA inhibitor H89 (10 μM) or cAMP analogue cAMPS-Rp (100 μM) for 30 minutes, followed by PG-901 treatment for 60 minutes. n=4–6 per group in each graph (C–I). Data are mean ± SEM. *P<0.05, **P<0.01 and ****P<0.0001 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc tests (A, C–E) or 2-way ANOVA and Šídák's post hoc tests (F–I). $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and $^{\#\#\#\#}$P<0.0001 for the main effect of PTX/ H-89/ cAMPS-Rp by 2-way ANOVA. cAMP; cyclic adenosine monophosphate, ELISA; enzyme-linked immunosorbent assay, JNK; c-Jun N-terminal kinase, PG-901; melanocortin 5 receptor selective agonist.

Since both α-MSH and PG-901 reduced cAMP level, it was hypothesised that MC5R is coupled with G_i receptor and thus further mechanistic experiments with H9c2 cells using the G_i protein inhibitor pertussis toxin were performed. However, treatment with pertussis toxin induced further reduction in phosphorylated JNK (**Figure 15F**) and downregulated *Nppb* and *Acta2* (**Figure 15G-H**), and it did not abolish the effects of PG-901, implying that the downstream effects of MC5R activation are not mediated *via* G_i signalling pathway. Since MC5R signalling can simultaneously couple to G_i- and G_s- pathways (Rodrigues et al., 2015), it was also investigated whether the effects of PG-901 could be reversed by blocking the G_s coupling. However, the PKA inhibitor H89 or the cAMP analogue cAMP-Rp, which antagonises cAMP-induced PKA activation, did not abrogate PG-901-induced reduction of p-JNK (**Figure 15I**) or changes in gene expression (data not shown). Collectively, these results indicate that the downstream effects of MC5R activation occur in a cAMP-independent manner.

To investigate whether MC5R regulates cardiomyocyte growth under hypertrophic conditions, H9c2 cells and NMCMs were treated with PG-901 combined with Ang II and protein synthesis rate was measured using the leucine incorporation method. PG-901 effectively blunted Ang II-induced increase in leucine incorporation in both H9c2 cells and NMCMs (**Figure 16A**). In addition, PG-901 treatment blunted many Ang II-induced changes in fibrotic gene expression, such as *Acta2* and *Tgfb1*, in H9c2 cells (**Figure 16B-C**).

Next, MC5R was silenced with siRNA and an increase in JNK phosphorylation (**Figure 16D**), upregulation of many hypertrophy- and fibrosis-related genes (**Figure 16F**), and enhanced leucine incorporation (**Figure 16E**) in *Mc5r*-silenced cells were observed. To test the dependency of the observed phenotype on JNK signalling, H9c2 cells were treated with the JNK inhibitor SP600125 prior to transfection with the *Mc5r*-targeting siRNA. JNK inhibition completely abolished the increase in leucine incorporation (**Figure 16G**) and the upregulation of *Nppb* and *Ctgf* (connective tissue growth factor) in *Mc5r*-silenced cells (**Figure 16H-I**).

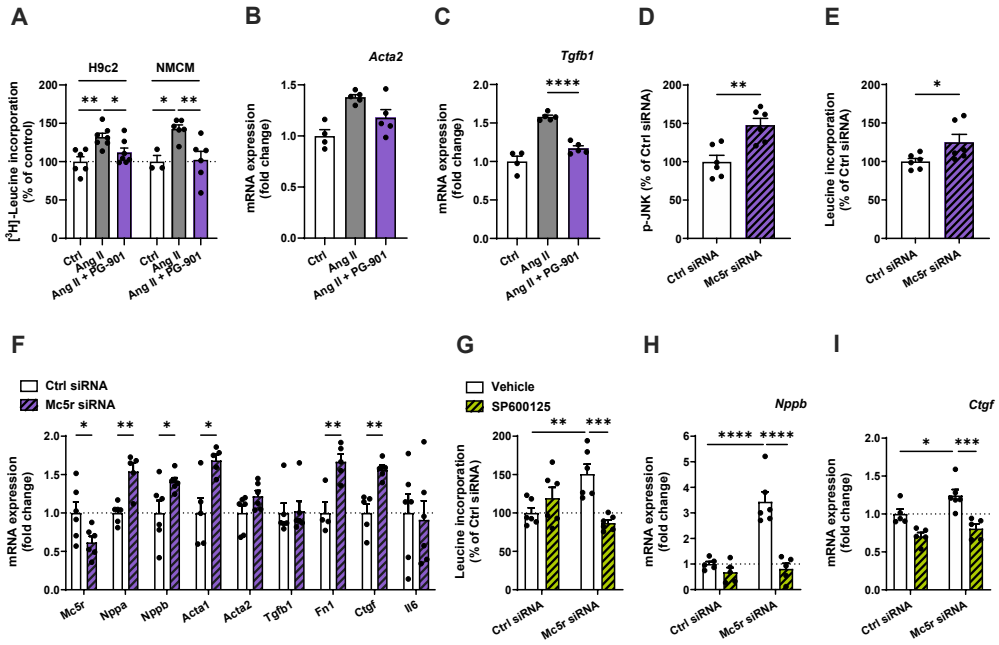


Figure 16. Cardiomyocyte hypertrophy is regulated via melanocortin 5 receptor signalling through JNK pathway. **(A)** [^{3}H]-Leucine incorporation assay in H9c2 cells and neonatal mouse ventricular cardiomyocytes (NMCM) treated with Ang II (0.1 μM) for 24 hours in the absence or presence of PG-901 (0.1 nM). **(B–C)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Acta2* (alpha-smooth muscle actin) and *Tgfb1* (transforming growth factor beta 1) in H9c2 cells treated with Ang II (0.1 μM) for 3 hours in the absence or presence of PG-901 (0.1 nM). **(D–F)** Quantification of p-JNK using ELISA assay in H9c2 cells (D) and [^{3}H]-Leucine incorporation (E) and qPCR analysis of the indicated genes (F) in NMCMs treated with control siRNA or Mc5r targeting siRNA for 24 hours. **(G-I)** [^{3}H]-Leucine incorporation (G) and qPCR analysis of *Nppb* (B-type natriuretic peptide) and *Ctgf* (connective tissue growth factor) mRNA expression (H-I) in H9c2 cells treated with or without the JNK inhibitor SP600125 (10 μM) for 30 minutes, followed by transfection with control siRNA or Mc5r targeting siRNA for 24 hours. n=3–7 per group in each graph from 2 independent experiments. Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc test (A–C) or unpaired Student's t-test (D–F) or 2-way ANOVA and Bonferroni's post hoc test (G–I). Ang II; angiotensin II, ELISA; enzyme-linked immunosorbent assay, JNK; c-Jun N-terminal kinase, PG-901; melanocortin 5 receptor selective agonist.

Based on the findings suggesting that MC5R in mouse and rat cardiomyocytes plays an anti-hypertrophic role, it was interesting to know if human cardiomyocytes also express MC5R and whether it is affected by hypertrophic stimuli or agonistic treatment. First, hiPSC-CMs were induced to hypertrophy by mechanical stretching for 24 or 48 hours or 24 hours of ET-1 treatment. Expression of *MC5R* mRNA was downregulated in both hypertrophy models, but with mechanical stretching only after prolonged stimuli (**Figure 17A, D**). In addition, hypertrophy-associated natriuretic peptide mRNA expressions were affected by

both models, even though stretching showed milder and distinct expression patterns with rapid upregulation of NPPB and delayed upregulation of NPPA (**Figure 17B-C, E**). To study whether MC5R is also functionally active in human cardiomyocytes, hiPSC-CMs were treated with PG-901 under baseline and hypertrophic conditions. Gene expression analysis revealed downregulation of many genes, including *TGFB1* and *FN1*, in PG-901-treated hiPSC-CMs (**Figure 17F-G**). Furthermore, PG-901 attenuated the ET-1-induced increase in proBNP

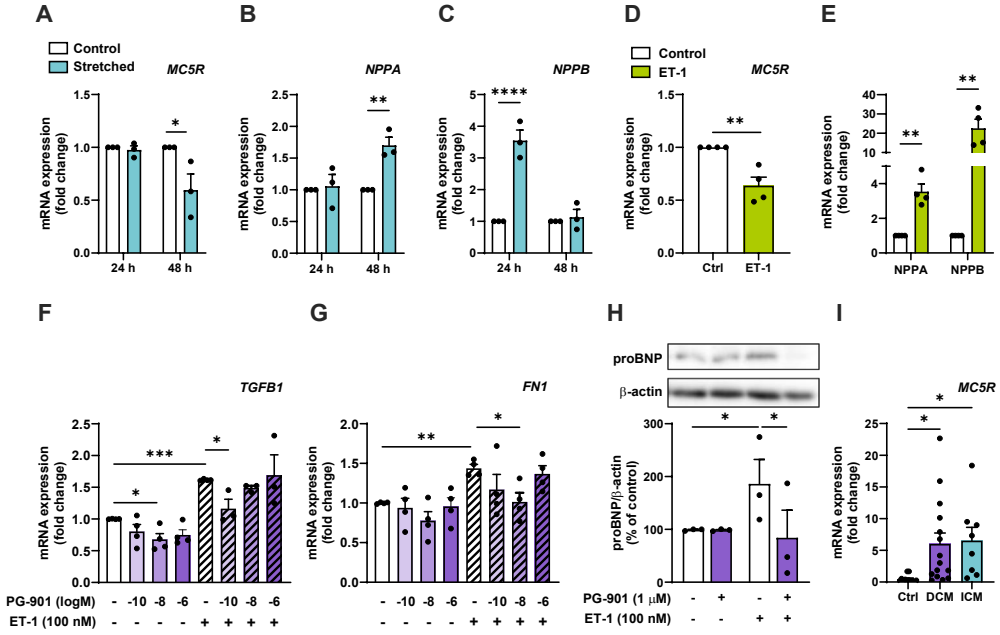


Figure 17. Melanocortin 5 receptor is expressed and functionally operative in human cardiomyocytes. **(A–E)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *MC5R* (melanocortin 5 receptor), *NPPA* (atrial natriuretic peptide) and *NPPB* (B-type natriuretic peptide) in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) that were mechanically stretched for 24 or 48 hours (A–C) or treated with endothelin-1 (ET-1) (100 nM) for 24 hours (D–E). **(F–G)** qPCR analysis of *TGFB1* (transforming growth factor beta 1) and *FN1* (fibronectin-1) mRNA expression in hiPSC-CMs treated with different concentrations of PG-901 for 24 hours in the absence or presence of ET-1 (100 nM). **(H)** Representative Western blots and quantification of proBNP protein expression in hiPSC-CMs treated with PG-901 (1 μM) for 24 hours in the absence or presence of ET-1 (100 nM). **(I)** qPCR analysis of *MC5R* mRNA expression in left ventricle samples from healthy control subjects (ctrl, n=13) and from patients with end-stage dilated (DCM, n=14) or ischemic (ICM, n=8) cardiomyopathy. Data are mean ± SEM. n=3–4 individual experiments/ batches of differentiation (A–H). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 for the indicated comparisons by randomised block ANOVA (using individual experiments and treatment as factors) and Dunnett's post hoc test (A–H) or by 1-way ANOVA and Dunnett's post hoc test (I). PG-901; melanocortin 5 receptor selective agonist.

protein expression in hiPSC-CMs (**Figure 17H**). Lastly, quantification of *MC5R* mRNA expression from LV samples of control subjects and patients with end-stage dilated (DCM) or ischemic cardiomyopathy (ICM) revealed significant upregulation of *MC5R* in DCM and ICM patients compared to control subjects (**Figure 17I**). Taken together, these results demonstrate that MC5R is present and functional in mouse, rat, and human cardiomyocytes, and it regulates cardiomyocyte hypertrophy through the JNK signalling pathway.

