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Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen

Departement Farmaceutische Wetenschappen

ROLE OF NON-MYOCYTE NRG1/ERBB4 SIGNALING IN CARDIAC REMODELING

ROL VAN NIET-MYOCYTE NRG1/ERBB4 SIGNALISATIE IN CARDIALE REMODELING

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Farmaceutische Wetenschappen aan de Universiteit Antwerpen te verdedigen door

LINDSEY DUGAUCQUIER

Promotors: Prof. Vincent FM Segers, MD PhD & Prof. Gilles W De Keulenaer, MD PhD

Antwerpen 2020

MEMBERS OF THE JURY

Prof. Guido De Meyer, PharmD PhD (Chair of the jury)
Laboratory of Physiopharmacology – University of Antwerp, Belgium

Prof. Ingrid De Meester, PharmD PhD (Chair of the jury)
Laboratory of Medical Biochemistry – University of Antwerp, Belgium

Prof. Vincent FM Segers, MD PhD (Promotor)
Laboratory of Physiopharmacology – University of Antwerp, Belgium

Prof. Gilles W De Keulenaer, MD PhD (Promotor)
Laboratory of Physiopharmacology – University of Antwerp, Belgium

Prof. Patrick D' Haese, MD PhD (Member)
Laboratory of Pathophysiology – University of Antwerp, Belgium

Prof. Carlo Gabriele Tocchetti, MD PhD (Member)
Center of Translational Medical Sciences – University of Naples Federico II

Prof. Anton Roks, PhD (Member)
Division of Vascular Medicine and Pharmacology – Erasmus Medical Center Rotterdam

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SCIENTIFIC CURRICULUM VITAE

NAME: Lindsey Dugaucquier

DATE OF BIRTH: March 12, 1991

EMAIL: lindsey.dugaucquier@gmail.com

EDUCATION

PhD in Pharmaceutical Sciences 2015–2020
University of Antwerp

Master of Science in Industrial Pharmacy 2014–2015
University of Antwerp, *Cum laude*

Master of Science in Drug Development: Pharmacist 2012–2014
University of Antwerp, *Cum laude*

Bachelor of Pharmaceutical Sciences 2009–2012
University of Antwerp, *Cum laude*

Additional educational courses

Statistical data analysis 2019
University of Antwerp

Teamwork and leadership Training 2019
University of Antwerp

Applied Communication Training 2017
University of Antwerp

Laboratory Animal Sciences (certificate category C) 2016
University of Antwerp

SCIENTIFIC ACTIVITIES

Theses

Master in Industrial Pharmacy – Laboratory of Physiopharmacology, University of Antwerp
"The protective effect of Neuregulin-1 in renal physiology"

Master in Drug Development – Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp
"Sialoadhesin and the macrophage, optimization of immunofluorescent stainings of the macrophage-specific receptor sialoadhesin"

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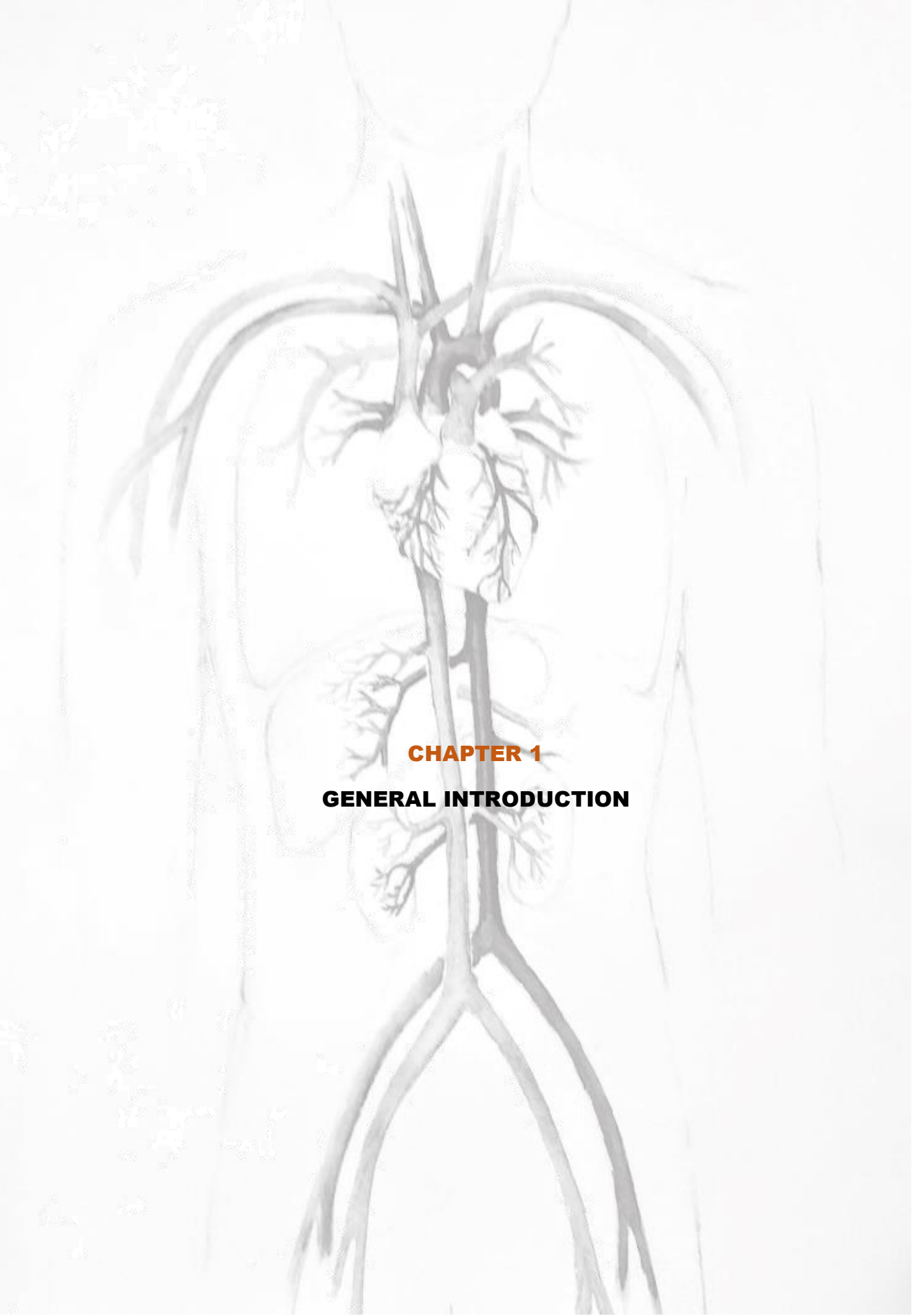
LIST OF ABBREVIATIONS

Ach	Acetylcholine
ADAM	a-disintegrin and metalloproteinase
ADHERE	Acute decompensated heart failure national registry
AKT	Protein kinase B
Ang II	Angiotensin II
ANGPT	Angiopoietin1
ATP	Adenosine triphosphate
BACE1	b-secretase1
BSA	Bovine serum albumin
CAD	Coronary artery disease
CCR2	C-C chemokine receptor 2
Cdh5	Cadherin 5
CHF	Chronic heart failure
CKD	Chronic kidney disease
CM	Cardiomyocyte
CM CSA	Cardiomyocyte cross-sectional area
COL1a1	Collagen 1a1
COL3a1	Collagen 3a1
COX-2	Cyclooxygenase-2
CRD	Cysteine-rich domain
CT	Cycle threshold
d	Diastole
DEANO	Diethylamine NONOate sodium salt hydrate
EC	Endothelial cell
ECM	Extracellular matrix
ECM	Experimental cerebral malaria
EDV	End-diastolic volume
EF%	Ejection fraction

EGF	Endothelial growth factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGM	Endothelial growth medium
eNOS	Endothelial nitric oxide synthase
ERBB	Erythroblastic leukemia viral oncogene homolog
ERK	Extracellular regulated kinase
ERT2	Estrogen receptor ligand-binding domain
ESV	End-systolic volume
ET1	Endothelin-1
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
FDR	False discovery rate
FGF	Fibroblast growth factor
FS%	Fractional shortening
FSP1/s100a4	Fibroblast-specific protein 1
GA	Gentamicin sulfate/ Amphotericin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGF2	Glial growth factor-2
hEGF	Human endothelial growth factor
hFGF	Human fibroblast growth factor
HFrEF	Heart failure with reduced ejection fraction
HMVEC	Human cardiac microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
HW/BW	Heart weight/body weight
i.p.	Intraperitoneal
IB4	Isolectin
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IGF1	Insulin-like growth factor-1

IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
KO	Knockout
LAD	Left anterior descending coronary artery
L-NAME	*N*$^{\Omega}$-nitro-L-arginine methyl ester
Lox	Locus of recombination
LPS	Lipopolysaccharide
LV	Left ventricular; left ventricle
LVID	Left ventricular internal diameter
LVPW	Left ventricular posterior wall
Lyz2	Lysozyme 2
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMP	Matrix metalloprotease / metallopeptidase
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
NRG	Neuregulin
P-AKT	Phospho-Protein kinase B
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P-ERK	Phospho-Extracellular regulated kinase
PI3K	Phosphatidyl inositol-3 kinase
PKG	Protein kinase G
PLGF	Placental growth factor
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene fluoride membrane

rNRG1	Recombinant neuregulin-1
RT-qPCR	Real time quantitative polymerase chain reaction
RV	Right ventricular; right ventricle
s	Systole
s.c.	Subcutaneous
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
STAT3	Signal transducer and activator of transcription 3
TAC	Transverse aortic constriction
TACE	Tumor necrosis factor-α converting enzyme
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
T2DM	Type 2 diabetes mellitus
VE-Cdh5-Cre^{+}	Vascular endothelium cadherin5 Cre-recombinase positive
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WB	Western blotting



CHAPTER 1

GENERAL INTRODUCTION

The heart and chronic heart failure

Since the 1990s, treatment of patients with chronic heart failure (CHF) has evolved significantly, but the incidence of CHF remains high and the prognosis poor. CHF is a major health problem, with a prevalence of 26 million people worldwide and over 200,000 people in Belgium. CHF has an average mortality of 26% within the year after diagnosis, particularly among patients of 65 years and older (1, 2). The current generation of drugs interferes with the renin-angiotensin-aldosterone system and of the adrenergic nervous system. However, hospitalization and rehospitalization of HF patients remains high and a curative treatment is not available (1, 3-5). Therefore, a new generation of drugs is currently in development or has already been introduced, including neuregulin-1 (NRG1).