5.3 The role of MC1R in experimental pathological and physiological cardiac remodelling

The finding that MC1R is expressed in the heart and affected by different hypertrophic stimuli led to the hypothesis that MC1R is involved in the regulation of pathological or physiological cardiac remodelling. The *in vitro* findings also supported this hypothesis, showing that MC1R activation promotes hypertrophic signalling. To test this hypothesis, experiments with MC1R-deficient mice were performed, and it was explored whether silencing of MC1R could evoke an opposite phenotype to that observed in LD211-treated cardiomyocytes.

5.3.1 Effects of MC1R deficiency in pathological cardiac hypertrophy

To investigate the role of MC1R in pathological cardiac remodelling, global MC1R-deficient mice ($Mc1r^{e/e}$) and their age-matched WT controls were first used and subjected to TAC surgery to induce LV pressure overload. Sham-operated mice were used as controls. Cardiac phenotyping after 8 weeks of sham or TAC operation showed no genotype difference in ventricular weight of sham-operated mice, but it revealed a milder increase in ventricular weight in $Mc1r^{e/e}$ mice compared to WT mice in response to pressure overload (**Figure 18A-B**). Echocardiography imaging after 8 weeks of the surgical operation showed impaired LV systolic function in pressure overloaded mice, measured by EF, but no genotype effect was observed in this regard (**Figure 18C**). Furthermore, MC1R deficiency had no significant effect on LV diameter or wall thicknesses as analysed by echocardiography (data not shown). At the molecular level, $Mc1r^{e/e}$ mice showed attenuated response to pressure overload as evidenced by qPCR, which revealed downregulation of hypertrophy- and fibrosis-associated marker genes such as *Nppa*, *Ctgf* and *Mmp2* (matrix metalloproteinase-2) in TAC-operated $Mc1r^{e/e}$ mice compared to TAC-operated WT mice (**Figure 18D, F-G**). Intriguingly, *Nppb* expression was upregulated in sham-operated $Mc1r^{e/e}$ mice and was not further increased after TAC operation (**Figure 18E**).

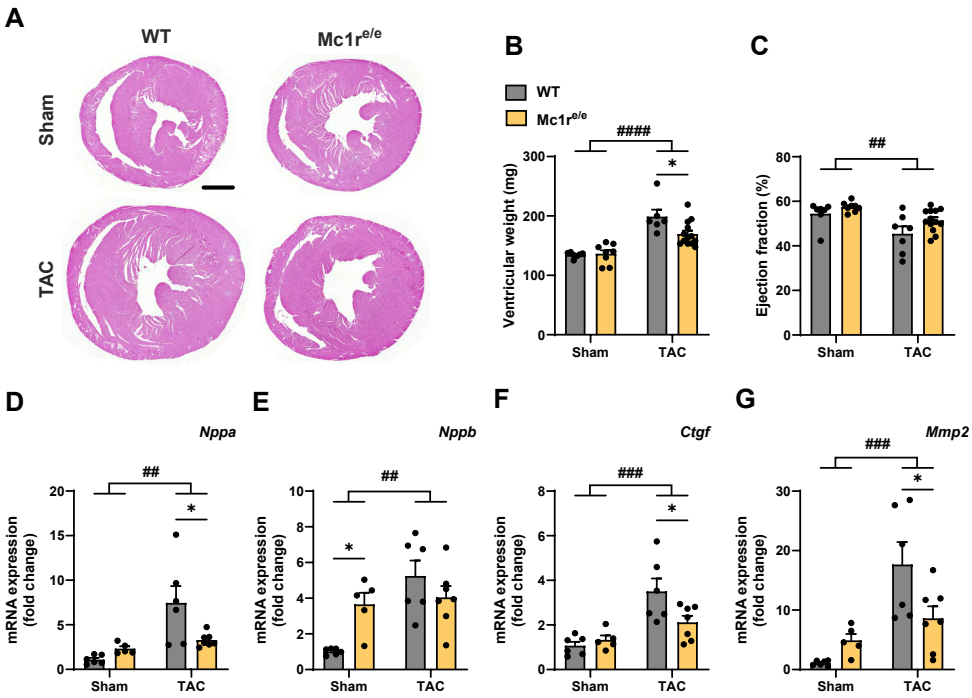


Figure 18. Global melanocortin 1 receptor deficiency blunts pressure overload-induced cardiac hypertrophy. **(A)** Representative hematoxylin and eosin-stained cardiac cross-sections showing the gross morphology of the heart of WT and Mc1r$^{e/e}$ mice at the end of the experiment after 8 weeks of sham or TAC operation. Scale bar, 1 mm. **(B-C)** Ventricular weight (B) and echocardiographic analysis of left ventricular ejection fraction (C) in WT and Mc1r$^{e/e}$ mice at the end of the experiment after 8 weeks of sham or TAC operation. n=7 in sham WT mice, n=8 in sham Mc1r$^{e/e}$ mice, n=6-7 in TAC WT mice, and n=13 in TAC Mc1r$^{e/e}$ mice. **(D-G)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppa* (atrial natriuretic peptide), *Nppb* (B-type natriuretic peptide), *Ctgf* (connective tissue growth factor) and *Mmp2* (matrix metalloproteinase 2) in the left ventricle of WT and Mc1r$^{e/e}$ mice after 8 weeks of sham or TAC operation. n=5-7 per group in each graph. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05 for the indicated comparisons by 2-way ANOVA and Šídák's post hoc test. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and $^{\#\#\#\#}$P<0.0001 for the main effect of TAC by 2-way ANOVA. Mc1r$^{e/e}$; melanocortin 1 receptor recessive yellow, TAC; transverse aortic constriction, WT; wildtype.

Since the global MC1R-deficient Mc1r$^{e/e}$ mice showed a blunted hypertrophic response to LV pressure overload, it was next investigated if the phenotype is primarily driven by disturbed MC1R signalling specifically in cardiomyocytes. To this end, a tamoxifen-inducible Mc1r-cKO mouse model was engineered, and these mice and their age-matched controls (Myh6-MCM) were subjected to TAC or sham operation. Cardiac phenotyping showed a milder response to pressure overload in Mc1r-cKO mice compared to Myh6-MCM mice (**Figure 19A**), as evidenced by lower ventricular weight after 8 weeks of TAC operation (**Figure 19B**). At the cellular level, TAC-challenged Mc1r-cKO mice showed a higher cardiomyocyte length-to-CSA ratio compared to Myh6-MCM mice (**Figure 19C**). Echocardiographic analyses at the end

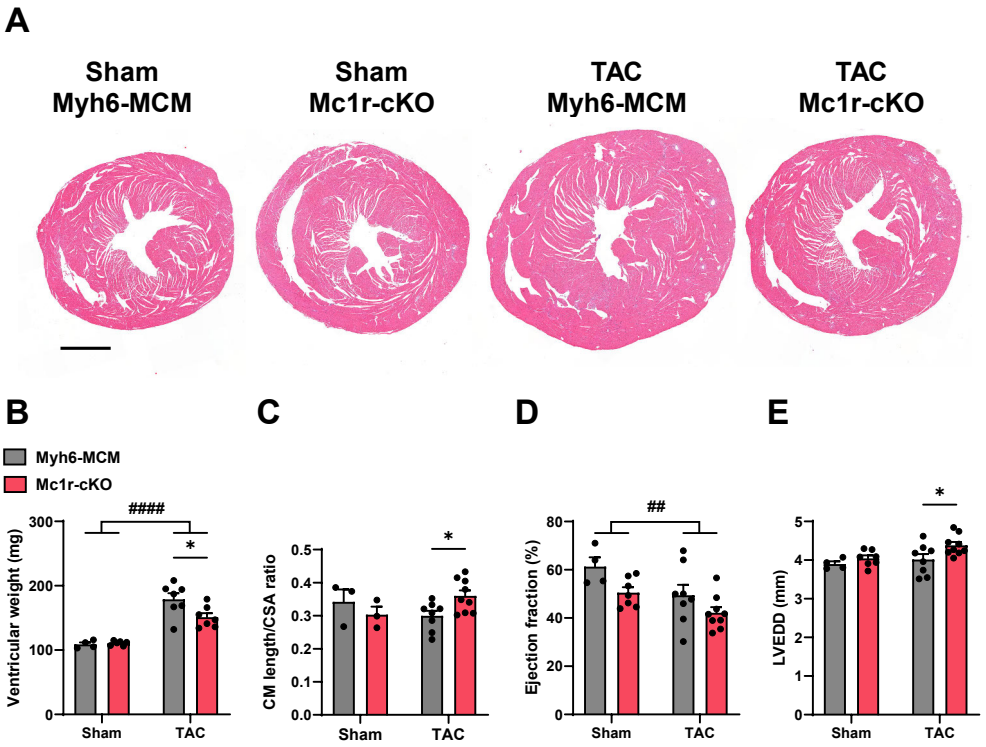


Figure 19. Cardiomyocyte-specific melanocortin 1 receptor knockout mice show reduced left ventricular hypertrophy but signs of compromised cardiac function after transverse aortic constriction-induced pressure overload. **(A)** Representative hematoxylin and eosin-stained cardiac cross-sections showing the gross morphology of the heart of control (Myh6-MCM) and Mc1r-cKO mice at the end of the experiment after 8 weeks of sham or TAC operation. Scale bar, 1 mm. **(B-E)** Ventricular weight (B), cardiomyocyte length to cross-sectional area ratio (C), and echocardiographic analysis of left ventricular ejection fraction (D) and left ventricular end-diastolic dimension (E) in Myh6-MCM and Mc1r-cKO mice at the end of the experiment after 8 weeks of sham or TAC operation. n=3-4 in sham Myh6-MCM mice, n=3-7 in sham Mc1r-cKO mice, n=7-8 in TAC Myh6-MCM mice and n=7-9 in TAC Mc1r-cKO mice. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05 for the indicated comparisons by 2-way ANOVA and Šídák's post hoc test. $^{\#\#}$P<0.01 and $^{\#\#\#\#}$P<0.0001 for the main effect of TAC by 2-way ANOVA. CM; cardiomyocyte, CSA; cross-sectional area, LVEDD; left ventricular end-diastolic dimension, Mc1r-cKO; cardiomyocyte-specific melanocortin 1 receptor knockout, Myh6-MCM; Myh6-MerCreMer transgenic, TAC; transverse aortic constriction.

of the experiment showed a general reduction in EF in Mc1r-cKO mice (P = 0.015 for genotype effect by 2-way ANOVA), but *post hoc* comparisons between the genotypes did not reach statistical significance (**Figure 19D**). Additionally, echocardiography revealed increased LVEDD in TAC-operated Mc1r-cKO mice compared to TAC-operated Myh6-MCM mice (**Figure 19E**).