The human heart is a highly structured pluricellular organ consisting of endothelial cells (ECs), cardiomyocytes (CMs), fibroblasts, inflammatory cells, and vascular smooth muscle cells (VSMC). ECs are the most abundant cell type accounting for 45% of the total cardiac cell population and 65% of the non-myocyte population (6, 7). Brutsaert *et al*. demonstrated the importance of cell-cell communication in the heart about 30 years ago (8-10). We now know that intercellular communication plays an important role during cardiac development, and is essential for cardiac homeostasis and in the failing heart. ECs are dynamic regulators and interact with nearby cells. Cross-talk between ECs and other cardiac cells is enabled by a dense capillary network of 3,000–4,000 capillaries/mm^2 (1, 7, 11, 12). In the normal adult myocardium, there is at least one capillary next to every CM and ECs directly communicate with adjacent CMs via a variety of soluble paracrine, autocrine, and endocrine factors (1, 11, 12).

A growing number of endothelial-derived cardio-active factors have been identified: nitric oxide (NO), endothelin-1 (ET1), angiotensin II (Ang II),

angiopoietins, prostaglandins, and growth factors like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and NRG1 play an important role in endothelium-CM cross-talk (1). Most endothelium-derived substances like NO and ET1 have both paracrine and autocrine properties, and endothelial dysfunction results in impairement of these substances and leads to adverse cardiac remodelling and dysfunction (1, 13, 14). NRG1, on the other hand, is expressed by ECs and binds in a paracrine manner on erythroblastic leukemia viral oncogene homolog (ERBB)4 and ERBB2 receptors on neighboring CMs (15). NRG1 is a member of the epidermal growth factor (EGF) family and is the most abundant in the cardiovascular system. The first evidence for the physiological activity of NRG1 was discovered by accident in human during the development of ERBB2-targeted therapy for breast cancer. Cardiotoxicity was induced in patients with metastatic breast cancer receiving the humanizred monocloanal anti-ERBB2 antibody trastuzumab (Herceptin®), especially when combined with anthracyclines (16-20). Multiple studies suggest the important role of NRG1/ERBB signaling during cardiac development, physiology, and pathophysiology (Figure 1). Treatment of various animal models with recombinant NRG1 (rNRG1) showed the therapeutic potential of NRG1. rNRG1 is able to improve several outcomes of HF, including left ventricular (LV) remodeling, ventricular fibrosis, cardiac performance, and animal survival. These promising preclinical animal studies have led to ongoing clinical trials. Two phase II clinical trials have stated that administration of rNRG1 is safe and improves LV function in patients with HF with reduced ejection fraction (HFrEF) (17, 21, 22).

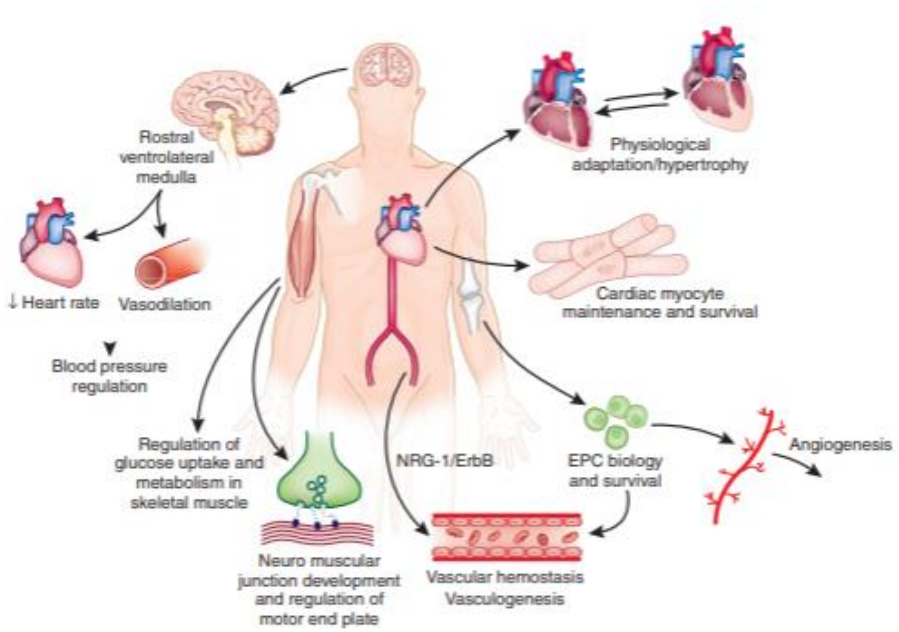


Figure 1: Biological and physiological role of NRG1 signaling in the heart. *After Odiete O et al., Circulation Research, 2012 (17). Various effects of NRG1 in the cardiovascular system. EPC, Endothelial progenitor cell.*

ERBB receptors are also expressed on ECs, theoretically allowing autocrine signaling of NRG1. However, the role of autocrine NRG1/ERBB signaling in ECs is less understood and will be discussed in depth in this thesis as endothelium-controlled signaling pathways are emerging as an important pathophysiological target in the treatment of HF. In addition, a recent studie by Vermeulen *et al.* from our laboratory showed the importance of paracine NRG1/ERBB signaling in inflammatory cells during Ang II–induced myocardial fibrosis. In this thesis, we examined NRG1/ERBB signaling in inflammatory cells in different models of cardiac remodeling.

Our laboratory has been studying the NRG1/ERBB pathway in the pathophysiology of HF since many years. We showed a direct role of ERBB2 in endothelium-CM cross-talk and in the preservation of myocardial integrity (23). Furthermore, our laboratory demonstrated that the NRG1/ERBB system is

activated, and plays a modulatory role, during physiological hemodynamic overload associated with pregnancy (24). We also showed that the NRG1/ERBB system was activated during the development of pacing induced HF (25) and that NRG1 is able to activate NO synthase in CMs and induces a negative inotropic effect in the cardiac muscle (26). Vandekerckhove *et al*. discovered therapeutic effects of rNRG1 in different organs of hypercholesterolemic type I diabetic mice, rNRG1 administration protects against the development of dilated cardiomyopathy, atherosclerosis, and nephropathy (27). Recently, Vermeulen *et al*. showed that NRG1 has antifibrotic and anti-inflammatory effects in the heart, skin, and lung (28).

In this thesis, we investigated the role of NRG1/ERBB4 signaling in non-myocytes, more specific in ECs and inflammatory cells during cardiac remodeling. We studied the underlying mechanisms of action using cell culture experiments and transgenic mouse models. Before going more into details about this research, we will focus on different key aspects of the NRG1/ERBB system.

NRG1 and the ERBB-receptor family

NRGs were first discovered in the brain almost 30 years ago and are encoded by the *Nrg1–4* genes. NRGs are cell-cell signaling proteins belonging to the EGF family, known to participate in cell proliferation, differentiation, and survival in many tissue types, including heart, lung, kidney, skeletal muscle, nervous system, and breast (29-31). Our laboratory studies NRG1 and this specific protein will be the focus of my present work.

NRG1 and its different isoforms

NRG1 is a member of 4 structurally related transmembrane polypeptides encoded by four genes (*Nrg1*, *Nrg2*, *Nrg3*, and *Nrg4*) and has been well-studied

in the cardiovascular system. NRG1 is synthesized as a transmembrane polypeptide and the most abundant source of NRG1 in the heart is the endothelium (15, 17, 23, 31). The *Nrg1* gene is located on chromosome 8 in both humans and mice, where it encodes for 21 exons resulting in over 31 protein isoforms. These different isoforms share a unifying EGF–like domain, which is essential for NRG1 ligand binding to the ERBB family of receptor tyrosine kinases. The EGF–like domain, is a protein domain comprised of six cysteine residues, which form 3-disulfide bonds. Alternative splicing of the EGF–like domain, based on different exon usages, leads to an α or β variant at its C-terminus. The NRG1β isoform has shown to be 10 to 100 more potent compared to the α variant (29, 30). N-terminal sequences distinguish NRG1 isoforms as either Type I, Type II, Type III, Type IV, Type V, or Type VI (Figure 2). NRG1 N-terminal regions can include a signal peptide, a kringle-like domain, a cysteine-rich domain, an immunoglobulin (Ig)-like domain, and a glycosylation region. Type I, IV and V contain a glycosylation region. Type I, II, IV and V consist of a Ig-like domain, typically ~80 amino acid residues forming 7-10 β sheets and serving different cellular functions, like molecular transport, adhesion, morphogenic control and cellular recognition. Type III contains a cysteine-rich domain (CRD), instead of an Ig-like domain, and has a intracellular N-terminal sequence. Type II contains a kringle-like domain, serving as a protein-protein interaction site. NRG2, NRG3, and NRG4 exhibit far less diversity in isoform N-terminal sequences (30, 31).

Release of NRG1 takes place at the plasma membrane when the N-terminal ectodomain of pro-NRG1 undergoes proteolytic cleavage by specific proteases. The juxtamembrane region, found between the EGF–like and the transmembrane domain, serves as a proteolytic site for a disintegrin and metalloproteases (ADAMs), such as tumor necrosis factor-α converting enzyme (ADAM17/TACE), ADAM10 and meltrin-β (ADAM19). NRG1 can also be cleaved at its cytoplasmic tail by a b-secretase1 (BACE1), which releases the

intracellular domain and can then translocate to the nucleus to promote expression of anti-apoptotic genes. The functional differences between these isoforms, and their action through either paracrine, autocrine, or juxtacrine communication, define their final biologic activity (15, 29-31).

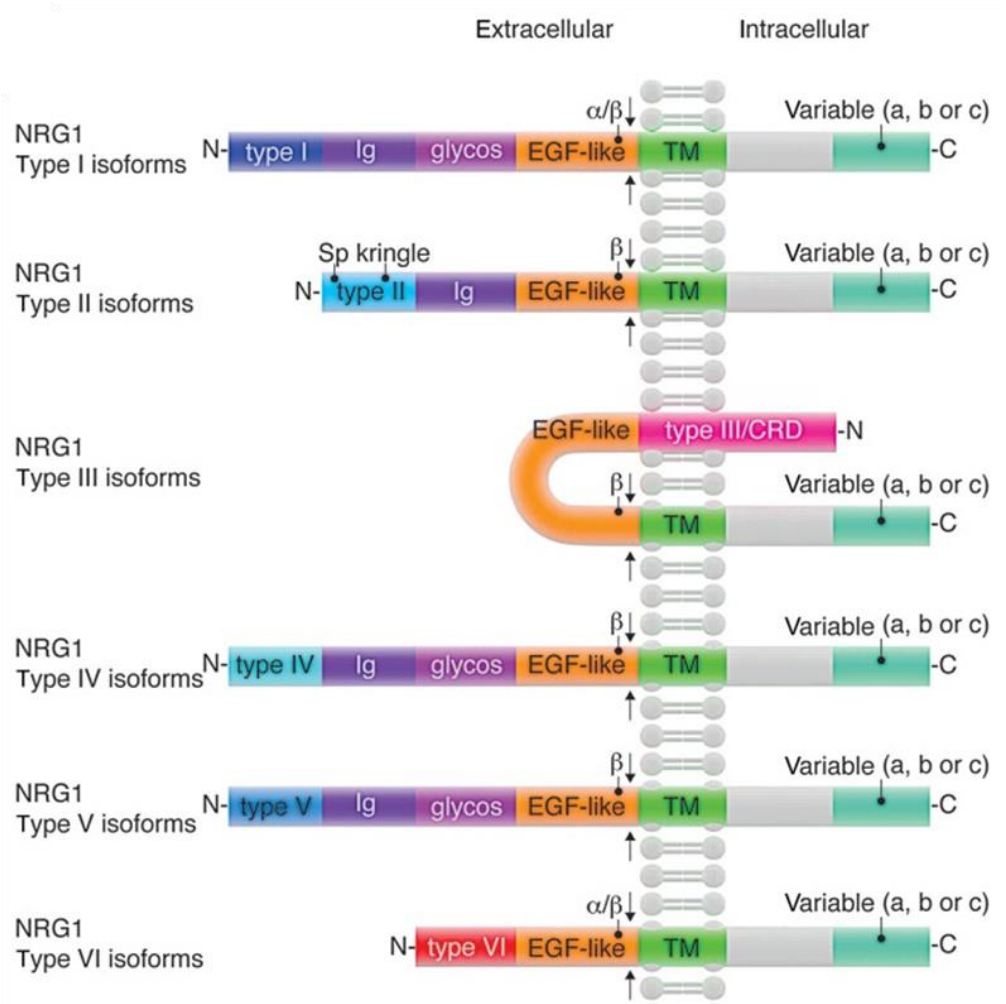


Figure 2: NRG1 isoforms. *Modified after Parodi EM et al., Cardivascular Research, 2014 (30). N-terminal sequences distinguish NRG1 isoforms as either Type I, Type II, Type III, Type IV, Type V, or Type VI. NRG1 N-terminal regions can include a signal peptide (sp), a kringle-like domain, a cysteine-rich domain, an immunoglobulin (Ig)-like domain, and a glycosylation region. Arrows indicate proteolytic cleavage sites. TM, transmembrane domain.*

ERBB family of receptor tyrosine kinases

NRG1 is synthesized as a 115 kDa transmembrane protein by the endothelium and released after proteolytic cleavage as a 30 kDa NRG1β fragment, after which it can mediate its effects through binding with a member of the ERBB family of receptor tyrosine kinases. The ERBB family consists of four closely related transmembrane tyrosine kinase receptors, including ERBB1 or EGF receptor (EGFR), ERBB2, ERBB3, and ERBB4 (31, 32). ERBB receptors transduce biological signals from the extracellular to the intracellular space after binding polypeptides of the EGF family, including NRG1. These receptors are strong mediators of cell growth, proliferation, survival, and organ development and repair (32, 33).

The overall structure of the ERBB receptor family consists of three functional domains: a glycosylated extracellular ligand binding domain, a transmembrane domain, and an intracellular domain containing tyrosine kinase (32). The glycosylated extracellular domain consists of four smaller subdomains of which two are leucine-rich ligand binding domains consisting of β-helix folds (I and III), the remaining two are cysteine-rich domains cross-linked by disulphide bonds (II and IV) (Figure 3A). The short transmembrane domain is rich in hydrophobic amino acids and is flanked on the intracellular side by a juxtamembrane region that has a regulatory role in downstream signaling. The intracellular catalytic protein tyrosine kinase (PTK) subdomain is a bilobed structure predicted to bind adenosine triphosphate (ATP). Its C-terminal region contains several tyrosine residues, which become phosphorylated upon receptor activation and which are important in further downstream effector binding (32). The structure of the 180 kDa ERBB receptors is archetypal. However, variations exists in terms of ligand binding and in protein tyrosine kinase activity.

NRG1 is not able to bind ERBB1, and thus this receptor does not lie within the focus of this thesis. ERBB2 has no known extracellular ligands and its extracellular domain normally assumes an “active” conformation. However, the PTK subdomain is inactive in its monomeric form, and heterodimerization is needed in order to induce phosphorylation of the tyrosine residues. Additionally, ERBB2 is the preferred heterodimerization partner of the other ERBB receptors, ERBB2 heterodimers have an increased ligand binding affinity and are more stable than other heterodimers (32, 34). ERBB3, on the other hand, has a catalytically inactive PTK subdomain. Heterodimerization with other ERBB members is essential for the phosphorylation of the tyrosine residues, and thus to become activated (Figure 3A). Furthermore, ERBB4 has an increased sensitivity to proteolytic cleavage by matrix metalloproteases (MMPs). This releases the ERBB4 extracellular domain as well as the 80 kDa intracellular domain, which may translocate to the nucleus and participate in transcriptional regulation (32, 35).

ERBB receptors normally exist as inactive monomers. Ligand binding to the glycosylated extracellular domain initiates rearrangement to an active “open” conformation, exposing the dimerization arm as shown in Figure 3B. Dimerization can occur between two different ERBB receptors (heterodimerization) or between two receptors of the same type (homodimerization). After receptor dimerization, transphosphorylation of the intracellular C-terminal tyrosine residues occurs by its receptor partner. Phosphorylation allows the recruitment and activation of downstream proteins and a signaling cascade is initiated (32, 33). To summarize, ligand binding and following receptor dimerization is essential for ERBB function and intracellular signaling through a complex and tightly controlled network of signaling pathways that drive and regulate many cellular functions (33).

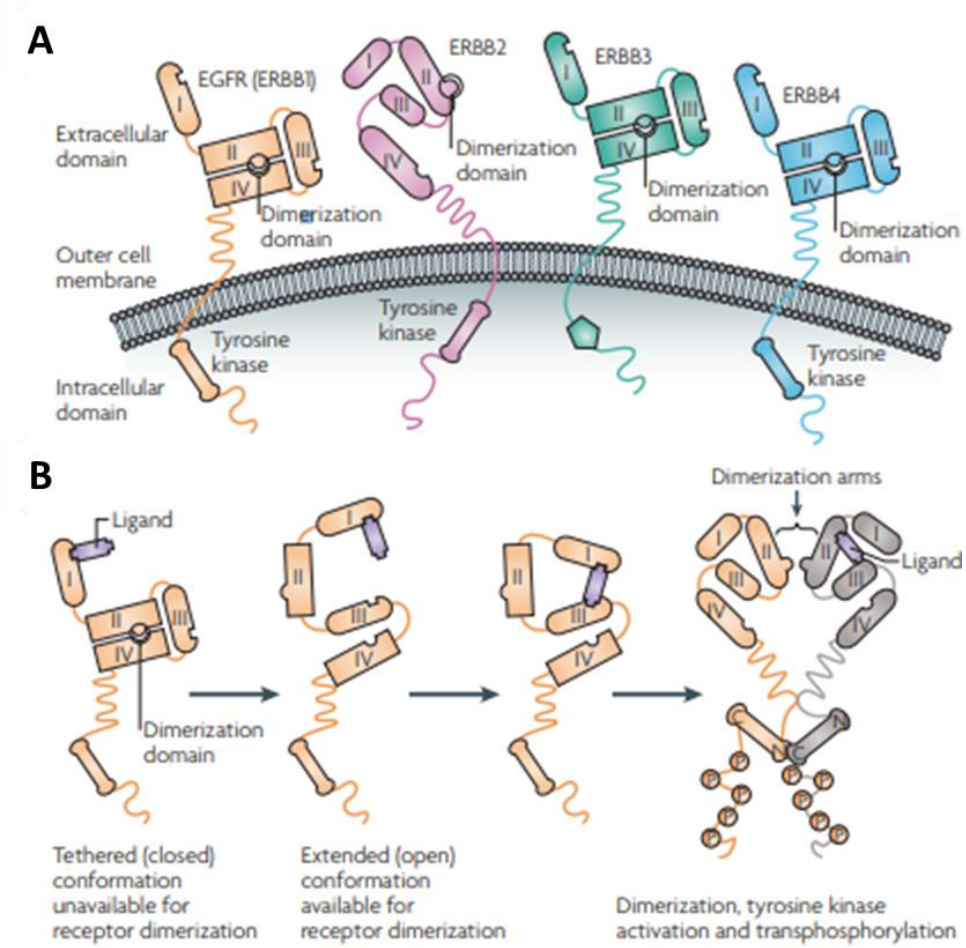


Figure 3: The ERBB family of receptor tyrosine kinases. *After Baselga J et al., Nature Reviews Cancer, 2009 (33). A: The ERBB family consists of four closely related transmembrane tyrosine kinase receptors, including ERBB1 or EGF receptor (EGFR), ERBB2, ERBB3, and ERBB4. The overall structure of the ERBB receptors consists of a extracellular ligand binding domain, a transmembrane domain, and an intracellular domain, containing the tyrosine phosphorylation. The extracellular domain consists of two leuine-rich ligand binding domains (I and III) and two cysteine-rich domains (II and IV). The intracellular domain consists of a bi-lobed protein tyrosine kinase subdomain. B: Ligand binding to the ERBB receptor initiates rearrangement to an active "open" conformation, followed by hetero- or homodimerization, and transphosphorylation.*

NRG1/ERBB signaling in the heart: implications in cardiac development, physiology, and pathophysiology

The heart is a pluricellular organ, consisting of cardiac ECs, CMs, fibroblasts, inflammatory cells, and VSMC. Cell-cell communication in the heart, and most important between ECs and CMs, is essential for cardiac performance. CMs depend on ECs not only for oxygenated blood supply but also for local protective signals. Cross-talk between these different cell types is mediated through endothelial-derived cardio-active factors, like NRG1 (1, 8, 12, 23, 36). NRG1 is highly expressed in the heart and is released by the endothelium. The generally accepted concept is that the cardiac effects of NRG1 are mediated by paracrine activation of ERBB4/ERBB2 receptors on CMs (17, 23), and that this activation is important during cardiac development, physiology and pathophysiology (15). This concept will be further described in this introduction and will be challenged in this thesis.

NRG1/ERBB signaling is essential for cardiac development

The NRG1/ERBB system is involved in cardiac development. Binding of NRG1 to ERBB4 drives dimerization with ERBB2, resulting in a signal transduction cascade that promotes proliferation and differentiation of CMs in the developing heart. Specific deletion of *Nrg1, Erbb2*, *Erbb3*, or *Erbb4* in transgenic mice leads to failure of cardiac development, including lack of ventricular trabeculation, leading to embryonic lethality (day 10.5). ERBB3 is expressed in the developing, but less in the adult heart and is detected in the invading mesenchyme and endocardial cardiac cushions. Deletion of *Erbb3* in transgenic mice results in malformation of cardiac cushion and inadequate valve formation, and in turn embryonic lethality (day 13.5) (37). In addition, NRG1/ERBB signaling is critical for the development of the cardiac conduction system. NRG1 stimulates differentiation of embryonic CMs into cells of the cardiac conduction system with

an increase in pacemaker current density, and transforms fetal CMs into cardiac pacemaker-like cells (Figure 4). Important for NRG1 activity in prenatal cardiac development is the Ig-like domain and the EGF–like domain, both mice with targeted deletion of the EGF–like domain and the Ig-like domain of NRG1 die in utero (15, 17, 29, 30).