Corroborating the finding of reduced ventricular weight, gene expression profiling revealed suppressed upregulation of hypertrophy-related genes, such as *Nppa* (**Figure**

20A), in the LV of TAC-operated Mc1r-cKO mice. However, the expression of *Nppb* was similarly upregulated in both genotypes after TAC operation (**Figure 20B**). Interestingly, the expression of *Myh6* and *Myh7* genes that encode two isoforms of cardiac contractility proteins α- and β-MHC, respectively, were upregulated in both sham- and TAC-operated Mc1r-cKO mice (**Figure 20C-D**). Cardiac mRNA levels of fibrosis-associated genes, including *Ctgf* and *Acta2*, were reduced in Mc1r-cKO mice compared to Myh6-MCM mice after TAC operation (**Figure 20E-F**). However, Picrosirius Red staining of cardiac cross-sections and quantification of the extent of LV fibrosis did not show a parallel reduction in TAC-operated Mc1r-cKO mice (**Figure 20G**).

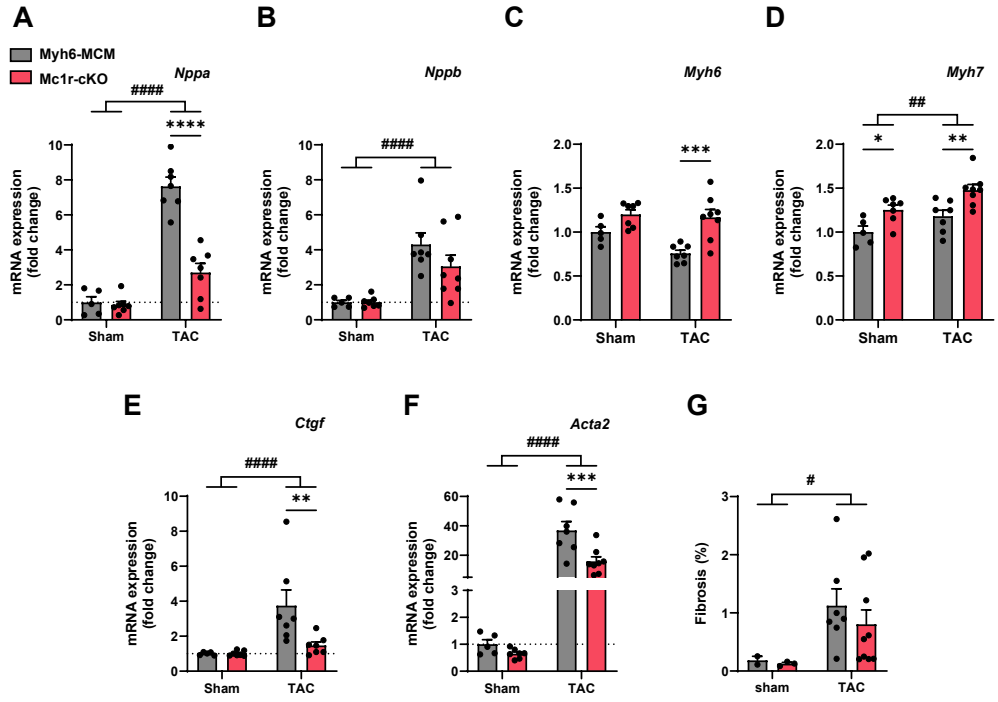


Figure 20. Cardiomyocyte-specific melanocortin 1 receptor knockout mice show signs of reduced hypertrophy and fibrosis at the gene level. **(A-F)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppa* (atrial natriuretic peptide), *Nppb* (B-type natriuretic peptide), *Myh6* (myosin heavy cahin-α), *Myh7* (myosin heavy chain-β), *Ctgf* (connective tissue growth factor) and *Acta2* (α-smooth muscle actin) in the left ventricle of control (Myh6-MCM) and Mc1r-cKO mice at the end of the experiment after 8 weeks of sham or TAC operation. n=5-8 per group in each graph. **(G)** Quantification of the extent of left ventricular fibrosis in Myh6-MCM and Mc1r-cKO mice at the end of the experiment after 8 weeks of sham or TAC operation. n=2 in sham Myh6-MCM mice, n=3 in sham Mc1r-cKO mice, n=7 in TAC Myh6-MCM mice and n=9 in TAC Mc1r-cKO mice. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 for the indicated comparisons by 2-way ANOVA and Šídák's post hoc test. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#\#}$P<0.0001 for the main effect of TAC by 2-way ANOVA. Mc1r-cKO; cardiomyocyte-specific melanocortin 1 receptor knockout, Myh6-MCM; Myh6-MerCreMer transgenic, TAC; transverse aortic constriction.

Additionally, testing whether the phenotype of Mc1r-cKO mice occurs independently of the chosen model of pathological cardiac hypertrophy was also intended. For this purpose, Mc1r-cKO mice and their age-matched Myh6-MCM controls were subjected to 4-week Ang II infusion via osmotic minipumps as another model of pathological cardiac hypertrophy. Consistent with the TAC-model, H&E-stained cardiac cross-sections and measurement of ventricular weight at sacrifice showed blunted hypertrophic growth of the heart in Ang II-infused Mc1r-cKO mice compared to Myh6-MCM control mice (**Figure 21A-B**). Although absolute ventricular weight did not reach statistical significance, the relative ventricular weight-to-body weight ratio was more evident and statistically significant (data not shown). LV fibrosis was significantly increased by Ang II infusion, but no genotype difference was observed in this regard, even though

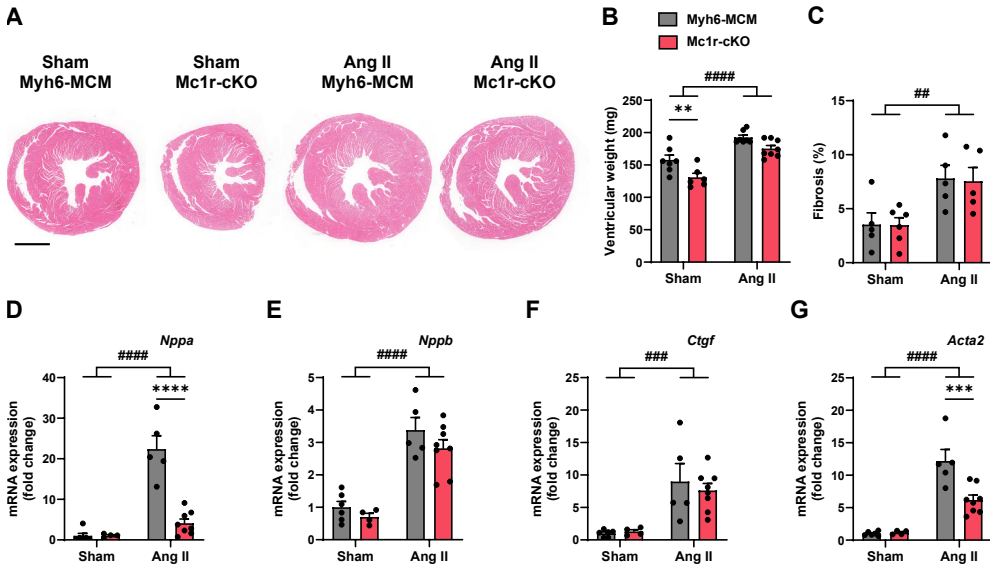


Figure 21. Cardiomyocyte-specific melanocortin 1 receptor knockout mice show reduced left ventricular hypertrophy after 4 weeks of angiotensin II infusion. **(A)** Representative hematoxylin and eosin-stained cardiac cross-sections showing the gross morphology of the heart of control (Myh6-MCM) and Mc1r-cKO mice subjected to sham operation or 4 weeks of Ang II infusion. Scale bar, 1 mm. **(B-C)** Ventricular weight (B) and extent of left ventricular fibrosis (C) in Myh6-MCM and Mc1r-cKO mice at the end of the experiment after 4 weeks of Ang II infusion or sham operation. n=5-7 in sham Myh6-MCM mice, n=6 in sham Mc1r-cKO mice, n=5-7 in Ang II Myh6-MCM mice and n=5-8 in Ang II Mc1r-cKO mice. **(D-G)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppa* (atrial natriuretic peptide), *Nppb* (B-type natriuretic peptide), *Ctgf* (connective tissue growth factor) and *Acta2* (α-smooth muscle actin) in the left ventricle of Myh6-MCM and Mc1r-cKO mice at the end of the experiment after 4 weeks of Ang II infusion or sham operation. n=4-8 per group in each graph. Data are mean ± SEM. Each dot represents an individual mouse. **P<0.01, ***P<0.001 and ****P<0.0001 for the indicated comparisons by 2-way ANOVA and Šídák's post hoc test. $^{\#\#}$P<0.01, $^{\#\#\#}$<0.001 and $^{\#\#\#\#}$P<0.0001 for the main effect of Ang II infusion by 2-way ANOVA. Ang II; angiotensin II, Mc1r-cKO; cardiomyocyte-specific melanocortin 1 receptor knockout, Myh6-MCM; Myh6-MerCreMer transgenic.

fibrotic gene *Acta2* was downregulated in the heart of Ang II-infused Mc1r-cKO mice compared to Myh6-MCM control mice, and other fibrosis-associated genes, such as *Ctgf*, showed a similar but nonsignificant decrease (**Figure 21C, F-G**). Altogether, qPCR analysis showed an mRNA expression profile coherent with the profile observed in TAC-operated Mc1r-cKO mice. Especially, Mc1r-cKO mice were devoid of the upregulation of *Nppa* after Ang II infusion, while *Nppb* was equally upregulated in Ang II-infused Myh6-MCM and Mc1r-cKO mice (**Figure 21D-E**).