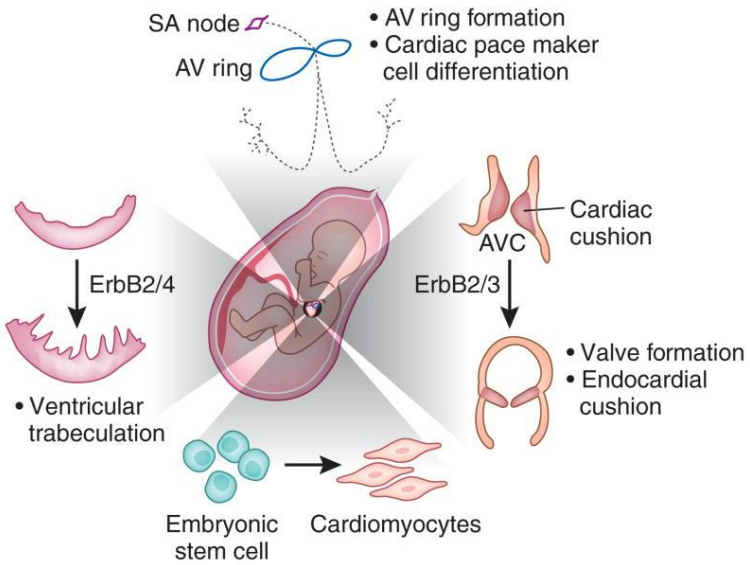


Figure 4: Role of NRG1/ERBB signaling in cardiac development. *After Odiete et al. Circulation Research, 2012 (17). NRG1/ErbB signaling is necessary for cardiac development. It plays an important role in the formation of cardiac cushion, endocardial cushion, development of the conduction system, valve formation, and ventricular trabeculation. SA indicates sinoatrial; AV, atrioventricular; AVC, atrioventricular canal.*

The role of NRG1/ERBB signaling in the adult heart

The NRG1/ERBB system is also crucial in the postnatal and adult heart. As previously mentioned, the importance of NRG1/ERBB signaling in postnatal cardiac homeostasis was first demonstrated by an unforseen side effect during the development of anti-ERBB2 therapies for cancer. In humans, the chemotherapeutic agent Trastuzumab can induce reversible cardiomyopathy as

a toxic side effect, most significant when given in combination with anthracyclines (17-19, 30, 38).

The cardioprotective role of the NRG1/ERBB system was further established by *Erbb2*- and *Erbb4*-null mice. Conditional CM–specific deletion of *Erbb2* and *Erbb4* have been examined in mice expressing myosin heavy chain promoter–regulated Cre-recombinase and floxed *Erbb* genes, which resulted in spontaneous dilated cardiomyopathy and higher sensitivity to cardiac stress. *Erbb2* conditional mutant mice displayed a lengthened ventricular repolarization time, suggesting additional impairment in the conduction system. CMs from conditional *Erbb4* deletion mice exhibited both delayed conduction and impaired contractility (17, 39, 40). Furthermore, heterozygous knockout (KO) of *Nrg1* in mice exacerbates doxorubicin-induced HF, which is associated with the depression of activation of the heterodimerization partner ERBB2 as well as different downstream pathways, like protein kinase B (AKT), and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways (41).

The role of the NRG1/ERBB system in physiological adaptation of the heart to changes in cardiac demand was also demonstrated by Lemmens *et al..* Administration of an ERBB1/ERBB2 tyrosine kinase inhibitor lapatinib resulted in inhibition of NRG1/ERBB signaling, followed by premature maternal death during pregnancy, increased pregnancy-induced LV dilatation, and reduced LV fractional shortening (FS%). This study showed that the NRG1/ERBB system has cardioprotective properties that are activated in conditions of physiological stress, such as ventricular overload and remodeling (24). In addition, it was found that ET1 and increased mechanical stress on the heart increases NRG1 expression, whereas Ang II and phenylephrine decrease NRG1 expression. This observation supports the role of NRG1 in cardiac homeostasis (23, 30). In addition, some studies show contrasting observations. For example, NRG1/ERBB signaling plays a role in cardiac hypertrophy. NRG1-treatment of

CMs *in vitro* has been shown to increase hypertrophy (42). However, in two month-old mice with left anterior descending coronary artery (LAD) ligation, administration of NRG1 was shown to decrease CM hypertrophy (43).

Furthermore, *in vitro* studies have demonstrated likely cellular mechanisms of how NRG1/ERBB signaling may regulate cardiac adaptation to stress. NRG1 is expressed as a transmembrane protein in the microvascular endothelium and endocardial ECs. After cleavage by MMP from the ADAM family, NRG1 binds the ERBB4 receptor on CMs. Heterodimerization with ERBB2 or homodimerization leads to activation of several distinct and interacting signaling cascades, including ERK1/2, mitogen activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K)/AKT, NO synthase (NOS) and Src/focal adhesion kinase (FAK). All of these signaling pathways are implicated in cellular responses, including cell survival, mitochondrial function, proliferation, growth, glucose uptake, sarcoplasmic reticulum calcium uptake, and focal adhesion formation in CMs (38, 44). More specific, NRG1 prevents CM apoptosis by inhibiting cytochrome C release and caspase-3 activation through a PI3K/AKT-dependent signaling pathway (45, 46). And NRG1 enhances NO production in ventricular myocytes through PI3K/AKT activation. The increased NO levels lead to protein kinase G (PKG) activation with the phosphorylation of phospholamban, which increases calcium uptake in the sarcoplasmic reticulum (47). In addition, NRG1 attenuates β-adrenergic responses in CMs, having negative inotropic effects and NRG1 is implicated in hypertrophic responses. Furthermore, it has been shown that NRG1 protects CMs from apoptotic cell death induced by oxidative stress and anthracyclines *in vitro*, demonstrating the modulatory role of NRG1/ERBB signaling in the cardiovascular system during stress (17, 23, 26, 29). NRG1 has also been shown to modulate cell survival in non-myocyte cells, including ECs, fibroblasts, and inflammatory cells. Liu *et al.* demonstrated that NRG1 attenuates experimental cerebral malaria (ECM) mortality by regulating ERBB4/AKT/signal transducer and activator of

transcription 3 (STAT3) signaling. The researchers determined that NRG1 protects against cell death and apoptosis of human brain microvascular EC (48). NRG1 also seems to play an important role in regulating the structure and function of the vasculature in many organs. This effect has been reported to be both dependent and independent of VEGF (49, 50). Furthermore, NRG1-induced PI3K/AKT signaling through phosphorylation of ERBB2 and ERBB3 increases cell growth and survival in cardiac fibroblasts (51). In addition, the NRG/ERBB systems plays a role in cell survival of macrophages and is able to control inflammatory processes. Schumacher *et* al. showed that proinflammatory activation of cultured murine and human macrophages induced ERBB4 expression. Subsequent treatment of activated proinflammatory macrophages with NRG4 induced apoptosis, which serves as an efficient clearance mechanism of proinflammatory macrophages from the tissue and is critical to prevent prolonged inflammation (52). A recent study at our laboratory showed that myeloid-specific deletion of *Erbb4* intensifies myocardial fibrotic response to Ang II (28), indicating the important role of NRG1/ERBB4 signaling in cardiac inflammatory cells.

Besides its proliferative role in cardiac development and its role in cell survival, NRG1/ERBB signaling enhances proliferation and regeneration of adult CMs. It was first stated that adult CMs are withdrawn from the cell cycle, and thus that the adult heart is a terminally differentiated organ. However, Bersell *et al.* suggested that NRG1 can induce mononucleated CMs to proliferate through activation the ERBB4 receptor and following activation of the PI3K pathway. Both the ERBB4 receptor and downstream PI3K pathway are required for CM cell cycle reentry and for the stimulation of the division of differentiated mononucleated CMs. Furthermore, NRG1-induced CM proliferation promotes myocardial regeneration following myocardial injury. Administration of NRG1 in two month-old mice with LAD ligation resulted in improved myocardial function, smaller infarct size, and attenuated myocardial hypertrophy (43). In contrast, a

study by Reuter *et al.* indicates that rNRG1 did not increase CM DNA synthesis in the normal or infarcted adult mouse heart, and that any protective effect on cardiac structure and function occurs independently of enhanced CM renewal (53). Polizzotti *et al.*, on the other hand, identified the neonatal period as a more effective time period for rNRG1-induced CM regeneration. This narrows the therapeutic window and could have implications for future clinical trials (54). In addition, a recent study of D'Uva et al. demonstrated that ERBB2 is necessary for both NRG1-induced CM proliferation and regeneration. They showed that ERBB2 is essential for the integrity and function of the heart and for NRG1-dependent CM proliferation up to the immediate neonatal period. In addition, they observed diminishing levels of ERBB2 one week after birth and a decreased NRG1-induced CM proliferation and regeneration. They further demonstrate that transient expression of constitutively activated ERBB2 after ischemic injury in juvenile or adult hearts triggered CM dedifferentiation, proliferation, neovascularization and CM redifferentiation, which leads to anatomical and functional regeneration. Taken together, this data showed that ERBB2 levels limit CM proliferation and regenerative plasticity postnatally (55).

Importance of NRG1/ERBB signaling in cardiovascular diseases

The importance of NRG1 in the postnatal heart is further endorsed by studies showing that NRG1/ERBB signaling changes during cardiac diseases. Various animal models have demonstrated important changes within cardiac NRG1/ERBB activation during the progression of CHF. In the setting of transverse aortic constriction (TAC)–induced pressure overload, expression of *Erbb2*/*Erbb4* mRNA in the LV increases during compensatory cardiac hypertrophy. However, after the transition to CHF, myocardial *Erbb2*/*Erbb4* mRNA and protein levels are decreased compared to control hearts (29, 56). In diabetic animals, myocardial NRG1 expression and *Erbb2/Erbb4* mRNA expression and activation are also reduced (57). Further, NRG1 levels are

increased according to disease severity. Increased NRG1 and decreased ERBB2/ERBB4 expression have been observed in ventricular tissue from patients with advanced HF. Especially in patients with ischemic HF, NRG1 shows an increased activity (58, 59). Ventricular levels of NRG1, as well as phosphorylated ERBB4 receptor levels, are increased in response to acute ischemic insults. Deleting the *Nrg1* gene in ECs aggravate ischemic insults (60). The initial robust increase in NRG1/ERBB activity occurs during development of concentric LV hypertrophy and is most likely the result of mechanical wall strain. The subsequent decline in NRG1/ERBB activity coincides with the development of pump failure and is accompanied by a downregulation in *Erbb2/Erbb4* mRNA levels (17, 29, 30). These observations support a role of NRG1/ERBB signaling during HF and identifies NRG1 as a potential biomarker with potential clinical applications.