5.3.2 Effects of MC1R deficiency in physiological cardiac hypertrophy

Secondly, it was investigated whether MC1R deficiency affects the hypertrophic response to physiological stimulus by subjecting $Mc1r^{e/e}$ mice and their WT controls to 5-week voluntary wheel running. Importantly, cumulative and average daily running distance did not differ between groups (data not shown), indicating equal hypertrophic stimulus between genotypes. After the 5-week exercise period, $Mc1r^{e/e}$ mice showed lower ventricular weight than WT mice (**Figure 22A**). Furthermore, echocardiography measurements revealed increased LVPW thickness in WT mice after voluntary wheel running and a tendency to compensatory decrease in LVEDD, responses that were completely lacking in $Mc1r^{e/e}$ mice, suggesting that they are not responsive to a physiological hypertrophy stimulus (**Figure 22B-C**). Notably, the

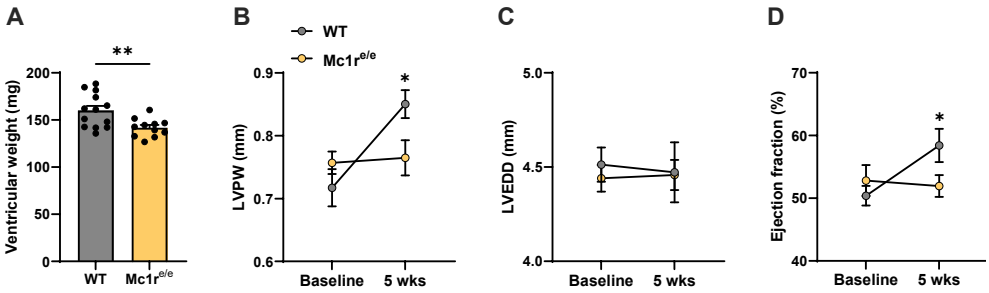


Figure 22. Melanocortin 1 receptor deficiency restrains physiological cardiac hypertrophy induced by voluntary wheel running. **(A)** Ventricular weight in WT and $Mc1r^{e/e}$ mice after 5 weeks of voluntary wheel running. n=13 in WT mice and n=11 in $Mc1r^{e/e}$ mice. **(B-D)** Echocardiographic analysis of left ventricular posterior wall thickness (B), left ventricular end-diastolic dimension (C) and left ventricular ejection fraction (D) in WT and $Mc1r^{e/e}$ mice at baseline and after 5 weeks of voluntary wheel running. n=10 mice per group in each graph. Data are mean ± SEM. Each dot represents an individual mouse. **P<0.01 vs WT mice by unpaired Student's t-test. (A) *P<0.05 vs WT mice at 5 weeks by 2-way repeated measures ANOVA and Šídák's post hoc test. (B-D) LVEDD; left ventricular end-diastolic dimension, LVPW; left ventricular posterior wall, $Mc1r^{e/e}$; melanocortin 1 receptor recessive yellow, WT; wildtype.

exercise-induced increase in LV systolic performance was also restricted in MC1R-deficient mice, as evidenced by lower EF in Mc1r$^{e/e}$ mice compared to WT mice after 5 weeks of running (**Figure 22D**).

Next, a similar voluntary wheel running experiment was performed with Mc1r-cKO and Myh6-MCM control mice to evaluate if cardiomyocyte-specific MC1R deficiency evokes a similar structural and functional response to aerobic exercise as global MC1R deficiency. Monitoring of running activity showed similar average and cumulative distance between genotypes (data not shown), demonstrating equal stimulus for physiological cardiac hypertrophy. There was no difference in ventricular weight between the genotypes at the end of the experiment (**Figure 23A**), but the exercise-induced increase in LVPW thickness was less evident in Mc1r-cKO mice, and the change in LVEDD went in opposite directions between the genotypes **Figure 23B-C**). Echocardiography also showed a lack of exercise-induced improvement in LV systolic performance in Mc1r-cKO mice after 5 weeks of running (**Figure 23D**). Collectively, these results demonstrate that MC1R deficiency blunts the hypertrophic response to physiological stimuli.

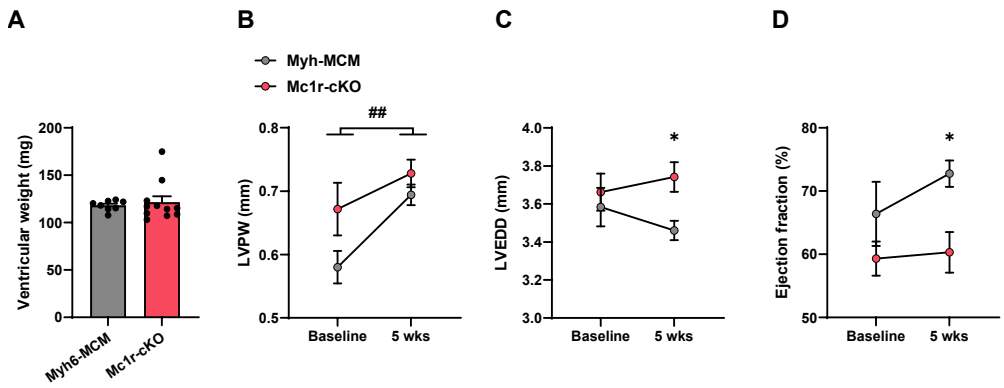


Figure 23. Cardiomyocyte-specific deletion of melanocortin 1 receptor blunts cardiac hypertrophic response to physiological stimuli induced by voluntary wheel running. **(A)** Ventricular weight in control (Myh6-MCM) and Mc1r-cKO mice after 5 weeks of voluntary wheel running. **(B-D)** Echocardiographic analysis of left ventricular posterior wall thickness (B), left ventricular end-diastolic dimension (C) and left ventricular ejection fraction (D) in Myh6-MCM and Mc1r-cKO mice at baseline and after 5 weeks of voluntary wheel running. n=8 in Myh6-MCM mice and n=11 in Mc1r-cKO mice in each graph. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05 vs Myh6-MCM mice at 5 weeks by 2-way repeated measures ANOVA and Šídák's post hoc test. ##P<0.01 for the main effect of exercise by 2-way ANOVA. LVEDD; left ventricular end-diastolic dimension, LVPW; left ventricular posterior wall, Mc1r-cKO; cardiomyocyte-specific melanocortin 1 receptor knockout, Myh6-MCM; Myh6-MerCreMer transgenic.

5.4 The role of MC5R in experimental pathological cardiac remodelling

The *in vitro* data showed that MC5R silencing aggravates the hypertrophic response of cardiomyocytes; on the other hand, activation of MC5R blunts the hypertrophic growth of cardiomyocytes. This data led to the hypothesis that MC5R has a role in regulating cardiac hypertrophy.

5.4.1 Cardiomyocyte-specific MC5R silencing affects the response to maladaptive cardiac remodelling

First, the effects of MC5R silencing in cardiomyocytes were investigated by generating an inducible, Mc5r-cKO mouse model by Cre-Lox technology. To induce pressure overload, these mice and their age-matched controls (Myh6-MCM and $Mc5r^{fl/fl}$) were subjected to TAC surgery, while another batch of mice underwent sham surgery and served as controls. 4 weeks after the surgery, TAC-operated Mc5r-cKO mice showed a subtle but significant increase in ventricular weight and thus hypertrophic response compared to TAC-operated control mice, while no genotype difference in ventricular weight was seen in sham-operated mice (**Figure 24A-B**). Supporting this finding, thickening of LVPW after TAC operation was enhanced in Mc5r-cKO mice (**Figure 24C**). Echocardiographic measurements did not reveal any change in EF after 4 weeks of TAC surgery (**Figure 24D**). However, more subtle analysis of cardiac function showed decreased endocardial FAC in TAC-operated Mc5r-cKO mice compared to Myh6-MCM mice, indicating deterioration of LV systolic function (**Figure 24E**).

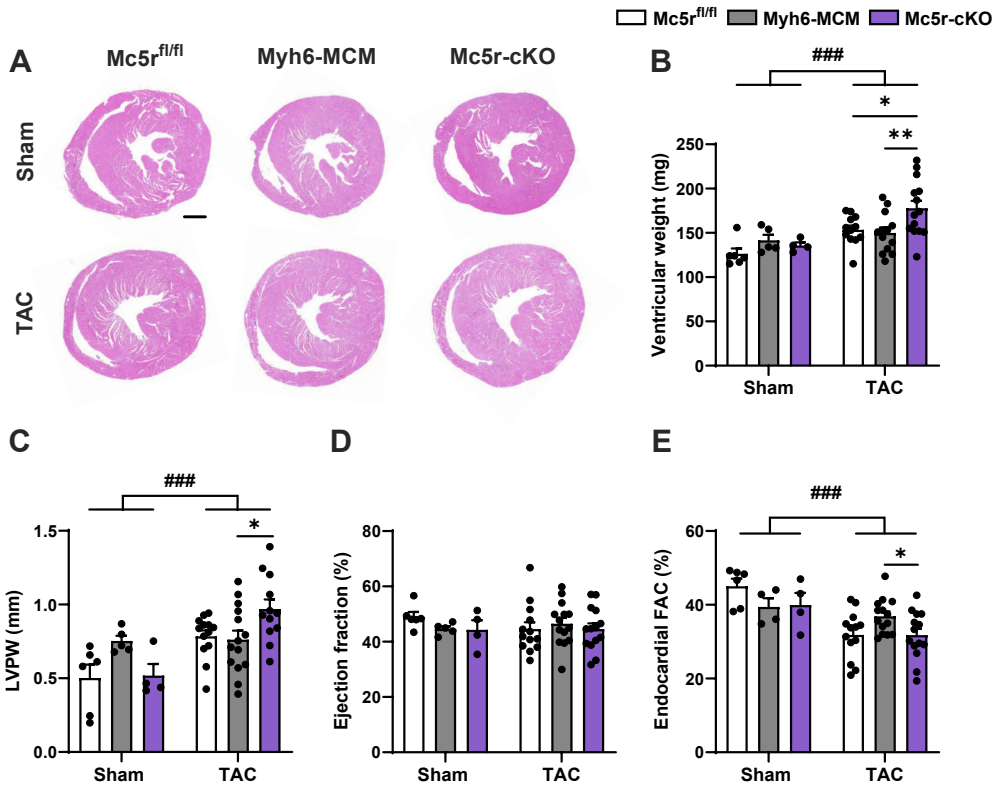


Figure 24. Cardiomyocyte-specific melanocortin 5 receptor-deficient mice show increased left ventricular hypertrophy and signs of compromised cardiac function after transverse aortic constriction-induced pressure overload. **(A)** Representative hematoxylin and eosin-stained cardiac cross-sections showing the gross morphology of the heart of control (Myh6-MCM and Mc5r$^{fl/fl}$) and Mc5r-cKO mice at the end of the experiment after 4 weeks of sham or TAC operation. Scale bar, 1 mm. **(B-E)** Ventricular weight **(B)** and echocardiographic analysis of left ventricular posterior wall thickness **(C)**, left ventricular ejection fraction **(D)** and endocardial fractional area change **(E)** in Myh6-MCM, Mc5r$^{fl/fl}$ and Mc5r-cKO mice at the end of the experiment after 4 weeks of sham or TAC operation. n=6 in sham Mc5r$^{fl/fl}$ mice, n=4-5 in sham Myh6-MCM mice, n=4 in sham Mc5r-cKO mice, n=12-13 in TAC Mc5r$^{fl/fl}$ mice, n=13-14 in TAC Myh6-MCM mice and n=12-14 in TAC Mc5r-cKO mice. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05 and **P<0.01 for the indicated comparisons by 2-way ANOVA and Dunnett's post hoc test. ###P<0.001 for the main effect of TAC by 2-way ANOVA. FAC; endocardial fractional area change, LVPW; left ventricular posterior wall, Mc5r-cKO; cardiomyocyte-specific melanocortin 5 receptor knockout, Mc5r$^{fl/fl}$; melanocortin 5 receptor floxed, Myh6-MCM; Myh6-MerCreMer transgenic, TAC; transverse aortic constriction.