Preclinical studies in animal models of HF have initiated clinical trials, which are currently ongoing and examine the effects and safety of NRG1 in patients with HF. To date, ClinicalTrials.gov has listed 5 trials examining NRG1 administration as a treatment for cardiovascular diseases (Table 1). One phase I randomized, double blind, placebo-controlled trial has been completed. 40 patients with LV systolic dysfunction and symptomatic HF received a single ascending intravenous (i.v.) dose of the glial growth factor-2 (GGF2) version of NRG1. This dose was well tolerated and dose-dependent improvement in LV ejection fraction (EF%) at 28 days was observed, which was sustained up to 90 days. A phase II randomized, double-blind, multi-center study demonstrated the role of NRG1 in improving cardiac function of CHF patients by increasing the EF%, and showed its role in reverse remodeling by decreasing end-systolic and end-diastolic volume (ESV, EDV). A total of 44 CHF patients received either placebo or rNRG1 in low, intermediate, or high dose (0.3, 0.6, or 1.2 $\mu g.kg^{-1}.day^{-1}$). At day 30, EF% was significantly increased, ESV and EDV were significantly decreased in the intermediate dose group. Both ESV and EDV continued to decrease at

day 90 (ChiCTR-TRC-09000414) (21). In addition, another phase II non-randomized, open, single group trial, including 15 patients with CHF, demonstrated improved acute and chronic hemodynamic outcomes. All study subjects received a daily i.v. infusion of rNRG1 over a period of 6 to 12 h, for 11 days. Acutely, cardiac output increased by 30%. Acute hemodynamics were sustained, and 12 weeks after the last dose, there was a 12% increase in ventricular systolic function (ACTRN12607000330448) (22). A larger phase II randomized, double-blind, placebo-controlled trial has been completed, in which 120 CHF patients received daily subcutaneous (s.c.) infusion of rNRG1 for 10 consecutive days to assess safety and efficacy of the NRG1 treatment (NCT01251406). Furthermore, a phase III randomized, double-blind, multi-center trial is ongoing and studies the survival rates of 1600 CHF patients after 10 days of rNRG1 treatment, followed by a weekly 10 min i.v. bolus from the 3rd week for 23 weeks (NCT03388593).

Table 1: Clinical studies examining NRG1 administration as a treatment for cardiovascular diseases.

Clinical study	Study number	Last Updated	Patients	Administration	Effect of NRG-1
Phase I, Randomized, Double-blind, Placebo Parallel controlled, Single ascending dose study	NCT01258387	July 2014 (Completed: March 2013)	40 patients with LV systolic dysfunction and symptomatic HF	Single i.v. dose of the GGF2 version of NRG-1	- Well tolerated - Dose-dependent improvement in LVEF at 28 days that was sustained up to 90 days
Phase II, Randomized, Double-blind, Placebo Parallel controlled study	NCT01251406	August 2014 (Completed: March 2014)	120 CHF patients	10-day rNRG1 s.c. infusion therapy	- Safety and efficacy of treatment
Phase II, Randomized, Double-blind, Multi-center, Placebo Parallel controlled study	ChiCTR-TRC-09000414	May 2015	44 CHF patients	10-day rNRG1 i.v. infusion therapy	- Significantly increased LVEF - Reduced ESV and EDV, which continued to decrease at day 90 - Reverse remodeling
Phase II, Non-randomized, Open, Single group study	ACTRN12607000330448	January 2019	15 CHF patients	11-day rNRG1 i.v. infusion therapy	- Improved acute and chronic hemodynamics
Phase III, Randomized, Double-blind, Multi-center, Placebo Parallel controlled study	NCT03388593	November 2018	1600 CHF patients	10-day rNRG-1 i.v. infusion therapy, followed by a weekly 10 min bolus from the 3rd week for 23 weeks	- No results posted

Conclusion

The NRG1/ERBB system plays an indispensable role during myocardial development, cardiac homeostasis, and cardiac pathophysiology. NRG1/ERBB signaling is critical during cardiac remodeling (61), it allows the heart to cope with physiological stress (24). It has been shown in both preclinical and clinical studies that administration of NRG1 attenuates cardiac remodeling and improves cardiac function in different models of HF (21, 22, 56, 58, 62). This research is still ongoing, and this thesis will add important knowledge regarding the role of non-myocyte NRG1/ERBB4 signaling in HF.

NRG1/ERBB signaling during HF and its comorbidities

Apart from studies on the effect of NRG1 on HF, recent studies have demonstrated therapeutic effects of NRG1 on different comorbidities of HF. These effects emphasize pleiotropic effects of NRG1 throughout the body and will be discussed further (Figure 5).

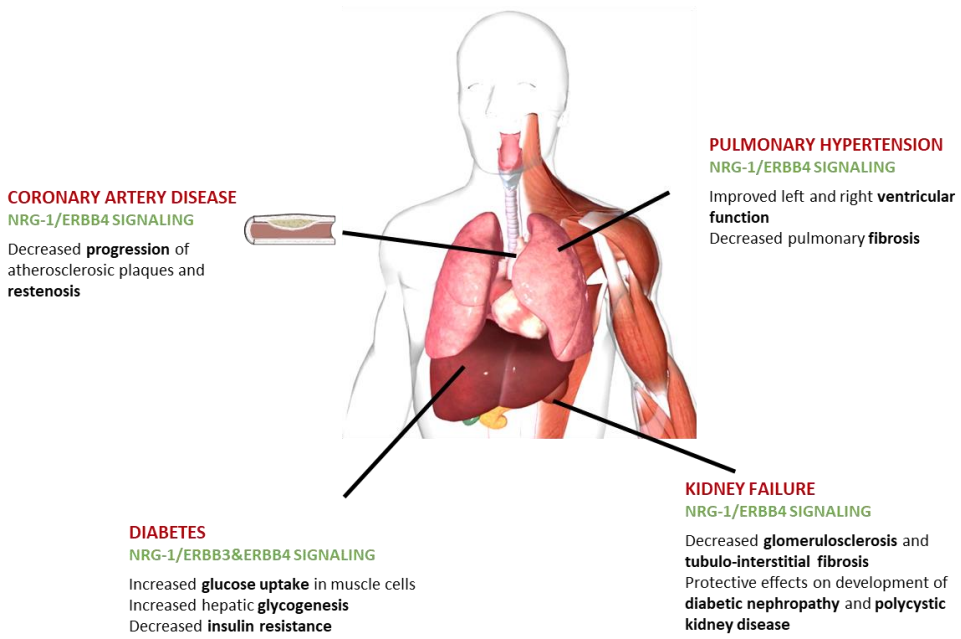


Figure 5: Pleiotropic activities of NRG1/ERBB signaling. *HF is associated with different comorbidities in which NRG1/ERBB signaling could play an important role.*

Coronary artery disease

Coronary artery disease (CAD) is the leading cause of mortality in the United States (63). CAD is a major cause of death and disability in developed countries. It causes about one-third of all deaths in people older than 35 years (64). Xu *et al.* were the first to discover the atheroprotective effects of NRG1 in CAD. NRG1 inhibits atherogenesis and suppresses macrophage foam cell formation (65). In addition, they proved a correlation between NRG1 plasma levels and disease severity. Patients with CAD showed significantly lower levels of NRG1 compared to patients with mild hypertension and healthy volunteers (65). Geisberg *et al.* confirmed that NRG1 plasma levels were inversely correlated with angiographic severity of CAD in patients undergoing cardiac catheterization (63). Also, Panutsopulos *et al.* suggest an important role of NRG1 in CAD and the expansion of atherosclerotic plaques (66). Diabetic ApoE null mice developed dense atherosclerotic places, NRG1 treatment prevented this by activation of the ERBB2 and ERBB4 receptors in the heart (27). Furthermore, NRG1 attenuates proinflammatory responses involved in CAD by decreasing the expression of different proinflammatory cytokines, like interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), MMP9, and cyclooxygenase-2 (COX-2) in monocytes (67).

Chronic kidney disease

Most patients with HF are 65 years or older, and often suffer from one or more comorbidities. A common comorbidity of HF is chronic kidney disease (CKD), and a study of 105,388 HF patients in the Acute Decompensated Heart Failure National Registry (ADHERE) reported that 60% of the patients suffered from CKD (68-70). Veikkolainen *et al.* suggested a role of ERBB4 in the kidney. They demonstrated that ERBB4 signaling modulates proliferation, polarization, and cellular functions critical for the development of epithelial ducts in the kidney

(71). At out laboratory, Vandekerckhove *et al*. showed receptor activation of ERBB2 and ERBB4 in the kidney upon NRG1 administration. Significant reduction in albuminuria, neutrophil gelatinase-associated lipocalin (NGAL) uria, glomerular fibrosis, and decreased expression of fibrotic markers after NRG1 administration in ApoE deficient mice were observed. This suggests protective actions of NRG1 in the kidney in a hypercholesterolemic type 1 diabetic mouse model (27, 72). Further, mice with global *Erbb4* deletion developed renal fibrosis in an accelerated manner in an obstructive nephropathy model. ERBB4 expression levels were increased in the early stages of renal injury, which endorses a compensatory role of ERBB4 signaling in the pathophysiology of the kidney. In more advanced stages of CKD ERBB4 expression levels were found to be inversely correlated to renal fibrosis in human fibrotic kidneys (73). The same research group demonstrated that deletion of *Erbb4* accelerates the progression of polycystic kidney disease in a mice. They stated that *Erbb4* deletion contributes to elevated cell proliferation and unbalanced cell apoptosis (74). In contrast to this study, Streets *et al*., reported *Erbb4* as a major factor driving cyst growth in autosomal dominant polycystic kidney disease (75). In conclusion, al data suggest the ERBB4 receptor as a therapeutic target in CKD.

Diabetes

Type 2 diabetes mellitus (T2DM) is a common comorbidity in HF patients. Patients with HF are exposed to a significant risk for either concurrently having diabetes or subsequently developing diabetes (76, 77). The prevalence of T2DM is four-fold higher in patients with HF, compared to patients without HF (78). In addition, T2DM worsens the prognosis of patients with HF by increasing the number of hospitalizations and mortality (79). Reversely, T2DM patients have a 75% higher risk of CV death or HF hospitalization (80). We can conclude that there is a bidirectional relation between HF and T2DM in which the NRG1/ERBB system can play an important role. Doggen *et al.* demonstrated that T2DM in

mice leads to deficient NRG/ERBB signaling in the LV and to delayed cardiac muscle relaxation. Chronic treatment with NRG1, but not with insulin, reversed these adverse cardiac outcomes (81). Consistent with these previous studies, Gui *et al.* showed a decreased ERBB2 and ERBB4 receptor expression and phosphorylation, as well as NRG1 protein synthesis, in a rat model of diabetic cardiomyopathy (57). In another study, researchers demonstrated that *Erbb4* deficient mice developed metabolic syndrome after 24 weeks on a medium-fat diet, indicated by the development of obesity and insulin resistance. In addition, they showed that NRG4 significantly increased glucose uptake in adipocytes isolated from wild-type mice. Glucose uptake was significantly decreased in adipocytes isolated from *Erbb4* deficient mice. These results support a role of ERBB4 in glucose homeostasis and lipogenesis (82). Salinas *et al*. linked the *Erbb4* gene to increased BMI, present in patients with obesity and diabetes (83). Furthermore, NRG1 showed glucose-lowering effects in pigs and mice (84). Different studies showed that administration of NRG1 induces glucose transport through translocation of glucose transporters in muscle cells (85), and reduces glucose levels in the liver by activating ERBB3/AKT signaling (84, 86-88). Li *et al*. provided additional evidence for a role of NRG1 in diabetic cardiomyopathy rats, NRG1 significantly improved heart function and reversed cardiac remodeling in diabetic cardiomyopathy rats (89). In conclusion, these data suggest a role of NRG1 in both HF and T2DM. Administration of NRG1 to HF patients with T2DM could be a potential therapeutic strategy. However, the anti-diabetic effects still need to be confirmed in human patients.