Supporting the finding of increased ventricular weight, gene expression analysis by qPCR revealed upregulation of hypertrophy-related genes *Nppa* and *Nppb* in the heart of TAC-operated Mc5r-cKO mice (**Figure 25A-B**). Furthermore, fibrosis-associated genes, such as *Ctgf* and *Mmp2*, were upregulated in Mc5r-cKO mice after TAC surgery (**Figure 25C-D**). Supporting these findings, Picrosirius Red staining

of cardiac cross-sections and quantification of the extent of perivascular and interstitial fibrosis revealed increased LV fibrosis in TAC-operated Mc5r-cKO mice (**Figure 25E**). These results, together with the *in vitro* data, indicate that dysfunctional MC5R predisposes to enhanced hypertrophic growth of the heart and impairs cardiac function.

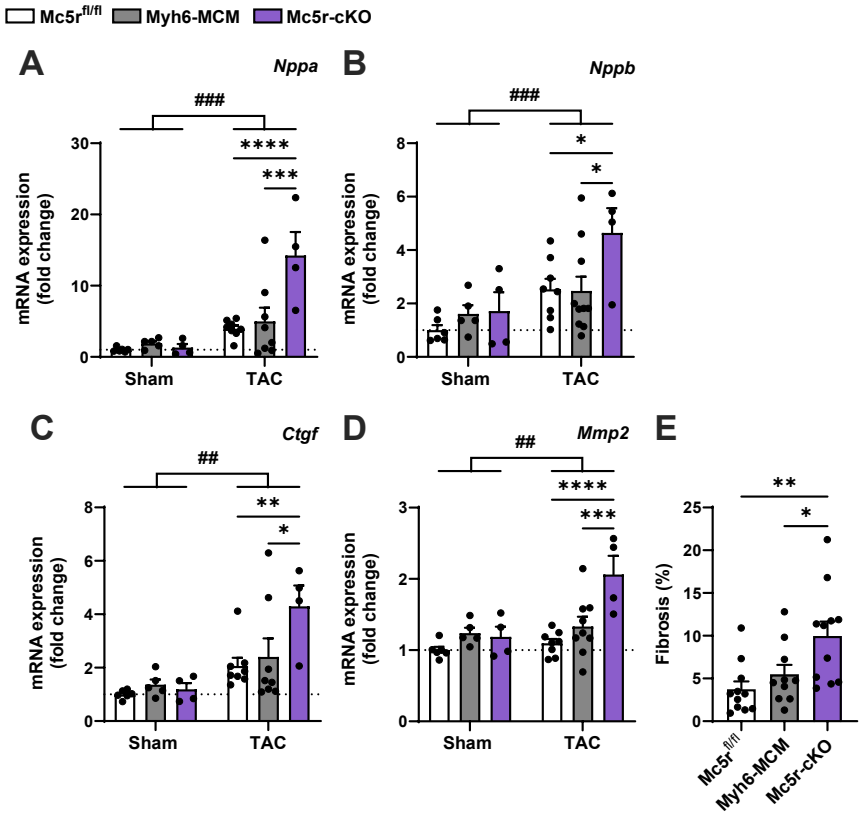


Figure 25. Cardiomyocyte-specific melanocortin 5 receptor-deficient mice show enhanced hypertrophy and fibrosis after transverse aortic constriction-induced pressure overload. **(A-D)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppa* (atrial natriuretic peptide), *Nppb* (B-type natriuretic peptide), *Ctgf* (connective tissue growth factor) and *Mmp2* (matrix metalloproteinase 2) in the left ventricle of control (Myh6-MCM and Mc5r$^{fl/fl}$) and Mc5r-cKO mice at the end of the experiment after 4 weeks of sham or TAC operation. n=6 in sham Mc5r$^{fl/fl}$ mice, n=5 in sham Myh6-MCM mice, n=4 in sham Mc5r-cKO mice, n=8 in TAC Mc5r$^{fl/fl}$ mice, n=8-10 in TAC Myh6-MCM mice and n=4 in TAC Mc5r-cKO mice. **(E)** Quantification of the extent of left ventricular fibrosis in Myh6-MCM, Mc5r$^{fl/fl}$ and Mc5r-cKO mice after 4 weeks of TAC operation. n=11 in TAC Mc5r$^{fl/fl}$ mice, n=10 in TAC Myh6-MCM mice and n=11 in TAC Mc5r-cKO mice. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 for the indicated comparisons by 2-way ANOVA and Dunnett's post hoc test. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 for the main effect of TAC by 2-way ANOVA. Mc5r-cKO; cardiomyocyte-specific melanocortin 5 receptor knockout, Mc5r$^{fl/fl}$; melanocortin 5 receptor floxed, Myh6-MCM; Myh6-MerCreMer transgenic, TAC; transverse aortic constriction.

5.4.2 Therapeutic potential of melanocortin system activation in pathological cardiac hypertrophy

The findings of local α-MSH production in the mouse heart and its decline in the failing heart raised a question about whether α-MSH could be protective against pathological cardiac hypertrophy. To investigate the therapeutic potential of α-MSH, C57Bl/6J mice were subjected to TAC surgery and randomly assigned to treatment groups receiving either a potent analogue of α-MSH (melanotan-II; MT-II) or vehicle (PBS) once daily. Sham-operated vehicle-treated mice were used as controls. After 8 weeks of pressure overload and a 6-week treatment period,

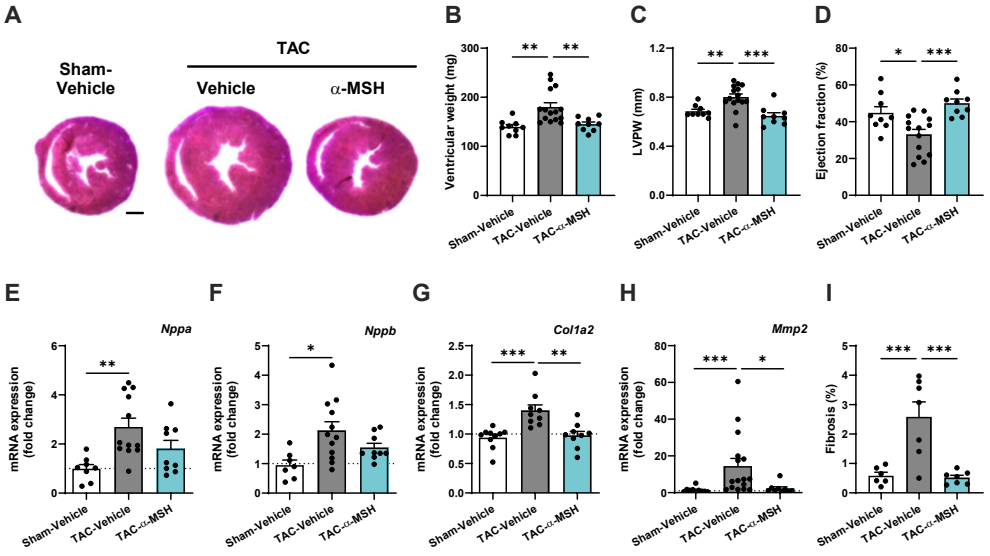


Figure 26. α-Melanocyte-stimulating hormone protects against pathological cardiac hypertrophy. **(A)** Representative hematoxylin and eosin-stained cardiac cross-sections showing the gross morphology of the heart of mice treated for 6 weeks with either vehicle or α-MSH analogue (melanotan II; MT-II) at the end of the experiment after 8 weeks of sham or TAC operation. Scale bar, 1 mm. **(B-D)** Ventricular weight **(B)** and echocardiographic analysis of left ventricular posterior wall thickness **(C)** and left ventricular ejection fraction **(D)** of vehicle or MT-II treated mice at the end of the experiment after 8 weeks of sham or TAC operation and 6 weeks of treatment. n=9 in sham-vehicle, n=14-15 in TAC-vehicle and n=9 in TAC-α-MSH. **(E-H)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppa* (atrial natriuretic peptide), *Nppb* (B-type natriuretic peptide), *Col1a2* (collagen type I, alpha 2) and *Mmp2* (matrix metalloproteinase 2) in the left ventricle of vehicle or MT-II treated mice at the end of the experiment after 8 weeks of sham or TAC operation and 6 weeks of treatment. n=7-10 in sham-vehicle, n=9-16 in TAC-vehicle and n=9 in TAC-α-MSH. **(I)** Quantification of the extent of left ventricular fibrosis of vehicle or MT-II-treated mice at the end of the experiment after 8 weeks of sham or TAC operation and 6 weeks of treatment. n=6 in sham-vehicle, n=7 in TAC-vehicle and n=7 in TAC-α-MSH. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05, **P<0.01 and ***P<0.001 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc test. α-MSH; α-Melanocyte-stimulating hormone, LVPW; left ventricular posterior wall, TAC; transverse aortic constriction.

cardiac phenotyping revealed reduced ventricular weight in α-MSH-treated TAC-mice compared to vehicle-treated TAC-mice (**Figure 26A-B**). An echocardiography imaging assessment of cardiac structure and function also showed that α-MSH treatment prevented TAC-induced LVPW thickening and reduction in EF (**Figure 26C-D**). Furthermore, gene expression analysis by qPCR further revealed downregulation of *Nppa* and *Nppb*, as well as fibrosis-related genes such as *Col1a2* (collagen type 1, alpha 2) and *Mmp2*, in α-MSH-treated TAC mice (**Figure 26E-H**). Additionally, LV fibrosis, quantified from Picrosirius Red-stained cardiac cross-sections, was significantly reduced after α-MSH treatment in TAC mice (**Figure 26I**). Taken together, these data indicate that α-MSH acts as an antihypertrophic regulator and protects against pathological cardiac hypertrophy.

Based on the *in vitro* finding that MC5R is the primary receptor for mediating the anti-hypertrophic effects of α-MSH, it was next investigated whether the MC5R-specific agonist PG-901 evokes similar effects compared to α-MSH in TAC-challenged mice. TAC surgery was performed on C57Bl/6N mice, since they are more prone to develop TAC-induced HF (Garcia-Menendez et al., 2013; Zi et al., 2019). Mice were treated with two different dose levels of PG-901 (0.005 or 0.5 mg/kg/day) or vehicle (0.5% DMSO in PBS) once daily. 5 weeks after TAC surgery and 4 weeks of treatment, cardiac phenotyping showed robust hypertrophic response in terms of ventricular weight and thickening of LVPW (**Figure 27A-C**), but no significant treatment effect was detected in these parameters. However, echocardiography showed significant improvement in EF with the low dose of PG-901 compared to vehicle treatment in TAC mice (**Figure 27D**). In addition, gene expression profiling revealed downregulation of the hypertrophy-associated gene *Nppa* and fibrosis-associated genes, including *Ctgf* and *Mmp2*, in TAC-operated mice treated with the low dose of PG-901 (**Figure 27E-H**). The extent of LV fibrosis was also reduced by the low-dose treatment with PG-901 (**Figure 27I**). Collectively, these results demonstrate that pharmacological targeting of MC5R partially mimics the effects of α-MSH also *in vivo* and provides anti-fibrotic regulation.