Pulmonary arterial hypertension

Pulmonary arterial hypertension is another common comorbidity of HF. During the natural progression of HF, LV dysfunction can lead to pulmonary hypertension, a progressive disorder characterized by pulmonary arterial remodeling (90). There is emerging evidence that this effect is attenuated by

treatment with NRG1. Mendes-Ferreira *et al*. demonstrated a decrease in pulmonary arterial and right ventricular (RV) remodeling in a rat model of monocrotaline-induced pulmonary hypertension and RV hypertrophy. NRG1 was able to restore pulmonary circulation, which suggests a role of NRG1/ERBB signaling in the pathophysiology of pulmonary arterial hypertension (91). Interestingly, Vermeulen *et al*., showed that NRG1 was able to reduce pulmonary fibrosis in a bleomycin-induced pulmonary fibrosis model in mice (28), further adding to the pleiotropic effect of NRG-1 in the pulmonary disease.

Conclusion

In conclusion, various studies showed the protective effect of NRG1 during HF. Interestingly, above studies demonstrated therapeutic effects of NRG1 on different comorbidities of HF, including CAD, CKD, T2DM, and pulmonary arterial hypertension. This research emphasizes pleiotropic effects of NRG1 throughout the body and adds important knowledge regarding the translational potential of rNRG.

Paracrine and autocrine NRG1/ERBB signaling in cardiac cells

The heart consists of CMs as well as non-myocytes like, ECs, fibroblasts, inflammatory cells, and VSMC. As shown in Figure 6, endogenous NRG1 promotes cardiac function by acting on both CMs and non-myocytes (30). Next, the actions of NRG1 on different cell types in the heart will be discussed.

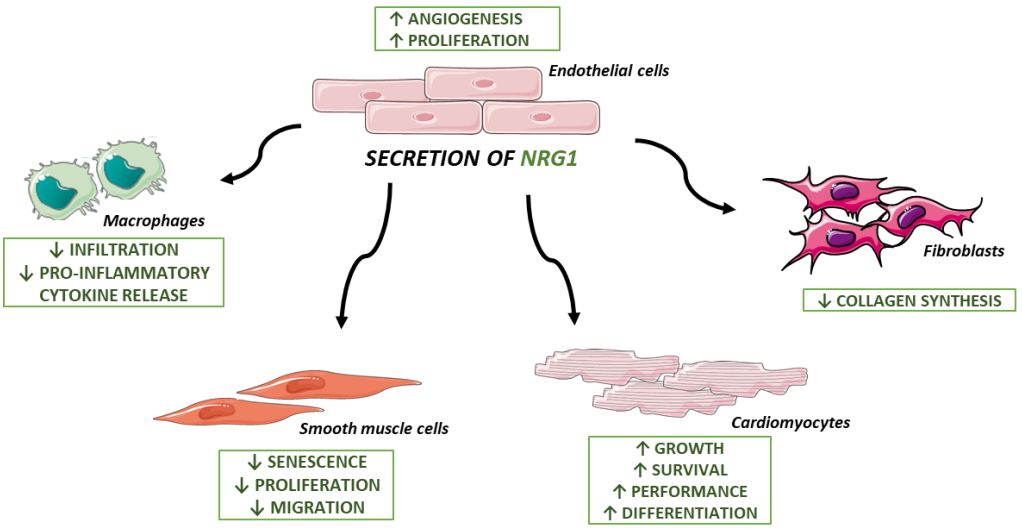


Figure 6: Effects of NRG1 on CMs and non-myocytes. *After secretion, NRG1 is able to act on both CMs and non-myocytes.*

Cardiomyocytes

Different studies showed the cardio-active function of NRG1 in CMs. Cote *et al.* showed that neonatal and adult rat ventricular myocytes in primary culture respond to rNRG1, with increased protein synthesis, fetal gene expression, survival, and inhibition of apoptotic cell death (92). Lemmens *et al.* demonstrated that the activation of the ERBB2 receptor by NRG1 is implicated in hypertrophic and anti-apoptotic responses in CMs. Furthermore, ERBB2/ERBB4 signaling participates in the paracrine survival and growth controlling effects of NRG1 on CMs (23). Also, NRG1 promotes glucose uptake in CMs, which could contribute

to the protective function in the heart (93). The important role in CM protection of EC–derived NRG1 is further demonstrated in a myocardial infarction (MI) model in endothelial-specific NRG1 KO mice. Deletion of NRG1 led to significantly decreased tolerance to ischemic insult (94).

Endothelial cells

Cardiac endothelium derived NRG1 is crucial for cardiac development and function. As mentioned before, targeted deletion of NRG1 and the ERBB receptors in mice results in endocardial cushion abnormalities, suggesting that these receptor-ligand interactions have important effects on vascular endothelial growth and development. Different ERBB receptors (ERBB2, ERBB3, ERBB4) are expressed on ECs and stimulation of ECs with NRG1 induces rapid calcium fluxes receptor tyrosine phosphorylation and cell proliferation. Furthermore, NRG1 is known to be released from ECs, more specific from endocardial endothelium and cardiac microvascular endothelium, and this growth factor is known to be indispensable for normal function of the adult heart through activation of ERBB4 on CMs (23). In addition, marked *in vitro* and *in vivo* angiogenesis was seen in response to NRG1. This effect has been reported to be both independent and dependent of VEGF (49, 50). Interleukin-6 (IL-6) and INF-γ cause rapid cleavage and release of transmembrane NRG1, followed by autocrine ERK activation and *in vitro* angiogenesis in a matrigel cord formation. This cytokine-activated MMP cleavage of NRG1 may play an important role in autocrine activation of EC signaling pathways, contributing to key biological effects (95). Hedhli *et al.* demonstrated that activation of the NRG1/ERBB pathway is crucial for vascular responses to stress: it is important for autocrine endothelial angiogenic signaling *in vitro* and endothelial production of NRG1 is required for angiogenesis and arteriogenesis induced by ischemic injury *in vivo*. Furthermore, exogenous administration of rNRG1 enhances this process, which indicates a potential role of NRG1 in vascular disease, like diabetes and

coronary artery disease. Further, endothelial-specific NRG1 deletion significantly impairs ischemia-induced angiogenesis, significant decrease in flow recovery after femoral artery ligation in the absence of endothelial NRG1 was seen using a scanning Doppler method. Moreover, impaired flow recovery after NRG deletion is accompanied by reduced small artery and capillary numbers. This indicates an important role of NRG1/ERBB signaling in regeneration of vessels (96).

Macrophages

Different studies suggest a regulating role of NRG1/ERBB signaling in macrophages. As mentioned before, Schumacher *et al*. showed that proinflammatory activation of cultured murine and human macrophages induced ERBB4 expression. Subsequent treatment of lipopolysaccharide (LPS)-activated proinflammatory macrophages with NRG4 induced apoptosis, which serves as an efficient clearance mechanism of proinflammatory macrophages from the tissue and is critical to prevent prolonged inflammation. This group confirmed anti-inflammatory effects of NRG4 in a mice model of colitis, administration of exogenous NRG4 during colitis reduced colonic macrophage number and ameliorated inflammation (52). This study adds important knowledge to our research, as NRG4 activates to the same ERBB4 receptor as NRG1. In addition, Ryzhov *et al*. studied the GGF2 activation of ERBB signaling *ex vivo*, which inhibited LPS-induced tumor necrosis factor-α (TNF-α) production in peripheral blood mononuclear cells. *In vivo*, the authors showed that a single dose of i.v. GGF2 reduced TNF-α expression in peripheral blood mononuclear cells of CHF patients, which was correlated with the expression of ERBB3 (97). In our laboratory, Vermeulen *et al.* showed that the anti-fibrotic effect of NRG1 in the heart is linked to anti-inflammatory activity of NRG1/ERBB4 signaling in macrophages. NRG1 attenuated myocardial macrophage infiltration and cytokine expression after 1 week Ang II exposure. In addition, NRG1 treatment

attenuated myocardial hypertrophy and fibrosis and improved passive ventricular stiffness after 4 weeks Ang II treatment and mice with myeloid-specific *Erbb4* deletion showed an increased myocardial fibrotic response to Ang II, relative to control mice. It was shown that the antifibrotic effect of NRG1 is at least partially mediated through inhibitory actions on macrophages, and is linked to ERBB4 receptor in macrophages. These antifibrotic effects were not only seen in the heart, but are also present in skin and lung (28).

Fibroblasts

Bersell *et al.* suggest involvement of fibroblasts in the cardioprotective actions of NRG1, as NRG1 administration following injury decreases scar size (43). In addition, Galindo *et al.* showed that GGF2, a secreted NRG which binds the ERBB receptors, attenuated myocardial fibrosis, altered extracellular matrix (ECM) structure, and reduced the number of myofibroblasts in a swine model of MI. This suggests a direct inhibitory effects of GGF2 on cardiac fibroblasts (98). At the laboratory of Physiology, microarray analysis of primary mouse fibroblasts showed a high responsiveness to NRG1 (28, 61).

Vascular smooth muscle cells

Clement *et al*. examined the role of NRG1 on VSMC function. NRG1 significantly decreased platelet-derived growth factor-stimulated VSMC proliferation and migration, suggesting attenuating effects of NRG1 on cellular growth. This makes NRG1 a potential therapeutic candidate for the treatment of restenosis and atherosclerosis (99). In addition, Shakeri *et al.* demonstrated modulatory functions of NRG1 on VSMC. Impaired NRG1/ERBB4 signaling by deletion of *Erbb4* on VSMC aggravates cellular senescence both *in vitro* and *in vivo* (100).