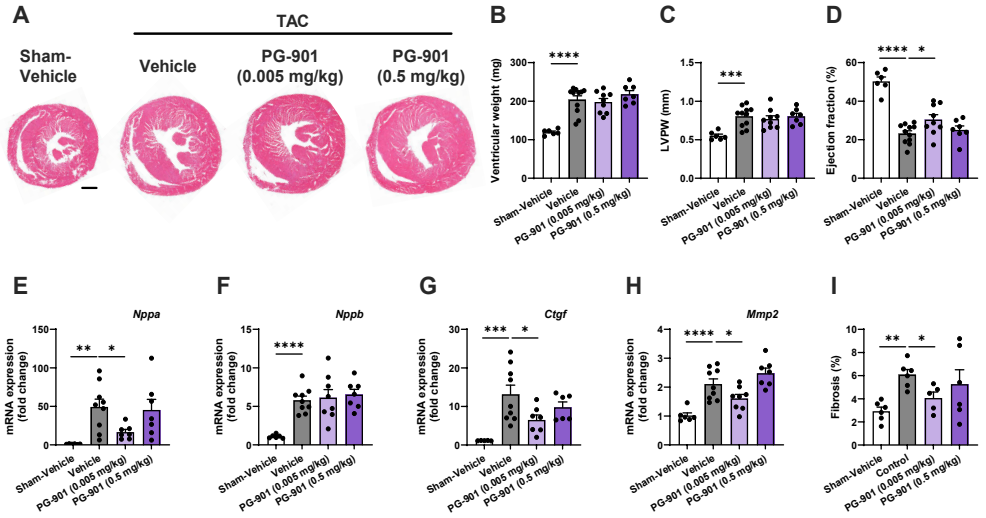


Figure 27. Melanocortin 5 receptor activation improves left ventricular systolic function and reduces cardiac fibrosis in mice after transverse aortic constriction-induced pressure overload. **(A)** Representative hematoxylin and eosin-stained cardiac cross-sections showing the gross morphology of the heart of mice treated for 4 weeks with either vehicle or PG-901 (0.5 or 0.005 mg/kg/day) at the end of the experiment after 5 weeks of sham or TAC operation. Scale bar, 1 mm. **(B-D)** Ventricular weight **(B)** and echocardiographic analysis of left ventricular posterior wall thickness **(C)** and left ventricular ejection fraction **(D)** of vehicle- or PG-901-treated mice at the end of the experiment after 5 weeks of sham or TAC operation and 4 weeks of treatment. n=6 in sham-vehicle, n=11 in TAC-vehicle, n=9 in TAC-PG-901 (0.005 mg/kg) and n=7 in TAC-PG-901 (0.5 mg/kg). **(E-H)** Quantitative real-time polymerase chain reaction (qPCR) analysis of *Nppa* (atrial natriuretic peptide), *Nppb* (B-type natriuretic peptide), *Ctgf* (connective tissue growth factor) and *Mmp2* (matrix metalloproteinase 2) in the left ventricle of vehicle- or PG-901-treated mice at the end of the experiment after 5 weeks of sham or TAC operation and 4 weeks of treatment. n=5-6 in sham-vehicle, n=9 in TAC-vehicle, n=7-8 in TAC-PG-901 (0.005 mg/kg) and n=6-7 in TAC-PG-901 (0.5 mg/kg). **(I)** Quantification of the extent of left ventricular fibrosis of vehicle- or PG-901-treated mice at the end of the experiment after 5 weeks of sham or TAC operation and 4 weeks of treatment. n=5-6 mice per group. Data are mean ± SEM. Each dot represents an individual mouse. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 for the indicated comparisons by 1-way ANOVA and Dunnett's post hoc test. LVPW; left ventricular posterior wall, PG-901; melanocortin 5 receptor selective agonist, TAC; transverse aortic constriction.

6 Discussion

6.1 Methodological considerations

6.1.1 *In vitro* models

Since primary mouse and hiPSC-derived cardiomyocytes are laborious, expensive, fragile and difficult to maintain in culture for long periods, and in the case of primary cells, isolation requires a sacrifice of laboratory animals, an alternative *in vitro* cardiomyocyte model was needed for the first-line screening of MCR-mediated effects. A widely used animal-free alternative in CV research is commercial cell line H9c2(2-1) (ATCC®, CRL-1446™), which was also used in the experiments of this thesis. These cells are a subclone of the original clonal cell line derived from embryonic BD1X rat ventricular heart tissue by a selective serial passaging method (Kimes & Brandt, 1976). H9c2 cells exhibit many of the properties of skeletal and cardiac muscle, including biochemical, morphological, electrical, and hormonal signalling properties (Hescheler et al., 1991; Kimes & Brandt, 1976). H9c2 cells are mononucleated cardiac myoblasts that have the ability to differentiate into myotubes with multiple nuclei, an elongated shape, and a parallel positioning when cultured in serum-reduced medium. Furthermore, by adding retinoic acid to low serum media, H9c2 cells can be differentiated to resemble the adult cardiac muscle phenotype (Ménard et al., 1999). However, despite the widespread use of these cells, several challenges related to them exist. For instance, the passage number of H9c2 cells can affect the reliability of the results, as the ageing of the cell line has been linked to poor reproducibility of studies (Witek et al., 2016). Since the experiments in this thesis required culturing cells for long periods and subdividing them multiple times, it might have affected the variability detected in the results within and between the experiments. Furthermore, a great deal of studies using H9c2 cells are performed using undifferentiated H9c2 myoblasts, which limits the relevance of the results, especially when compared to the experiments conducted with primary cardiomyocytes. We encountered the need to use undifferentiated H9c2 cells as well, since the attempts at serum deprivation and retinoic acid-mediated differentiation resulted in negative changes in cell morphology and viability. Although cell differentiation is likely to be more crucial in cardiotoxicity studies (Branco et al.,

2012), and undifferentiated H9c2 cells can respond similarly to hypertrophic stimuli compared to primary cardiomyocytes (Watkins et al., 2011), this limitation could partly explain differences in responses between H9c2 cells and primary cardiomyocytes detected in some of the results.

After screening the main effects of MCRs activation and the involved intracellular pathways, and optimising experimental conditions with H9c2 cells, the aim was to further validate the results by performing experiments with physiologically more relevant primary mouse cardiomyocytes. Primary cardiomyocytes are a widely used model to study, for instance, cardiac hypertrophy and drug-induced effects. The use of neonatal rat primary cardiomyocytes is probably a more prevalent model in widespread use, but NMCMs were selected for use in the studies of this thesis. Besides the greater availability of newborn mice, this choice provided better predictability for translation from *in vitro* to *in vivo* experiments, as it utilised the same species. Although clear instructions and commercial kits are available for isolating primary cardiomyocytes, many challenges remain in their culturing. Major technical limitations include attaining a high yield of cells, obtaining viable cells that contract spontaneously in culture and performing a highly pure cardiomyocyte culture without non-myocyte cells, such as fibroblasts and endothelial cells. This limited yield of spontaneously contracting, viable cardiomyocytes and the inevitable heterogeneity of cell populations influenced the number of technical replicates and power in the studies. This potentially explains, at least in part, the variability of the results within the individual experiments and between the experiments. One limitation of performing experiments with NMCMs and interpreting the results is that the findings reflect responses in the neonatal heart and may thus differ from responses in adult cardiomyocytes. This could have been one factor influencing the discordance of the detected antihypertrophic effect of PG-901 treatment, which was present in NMCMs but absent in mice. However, the results gained from *in vitro* studies quite effectively reflected the *in vivo* findings.

6.1.2 Genetic animal models

First, to investigate the effects of MC1R on cardiac function and structure, recessive yellow mice ($Mc1r^{e/e}$), which lack functional MC1R due to a single base deletion mutation in the *Mc1r* gene and are therefore characterised by yellow coat colour, were obtained. $Mc1r^{e/e}$ mice are widely used as an experimental tool to model global MC1R deficiency (Robbins et al., 1993). However, there are some challenges related to the use of $Mc1r^{e/e}$ mice in cardiac research. First, MC1R deficiency is constitutive, meaning that it is present from birth and can therefore have developmental effects and lead to possible compensatory responses later in life. Second, it has been demonstrated that $Mc1r^{e/e}$ mice also have a vascular phenotype, including arterial

stiffness and endothelial dysfunction (Rinne et al., 2015), which can indirectly influence cardiac structure and function as well. Furthermore, MC1R is expressed in cells of the immune system and thus involved in the regulation of inflammatory responses (Catania et al., 2004), which can also have an indirect effect on cardiac remodelling.

To overcome these challenges and to study whether the observed cardiac phenotype of $Mc1r^{e/e}$ mice was dependent on dysfunctional MC1R signalling in cardiomyocytes, an inducible Mc1r-cKO mouse model was generated by crossing MC1R floxed mice with tamoxifen-inducible Myh6-MerCreMer transgenic mice. The Mc5r-cKO mouse model was also generated with the same strategy. The phenotypes of both controls, Cre-negative and MCR homozygous mice and Cre-positive and MCR WT mice were first evaluated. Since the Cre-negative and MCR homozygous control mice showed no substantial phenotype or significant difference compared to Cre-positive and MCR WT control mice (referred to as Myh6-MCM), the Cre-positive control was chosen for future studies due to Cre-related concerns. In the Myh6-MerCreMer model, Cre is fused to mutated estrogen receptor ligand-binding domains insensitive to endogenous estrogens but sensitive to tamoxifen. After induction with tamoxifen, the Cre fusion protein is expressed in cardiomyocytes under the control of the human α-myosin heavy chain gene promoter (Hall et al., 2011; Sohal et al., 2001). This model is widely used to temporally control gene deletions in cardiomyocytes, and the inducibility of Cre expression enables the avoidance of harmful developmental effects that have been reported to occur due to constitutive Cre expression in the heart (Buerger et al., 2006; Sohal et al., 2001). Although this is a widely used model, certain disadvantages need to be considered. For instance, tamoxifen is a cardiotoxic substance and tamoxifen-induced Cre expression in the heart can lead to a transient reduction in systolic function (Hall et al., 2011). Therefore, a thorough literature review and a pilot study were performed to optimise tamoxifen dosing and to test how mice recover after it. As a result, tamoxifen dosing was required to further lower and fractionate on consecutive days, compared to previously reported dosing schemes, to avoid the Cre-mediated cardiotoxicity. With the chosen dosing scheme, efficient Cre-Lox recombination was achieved in the heart. However, analysis of genomic DNA samples and protein expression in the heart showed approximately a 50% reduction in cardiac MCR level. This insufficient recombination and partial MCR knockout could explain the subtle phenotype of the mouse models presented in this thesis, and thus also underestimates the effect sizes and significance of MC1R and MC5R in cardiac remodelling. In the case of Mc5r-cKO mice, another possible factor limiting the observed effect of genetically-induced MC5R deficiency on cardiac hypertrophy is that TAC-induced pressure overload itself was found to reduce the dimer form of MC5R. Furthermore, the background strain of used floxed mice was C57Bl/6J, which is known to be less

susceptible to developing TAC-induced HF compared to C57Bl/6N mice (Garcia-Menendez et al., 2013; Zi et al., 2019). This might, at least in part, explain the subtle phenotype of the mice after 4 or 8 weeks of TAC surgery.

6.1.3 Pharmacological activation of the melanocortin system

Because α-MSH is a linear peptide hormone, it is rapidly degraded by proteolytic enzymes, resulting in a short half-life and duration of action. For this reason, a more potent and more stable analogue of α-MSH, MT-II (Al-Obeidi et al., 1989), was used in the mouse experiments. Since α-MSH and MT-II have a unique ability to activate all MCR subtypes, it also complicates the interpretation of the results. For instance, in the *in vitro* experiments, it was found that the responses to α-MSH were variable, which could be explained by the subsequent findings that activation of MC1R promoted cardiomyocyte hypertrophy, while activation of MC5R mediated anti-hypertrophic effects. If these receptors are counteractive for each other in the heart, it can be speculated that the balance between MC1R and MC5R in the heart defines how the α-MSH affects cardiac remodelling. Furthermore, since the dimer form of MCRs is biologically more active, it might be possible that different MCR subtypes form dimers with each other in the heart, which further defines how melanocortin system activation influences cardiac remodelling.

For specific MC1R activation in cultured cardiomyocytes, the MC1R selective agonist LD211, which is modified from MT-II by multiple *N*-methylation technology and is able to bind and activate only the MC1R subtype, was used (compound 28 in the original publication) (Doedens et al., 2010). To selectively activate MC5R, PG-901, which has been characterised as the first full agonist at the human MC5R and a full antagonist at the human MC3R and MC4R, was used (Grieco et al., 2002). The cyclic structure of PG-901 makes it more resistant to proteolytic degradation and thus biologically more stable than α-MSH, which most likely explains the more sustained effects of PG-901. Furthermore, the EC_{50}-value of PG-901 was demonstrated to be below 0.1 nM in the cAMP assay in cells stably expressing the human MC5R, making it markedly more potent than MT-II (Grieco et al., 2002). This was consistent with the findings presented in this thesis and original publication, showing the strongest effects at subnanomolar concentrations of PG-901 *in vitro* and the lower dose of PG-901 in TAC-challenged mice. However, the lack of anti-hypertrophic effect of PG-901 *in vivo* was discordant with the phenotype of α-MSH-treated mice and the observed anti-hypertrophic effect of PG-901 *in vitro*. This raises a concern that PG-901 might evoke an off-target effect, which, in turn, could mask the potential anti-hypertrophic effect of MC5R activation *in vivo*. Since the binding properties of PG-901 have been studied in cells expressing human MCRs, PG-901 may act differently in mice; it may not be a full antagonist at

the mouse MC3R and MC4R. Since the binding properties of mouse MCR subtypes have not been reported, it remains open whether PG-901 interacts with other MCRs in the mouse heart beyond activating MC5R, thereby influencing the observed effects.

6.1.4 Animal models of cardiac hypertrophy and echocardiography evaluation of cardiac function

To investigate the roles of MC1R and MC5R in pathological cardiac hypertrophy, mice were subjected to either TAC surgery or Ang II infusion. Although both are models of pathological cardiac hypertrophy, the aim was to substantiate that MCR-related effects are model-independent and, therefore, two different models were employed. Generally, TAC is described as a model of pressure overload, maladaptive cardiac remodelling and impairment of systolic and diastolic cardiac function, which leads to HF, classically to HFrEF. On the other hand, Ang II infusion increases blood pressure and induces hemodynamic pressure overload, which is followed by cardiomyocyte hypertrophy, but rarely ends up in HF. The results demonstrate that both models induced clear hypertrophy and fibrosis, and TAC also deteriorated cardiac function. This provides important information that the lack or presence of genotype effect was not due to an invalid disease model, but due to other factors. Similarly, the results demonstrated that the 5-week voluntary wheel running induced a physiological hypertrophic response in the heart as evidenced by the increased ventricular weight-to-body weight ratio in exercised mice compared to sedentary mice, proving the validity of the model (data presented in the original publication II). Additionally, the genotype effects seen in the results were clearly attributable to the genotype itself, as the physiological stimulus was consistent between the WT and MC1R-deficient mice (data presented in the original publication II).

To evaluate the influences of hypertrophy models employed, transthoracic echocardiography, a valuable and extensively applied instrument for noninvasive serial assessment of cardiac structure and function in mice, was used. Despite its wide use, there are many challenges. The small cardiac size and high heart rate of mice have initially led to performing echocardiography under anaesthesia, since it provides sedation and immobility of mice, facilitating the reliable data acquisition; on the other hand, it is known to alter heart rate, chamber dimensions and systolic function (Roth et al., 2002; Rottman et al., 2003). However, imaging of conscious mice is not unproblematic either: it requires more technical skills, consequently increasing interobserver variability, and restraining of mice raises the heart rate (Syed et al., 2005). Most echocardiography is performed under anaesthesia, as was done in the presented studies. Anaesthesia might have influenced the results, as

another sonographer's shortage of technical expertise at the beginning resulted in longer imaging times and prolonged anaesthetic effects. However, inhalated isoflurane anaesthesia is preferred for imaging studies requiring relatively long anaesthesia periods and has not been shown to have adverse effects at the 1.5% maintenance level (Constantinides et al., 2011). The interobserver variability was controlled using two sonographers whose recordings were well in line, and the intraobserver variability was minimised by analysing three parallel recordings and forming an average for each parameter. Furthermore, echocardiography analyses were done by the same blinded person throughout the studies, thus minimising the bias and variability between analysts. Consequently, these actions were not limitations of our results but rather tried to enhance their reliability. On the other hand, although echocardiography is a valuable tool in both preclinical and clinical research, it only provides a reliable evaluation, not a definitive diagnosis. This limitation of the method hindered the interpretation of the results regarding the systolic and diastolic function of the heart. Based on the results, relying only on measurements of echocardiography parameters, the development of diastolic dysfunction in the Mc1r-cKO mice could not be concluded. Without other evidence, like invasive intracardiac ventricular filling pressure measurement or measurement of atrial volume, the interpretation was restricted to milder conclusions.

6.2 Novel role of the melanocortin system in the heart

The melanocortin system has been under intensive research for the past decades and has been shown to regulate many diverse physiological functions, but its role in the heart has remained elusive. This thesis revealed that a functional melanocortin circuit exists in the heart and regulates cardiac remodelling. It was observed that α-MSH is produced in the mouse heart with declining levels in pressure-overloaded and failing hearts. In addition, since plasma α-MSH concentration was not changed in TAC-challenged mice, it is plausible that local α-MSH production in the heart regulates cardiac remodelling in an autocrine or paracrine manner. Furthermore, novel roles for MC1R and MC5R signalling pathways in the hypertrophic remodelling of the myocardium were uncovered.

6.2.1 Importance of MC1R integrity in normal cardiac function

Due to the observation that MC1R is expressed in the mouse heart and downregulated in response to hypertrophic stimuli, *in vitro* studies using H9c2 cells and NMCMs were performed. It was discovered that selective MC1R activation

promoted cellular growth and induced phosphorylation of CREB and p38 signalling pathways. Further mechanistic experiments revealed that selective inhibition of CREB, but not p38, blocked the LD211-induced increase in protein synthesis, suggesting that MC1R-mediated hypertrophic signalling occurs in a CREB-dependent manner. However, further studies are needed to investigate whether changes in CREB signalling affect the observed cardiac phenotype of Mc1r-cKO mice *in vivo*. Apart from murine cell models, hiPSC-CMs showed downregulation of the *MC1R* gene in response to stretching or pharmacological treatment with ET-1, demonstrating that MC1R also exists in human cardiomyocytes.

To study whether MC1R is involved in pathological cardiac remodelling, Mc1r$^{e/e}$ mice were subjected to TAC surgery. It was discovered that global MC1R deficiency resulted in a milder increase in ventricular weight compared to WT controls, along with a reduced upregulation of genes associated with hypertrophy and fibrosis in the heart following TAC-induced pathological hypertrophy. Since MC1R is also expressed in other cell types, such as endothelial cells or fibroblasts, it is possible that MC1R deficiency in non-cardiomyocytes could influence the observed phenotype. For example, Mc1r$^{e/e}$ mice have endothelial dysfunction and increased arterial stiffness (Rinne et al., 2015), which can predispose these mice to increased cardiac afterload and possibly explain the increased expression of *Nppb* in the heart of sham-operated Mc1r$^{e/e}$ mice.

To clarify whether the phenotype of Mc1r$^{e/e}$ mice was driven by the lack of functional MC1R in cardiomyocytes, inducible Mc1r-cKO mice were generated, and mice were subjected to TAC surgery. Cardiac phenotyping of these mice revealed attenuated cardiac hypertrophic response and markedly reduced *Nppa* expression compared to TAC-operated control mice, similar to what was seen with Mc1r$^{e/e}$ mice. Although the expression of *Nppb* was not significantly reduced in the heart of TAC-challenged Mc1r-cKO mice compared to their controls, the expression was unchanged in sham-operated Mc1r-cKO mice, differentiating the phenotype from Mc1r$^{e/e}$ mice and suggesting that global deficiency of MC1R influences the phenotype of Mc1r$^{e/e}$ mice. Furthermore, a similar cardiac phenotype was observed in Ang II-infused Mc1r-cKO mice, demonstrating that attenuation of pathological cardiac hypertrophy is not dependent on the chosen model.

However, despite the reduced ventricular weight and changes in cardiac gene expression profile, systolic or diastolic LV function was not improved in Mc1r-cKO mice. On the contrary, cardiomyocyte-specific MC1R deficiency led to LV dilatation and signs of reduced LV function after TAC surgery. Since fibrosis-associated genes were downregulated and the extent of LV fibrosis tended to be reduced in Mc1r-cKO mice, changes in fibrosis are unlikely to explain the functional deterioration. Previous studies on mice have revealed that genetic deletion of *Nppa* or its target receptor NPR-A amplifies cardiac hypertrophy and LV dilation, subsequently

impairing LV systolic function following TAC-induced pressure overload, while deletion of *Nppb* does not affect susceptibility to cardiac hypertrophy (Knowles et al., 2001; Tamura et al., 2000; Wang et al., 2003). Based on this evidence, decreased *Nppa* expression in Mc1r-cKO mice might be responsible for the observed LV dilation and compromised cardiac function. However, a more likely decrease in *Nppa* is merely a sign of reduced LV weight since the activation of MC1R per se had no effect on *Nppa* in cultured NMCMs. Alterations in the cardiomyocyte length and length-to-width ratio, an architectural hallmark of LV dilation and progression of HFrEF (Katz & Rolett, 2016), could provide an alternative explanation for altered LV geometry and performance. In TAC-challenged Mc1r-cKO mice, cardiomyocyte lengthening occurred without an appropriate increase in CSA, resulting in an increased length-to-CSA ratio. This indicates a lack of concentric LV remodelling during the compensated phase of TAC-induced pressure overload, which could predispose Mc1r-cKO mice to LV dysfunction. Furthermore, changes in the expression of *Myh6* and *Myh7* genes, which encode cardiac function and energy regulation proteins MHC-α and -β, respectively, may contribute to the observed phenotype of Mc1r-cKO mice. Although the relative expression of MHC-α and -β is species-specific and tightly regulated by developmental stage, a comparable shift in their relative expression, characterised by MHC-β increase and MHC-α decrease, occurs in the failing human and mouse heart. This shift serves not only an energy-conserving function but also exerts detrimental effects on cardiac performance. (Gupta, 2007; Hamdani et al., 2008; Miyata et al., 2000) Thus, the observed upregulation of MHC-β (encoded by the *Myh7* gene) in Mc1r-cKO mice could be a causative factor for the functional deficits. Furthermore, prior experimental evidence has demonstrated that overexpression of MHC-β is also associated with a modest increase in LVEDD (Krenz et al., 2004), a phenotype that was similarly observed in Mc1r-cKO mice. In addition, increased LVEDD could be a predisposing factor for reduced EF in Mc1r-cKO mice. Together, this evidence from the literature and experiments on Mc1r-cKO mice suggests that increased MHC-β may be a causative factor in LV structural and functional changes. Nevertheless, these observations alone are insufficient to draw conclusions about the functional deficits and the antihypertrophic effect of MC1R deficiency, and further studies are warranted.