Conclusion

The heart is a pluricellular organ, in which cardiac remodeling comprises cellular changes in different cell types in the heart—ECs, CMs, fibroblasts, inflammatory cells, and VSMC. NRG1/ERBB signaling is critical during cardiac remodeling (61), it allows the heart to cope with physiological stress (24). It has been shown in both preclinical and clinical studies that administration of NRG1 attenuates cardiac remodeling and improves cardiac function in different models of HF (21, 22, 56, 58, 62). The generally accepted concept is that the cardiac effects of NRG1 are mediated by paracrine activation of ERBB4/ERBB2 receptors on CMs (17, 23). However, above studies suggest that NRG1 modulates macrophages, which contributes to its anti-inflammatory and anti-fibrotic actions. This indicates that paracrine NRG1/ERBB4 signaling in cardiac non-myocytes is equally important. At our laboratory, Vermeulen et al. demonstrated that NRG1 has direct effects on macrophages and decreases inflammation and fibrosis in a model of Angiotensin II (Ang II)–induced myocardial fibrosis (28). Here, we aimed at further investigating the regulatory role of the NRG1/ERBB4 system in myeloid cells on myocardial macrophage density and cardiac remodeling by studying the ***effects of myeloid-specific Erbb4 deletion*** *in two different models of cardiac remodeling. The role of ERBB receptors in ECs during cardiac remodeling, on the other hand, is less understood. NRG1/ERBB signaling in ECs itself point out an important autocrine communication loop with angiogenic effects. However, the effects of the endothelial NRG1/ERBB loop involved in cardiac remodeling remains unclear, therefore also* ***endothelial NRG1/ERBB4 signaling*** *is further investigated in this thesis. This research should further establish the non-myocyte mechanisms of NRG1/ERBB4 signaling in HF, and the ERBB4 receptor as a key therapeutic target in HF.*

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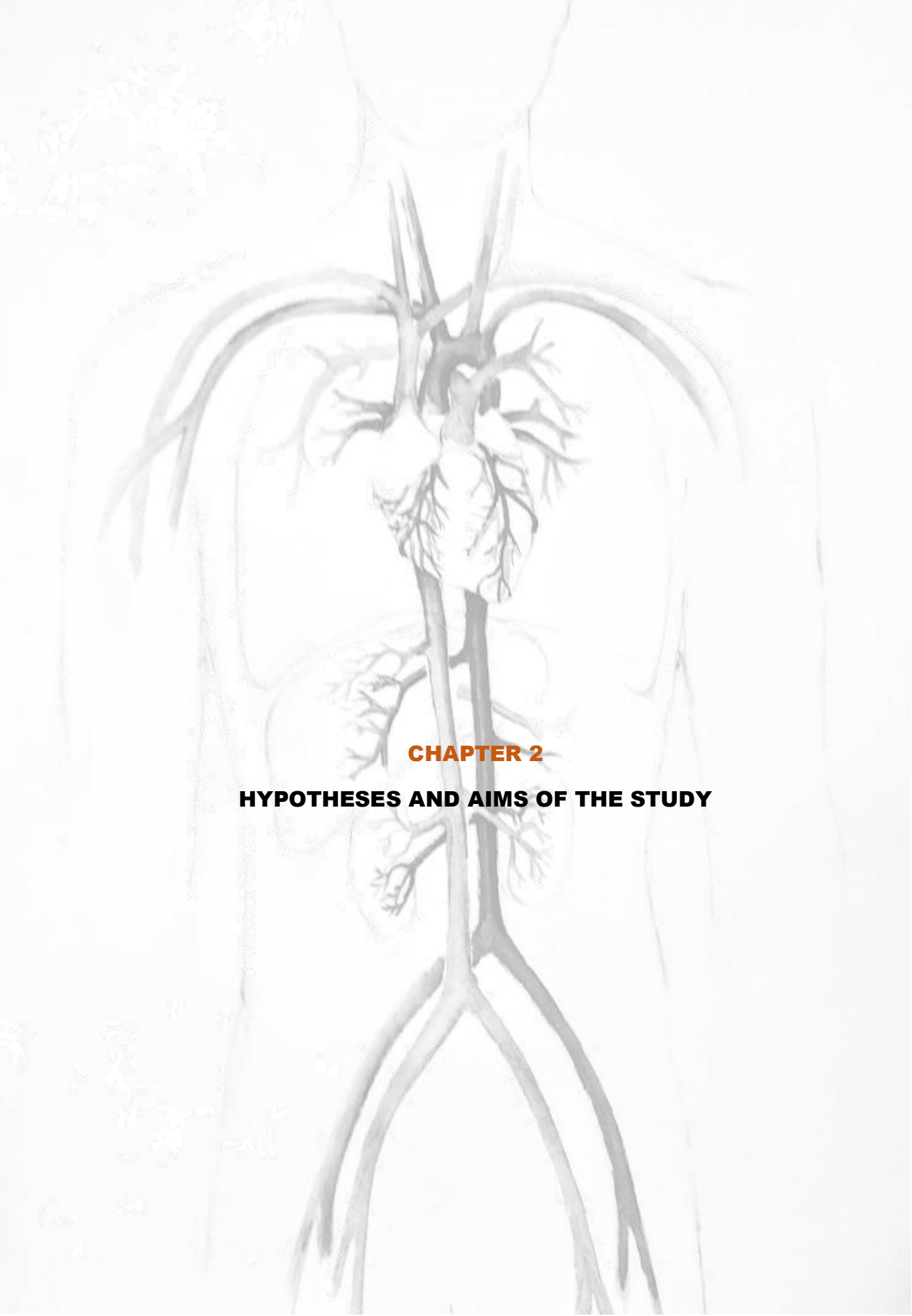


CHAPTER 2

HYPOTHESES AND AIMS OF THE STUDY

Our laboratory has been studying the NRG1/ERBB pathway in the pathophysiology of HF for almost 2 decades. During exploration of ERBB tyrosine kinase signaling in the heart, the central role of ERBB2 signaling in CMs has become evident. Paracrine activation of ERBB4/ERBB2 receptors on CMs contributes to the cardioprotective effects of rNRG1, but it is unclear to what extent this scenario explains the full picture. ERBB2, ERBB3 and ERBB4 receptors are also expressed by non-myocyte cardiac cells, including inflammatory cells, fibroblasts and ECs. In addition, a recent study at our laboratory by Vermeulen *et al*. showed that myeloid-specific deletion of *Erbb4* intensifies myocardial fibrotic responses to Ang II. This indicates that paracrine NRG1/ERBB4 signaling in cardiac non-myocytes is equally important. Here, we aimed at further investigating the regulatory role of the NRG1/ERBB4 system in both ECs and inflammatory cells during cardiac remodeling in different models of cardiac injury/overload. **The central hypothesis of this thesis is that NRG1/ERBB4 receptor signaling in non-myocytes contributes to the beneficial effects of NRG1 during cardiac remodeling** (Figure 7). This central hypothesis can be subdivided in two separate hypotheses, which will be further elaborated using different strategies:

We hypothesize that <u>ERBB4-dependent paracrine NRG1 signaling in myeloid cells</u> plays a beneficial role during cardiac remodeling. Here, we will expand on previous observations by Vermeulen *et al.* and test the hypothesis in two different models of cardiac remodeling – TAC, and MI – in both *Erbb4*$^{F/F}$ *LysM-Cre*$^{+/-}$ mice and control littermates. (i) TAC or sham surgery was performed, and mice were euthanized after 3 days to 20 weeks (n=35-45 per time point), (ii) MI was induced by LAD ligation, with a follow-up of 5 days (n=35) or 4 weeks (n=38) to study early and late cardiac responses induced by MI. The natural history of disease in the transgenic model was compared to the control littermates. Mice were phenotyped by echocardiography and

immunohistochemistry for CM cross-sectional area (CSA), myocardial macrophage density, and fibrosis.

We hypothesize that <u>ERBB4-dependent autocrine NRG1 signaling in ECs</u> plays a beneficial role during cardiac remodeling. First, we confirmed the responsiveness of ECs to NRG1 *in vitro.* Changes in endothelial signaling pathways and transcriptome were analyzed using RNA-sequencing, and *in vitro* angiogenic effects of NRG1 were studied. Additionally, we generated an EC–specific *Erbb4* KO mouse to specifically eliminate endothelial autocrine ERBB4 signaling without affecting paracrine NRG1/ERBB4 signaling in other cardiac cell types. We tested whether this specific conditional deletion of the endothelial ERBB4 receptor, and thus impaired autocrine ERBB4 signaling, changes cardiac remodeling in three different models of cardiac overload—TAC–induced pressure overload, high dose Ang II–induced hypertension, and MI—in both *Erbb4*$^{F/F}$ *VE-Cdh5-Cre*$^{+}$ mice and control littermates. (i) TAC or sham surgery was performed, and mice were euthanized after 8 (n=45) or 20 weeks (n=45), (ii) Ang II was given for 4 weeks using micro-osmotic pumps (1000 ng.kg-1.min-1; n=40), (iii) MI was induced by LAD ligation, with a follow-up of 4 weeks (n=40). Again, the natural history of disease in the transgenic model was compared to the control littermates. Mice were phenotyped by echocardiography and immunohistochemistry for CM CSA, fibrosis and capillary density.

These hypotheses have a major impact on the translational significance of ERBB pathways in CHF. Moreover, CHF is a complex disease because of the complex interaction between different cell types *in vivo* and the sequential phases of tissue remodeling. Accordingly, *in vivo* transgenic approaches are the most precise way to dissect the role of a particular receptor in a particular cell type during cardiac remodeling in CHF.