Additionally, $Mc1r^{e/e}$ mice showed a blunted hypertrophic response to 5-week voluntary wheel running, a model of physiological hypertrophy. These mice also had lower EF when compared to control mice after the exercise period, suggesting that global MC1R deficiency prevents exercise-induced improvement in LV systolic function. Likewise, Mc1r-cKO mice showed an attenuated hypertrophic response, a lack of exercise-induced improvement in EF, and increased LVEDD after 5-week voluntary wheel running, indicating that MC1R deficiency is not beneficial for physiological cardiac remodelling. Collectively, these findings demonstrate that

selective MC1R activation stimulates cardiomyocyte growth, and MC1R deficiency in cardiomyocytes blunts pathological and physiological cardiac hypertrophy but simultaneously leads to LV dilatation and compromised cardiac function, uncovering a novel role for MC1R in cardiac remodelling.

6.2.2 Cardioprotective role of MC5R activation

The initial observations revealed that the expression of MC5R and α-MSH in the mouse heart changed in parallel, with declining levels in severely hypertrophied and failing hearts. These results suggest that exhaustion of α-MSH production simultaneously compromises the integrity of MC5R. *MC5R* expression was also downregulated in stretched or ET-1-stimulated hiPSC-CMs, while an opposite effect, i.e. increased *MC5R* expression, was observed in human DCM and ICM patient samples. This inconsistent finding may have occurred due to many reasons. For example, the age of subjects, the aetiology of disease, chronic versus acute effects and interfering signals from non-myocytes in the case of heart lysates could explain these discordant results. Nevertheless, these findings demonstrated that MC5R is also expressed in the human heart.

Subsequent *in vitro* experiments revealed that α-MSH mediates anti-hypertrophic and anti-fibrotic regulation in cardiomyocytes by activating MC5R, since the MC5R selective agonist PG-901 mimicked the effects of α-MSH and MC5R antagonism reversed the α-MSH-induced effects. H9c2 cells, NMCMs and hiPSC-CMs all responded to the treatment with PG-901 in a cohesive way, resulting in downregulation of fibrosis-associated genes and reduction of protein synthesis. These results proved that cardiomyocytes express functional MC5R and demonstrated a novel role for this MCR subtype in cardiomyocyte hypertrophy.

In terms of intracellular signalling mechanisms, it has been previously shown that MC5R activation can engage two different and parallel signalling pathways, G_s/cAMP/PKA and G_i/ERK1/2 (Rodrigues et al., 2015). In cultured cardiomyocytes, MC5R activation reduced cAMP level, indicating G_i coupling, but further mechanistic experiments showed that the downstream effects occurred independently of G_i signalling. However, MC5R activation consistently reduced phosphorylated JNK in cardiomyocytes. Although intracellular signalling of MCRs has been rarely linked to the JNK pathway and its inhibition, a study demonstrates that melanocortin signalling via MC5R can inhibit JNK activity and inflammatory responses in mouse adipocytes (Liu et al., 2017). The exact molecular mechanisms for the JNK inhibition remain unclear, but the observations in cardiomyocytes imply it to be driven by a cAMP-independent signalling cascade. The role of JNK signalling in cardiac hypertrophy appears controversial, since *in vitro* studies suggest a prohypertrophic role for JNKs, while *in vivo* studies have shown either promotion

or attenuation of cardiac growth when JNK or its upstream regulator has been silenced (Liang & Molkentin, 2003). Nevertheless, *in vitro* experiments presented in this thesis demonstrate that MC5R regulates hypertrophic growth of cardiomyocytes in a JNK-dependent manner. However, further studies are clearly needed to investigate whether MC5R activation also reduces JNK phosphorylation *in vivo* and whether it has a direct effect on hypertrophic growth of the heart.

In good agreement with the declining level of α-MSH in the failing heart and the beneficial anti-hypertrophic and anti-fibrotic effects of α-MSH in cultured cardiomyocytes, repeated α-MSH administration reduced ventricular weight and LV fibrosis and improved LV systolic function in TAC-operated mice, demonstrating that α-MSH treatment protects against pathological cardiac remodelling. However, since other cells in the heart, such as fibroblasts, endothelial cells and macrophages, are also essential effector cells in pathological cardiac remodelling and are all known to express MCRs, the involvement of these non-cardiomyocytes as mediators of the anti-hypertrophic and anti-fibrotic effect induced by α-MSH cannot be excluded. Despite this uncertainty, the *in vitro* experiments with H9c2 cells and NMCMs proved that cardiomyocytes are responsive to α-MSH and show reduced hypertrophic growth after α-MSH treatment, suggesting that the anti-hypertrophic effect of α-MSH *in vivo* is primarily mediated by cardiomyocytes. Since MC5R mediated α-MSH effects *in vitro*, it was investigated whether PG-901 mimics the effects of α-MSH *in vivo* as well, and similar anti-fibrotic regulation and improvement in LV systolic function were discovered. The anti-hypertrophic effect evoked by α-MSH, however, was lacking with PG-901 treatment in TAC-operated mice, although gene expressions of *Nppa* and *Ctgf* were downregulated. ANP is a well-known hypertrophy marker, and CTGF is recognised for stimulating hypertrophic growth of cultured cardiomyocytes, in addition to its role in regulating fibrosis (Hayata et al., 2008; Yoon et al., 2010). Altogether, PG-901 was found to prevent hypertrophy *in vitro*, although *in vivo* it remains to be further clarified.

Corroborating the regulatory role of MC5R in cardiac remodelling and the favourable anti-hypertrophic and anti-fibrotic effects of MC5R activation, cardiomyocyte-specific *in vitro* and *in vivo* silencing of MC5R demonstrated upregulation of hypertrophy- and fibrosis-associated genes. Furthermore, the loss-of-function studies in mice demonstrated that cardiomyocyte-specific MC5R deficiency aggravated TAC-induced cardiac hypertrophy and fibrosis, and led to deterioration of systolic function. Collectively, these results demonstrate that MC5R activation favourably regulates cardiac growth and function, while silencing of MC5R promotes pathological cardiac remodelling.

6.3 Translational aspects and therapeutic prospects in the future

Importantly, studies presented in this thesis uncovered novel functional roles for MC1R and MC5R subtypes in the heart, which merit future research in drug discovery in this field. MC1R is a well-known genetic determinant of skin and hair colour, and is highly polymorphic in humans. It has been shown that humans carrying loss-of-function variants of MC1R display not only a red hair and fair skin phenotype but also an unfavourable vascular phenotype characterised by increased arterial stiffness and endothelial dysfunction (Rinne et al., 2015). However, the impact of the various single-nucleotide polymorphisms of *MC1R* on cardiac structure and function has not been studied. In this thesis project, it was observed that MC1R deficiency in cardiomyocytes prevented cardiac hypertrophy but compromised cardiac function, while selective activation of MC1R promoted cardiomyocyte hypertrophy *in vitro*. These results might have clinical relevance and suggest that loss-of-function mutations of *MC1R* could be associated with changes in cardiac structure and function. Even if therapeutic targeting of MC1R would not prove to be efficient in the care of human HF, it is important to consider and investigate the possible cardiac effects of genetic MC1R deficiency and MC1R-activating drugs. This is particularly important, since analogues of naturally occurring α-MSH with agonistic activity at MC1R (Scenesse®, Vyleesi® and Imcivree®) (Montero-Melendez et al., 2022) have been recently approved for clinical use in different therapeutic areas.

MC5R was previously found to be abundantly expressed in the skin, adrenal and sebaceous glands and skeletal muscle, while only low level of *Mc5r* mRNA was found in the heart without any elucidation of its functional significance (Fathi et al., 1995; Labbé et al., 1994). The results of this thesis demonstrated that functional MC5R exists in mouse, rat, and human cardiomyocytes, and that MC5R activation protects against pathological cardiac remodelling. Thus, MC5R may serve as a potential therapeutic target for the management of HF. Furthermore, even if future drug research does not support the development of MC5R-targeted therapies for human HF, our results provide important safety information related to MC5R activation. Based on our results, it is expected that drugs with agonistic activity at MC5R have a favourable cardiac safety profile. This is particularly important because the aforementioned analogues of naturally occurring α-MSH also activate MC5R, and more of these kinds of drugs, as well as MCR subtype-selective drugs, are in the pipeline of different pharma companies (ClinicalTrials.gov) (Montero-Melendez et al., 2022).

7 Summary and Conclusions

This thesis investigated the undefined role of the melanocortin system in the heart and evaluated the functional roles of MC1R and MC5R in regulating cardiac remodelling using *in vitro* cardiomyocyte models and experimental disease models.

The main conclusions were:

1. α-MSH is produced in the mouse heart, with declining levels in pressure-overloaded and failing hearts, and with simultaneously unchanged plasma α-MSH levels, indicating that pathological cardiac remodelling affects local α-MSH production in the heart. Systemic administration of α-MSH protected mice against pathological hypertrophy and HF.
2. MC1R is expressed in the mouse heart and downregulated in response to pathological cardiac remodelling. MC1R deficiency attenuated pathological and physiological cardiac hypertrophy in mice, but simultaneously led to LV dilatation and compromised cardiac function, while pharmacological activation of MC1R promoted hypertrophy in cultured cardiomyocytes.
3. MC5R is expressed and functionally active in mouse, rat, and human cardiomyocytes. Pharmacological activation of MC5R favourably regulated hypertrophic and fibrotic responses in cultured cardiomyocytes and protected against fibrosis with a positive influence on cardiac function in mice. Furthermore, MC5R mediated cardiac α-MSH effects. In contrast, MC5R deficiency aggravated pathological hypertrophy and fibrosis and deteriorated cardiac systolic function in mice.

These findings demonstrate for the first time the existence of an active melanocortin system in the heart, which plays a crucial role in the regulation of hypertrophic and fibrotic remodelling of the heart, along with potential for pharmacological targeting.

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