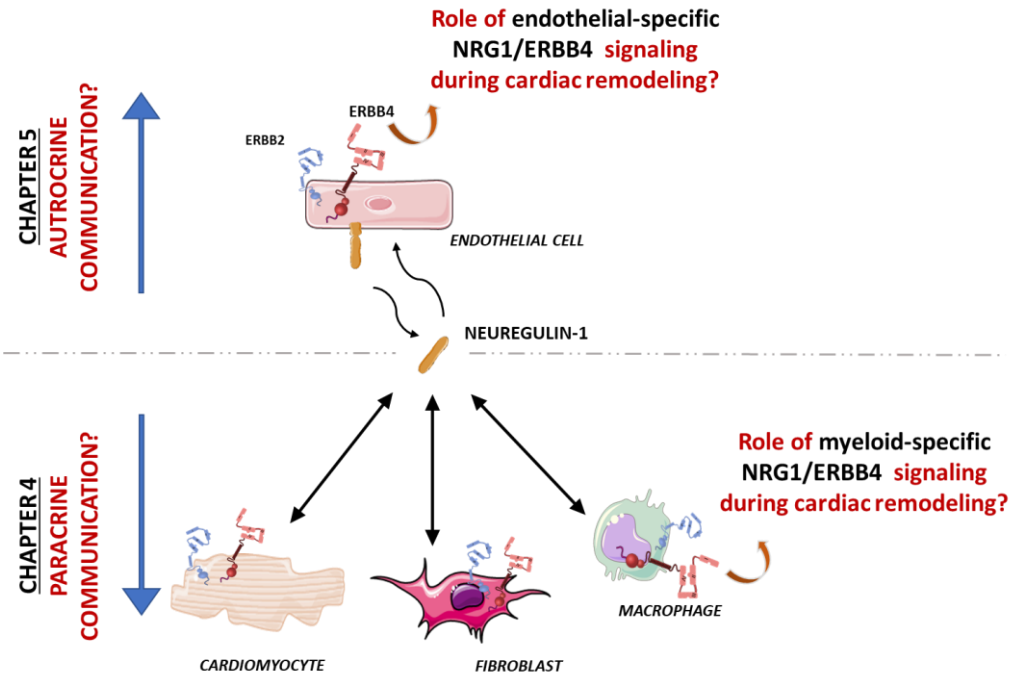
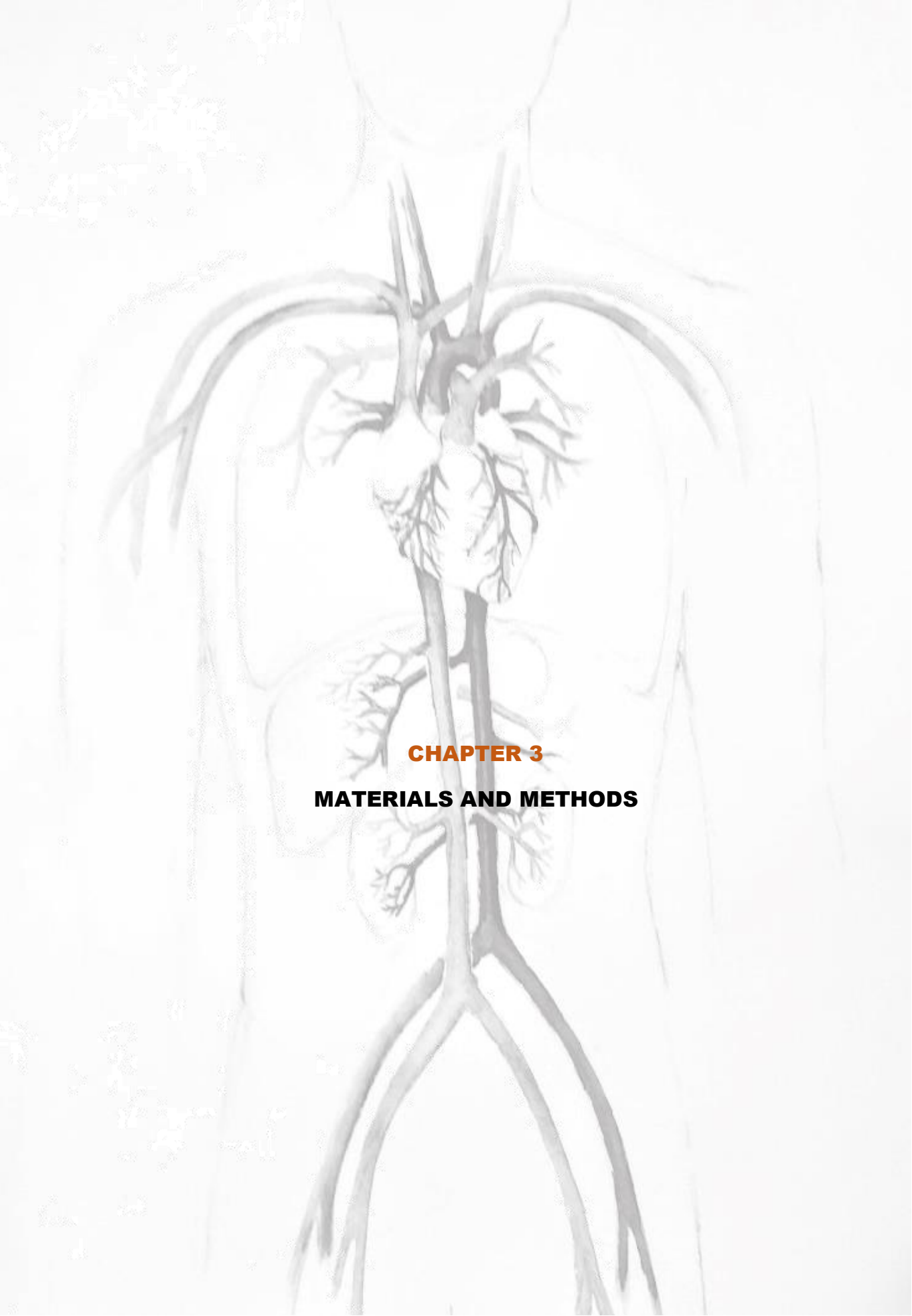


Figure 7: Central hypothesis: NRG1/ERBB4 receptor signaling in non-myocytes contributes to the beneficial effects of NRG1 during cardiac remodeling. *NRG1 is a paracrine cardioprotective growth factor secreted by endothelial cells (ECs) in conditions of cardiac overload and injury. The generally accepted concept is that the cardiac effects of NRG1 are mediated by paracrine activation of ERBB4/ERBB2 receptors on CMs, but it is unclear to what extent this scenario explains the full picture. Therefore, both autocrine and paracrine NRG1/ERBB4 signaling in non-myocytes will be studied in this thesis.*



CHAPTER 3

MATERIALS AND METHODS

Animal models

All experiments were approved by the institutional ethics committee and conform to the Guide for the care and Use of Laboratory Animals, 8th edition published by the US National Institutes of Health in 2011. Mice were housed in the central animal care facility of the University of Antwerp and were given food and water *ad libitum*. All used animals were randomly assigned in treatment groups. EC–specific deletion and myeloid-specific deletion of *Erbb4* in mice was examined in different models of cardiac overload and remodeling: TAC–induced pressure overload, Ang II–induced myocardial fibrosis, and LAD ligation–induced MI. All animals were randomly assigned to treatment groups.

Transgenic mice

Endothelial-specific Erbb4 gene deletion

Mice with endothelial-specific *Erbb4* gene deletion were generated by crossbreeding tamoxifen-inducible vascular endothelium cadherin5 Cre-recombinase positive (*VE-Cdh5-Cre*$^{+}$) mice (*Cdh5Tg(Cdh5cre/ERT2)CIVE23Mlia; UCLA, Dr. Iruela-Arispe*) with *C57BL/6-Erbb4*$^{F/F}$ mice, carrying LoxP-flanked *Erbb4* alleles (*B6;129-Erbb4tm1Fej/Mmucd, #010439-UCD,MMRRC; Lox: Locus of recombination*) (Figure 8). *Cdh5Tg(Cdh5cre/ERT2)CIVE23Mlia* is an inducible Cre-recombinase line. Inducible control of Cre-activity has been achieved by fusing sequences from the Cre-gene with sequences encoding for a mutant form of the estrogen receptor ligand-binding domain (ERT2). ERT2 functions as a specific receptor for tamoxifen and is unresponsive to natural estrogens or other physiological steroids. Cre-ERT2 expression is directed to ECs by a VE-Cdh5 promoter. Previously, validation of efficiency and specificity of *VE-Cdh5-Cre* activity has been extensively studied (1-3). Here, Cre-antibody staining did not

show any positivity in CM (data not shown). When crossed with *C57BL/6-Erbb4$^{F/F}$* mice and treated with tamoxifen (2 mg.day^{-1}, for 5 consecutive days) at the age of 6 weeks, Cre-mediated recombination will result in deletion of *Erbb4* in the endothelium (*Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice*). *Erbb4$^{F/F}$ VE-Cdh5-Cre^{+}* mice treated with vehicle will serve as controls (1).

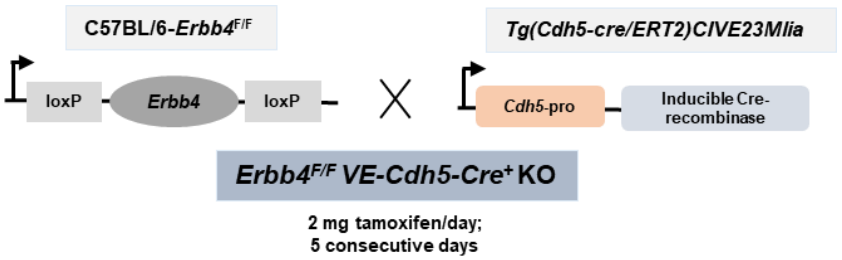


Figure 8: Endothelial-specific Erbb4 gene deletion. *The use of Cre/loxP technique generating tamoxifen-inducible Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice (EC-KO).*

Myeloid-specific Erbb4 gene deletion

Mice with myeloid-specific *Erbb4* gene deletion were generated by crossbreeding myeloid-specific LysM-Cre^{+} mice (*B6.129P2-Lyz2tm1(cre)Ifo/J; Jackson Laboratories*) with *C57BL/6 Erbb4$^{F/F}$* mice, carrying LoxP-flanked *Erbb4* alleles (*B6;129-Erbb4tm1Fej/Mmucd, #010439-UCD,MMRRC*) (Figure 9). The LysM-Cre knock-in allele has a nuclear-localized Cre-recombinase inserted into the first coding ATG of the lysozyme 2 gene (*Lyz2*), placing Cre-expression under the control of the endogenous *Lyz2* promoter. When crossed with *C57BL/6-Erbb4$^{F/F}$* mice, Cre-mediated recombination results in deletion of *Erbb4* (*Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice*) in the myeloid cell lineage, including monocytes, mature macrophages, and granulocytes (neutrophils, eosinophils, basophils). *Erbb4$^{+/+}$ LysM-Cre$^{+/-}$* mice will serve as controls (4).

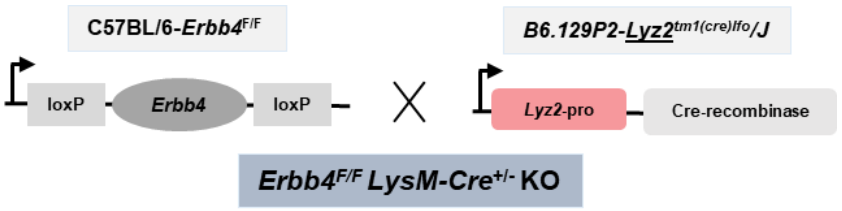


Figure 9: Myeloid-specific Erbb4 gene deletion. *The use of Cre/loxP technique generating Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice (Mye-KO).*

In vivo models of cardiac remodeling

Transverse aortic constriction – Myocardial infarction Recommendations for future PhDs

I'm proud to say that I was the first to implement a TAC and MI model in mice at our laboratory and went from a surgical mortality rate of 100% to only 5%.

What were the most commonly encountered difficulties for both models and what would I recommend to future PhDs? Regarding the **TAC model**, I would recommend using a blunted 27-gauge needle and, most importantly, performing TAC by a single researcher to obtain a consistent constriction. On the other hand, to avoid severe bleedings one should cut straight down the center of the sternum, and carefully close it using an interrupted suture pattern. Regarding the **MI model**, I would recommend to use blunt forceps at the 3rd intercostal space between 3rd and 4th ribs. Touching the internal thoracic artery should be avoided as there is danger of bleeding. In addition, it is important to carefully remove the pericardium and pull it apart without harming the heart and lungs, as this could severely hamper the post-surgical recovery.

Finally, the most important recommendation **do not give up** after the first few weeks of struggling, **keep calm,** and **practice every day**.

Transverse aortic constriction: pressure overload-induced HF

TAC was performed in 12-week old mice with endothelial-specific and myeloid-specific deletion of *Erbb4* (n = 8–15 per group; *Erbb4*$^{F/F}$ *VE-Cdh5-Cre*$^{+}$ mice with or without tamoxifen treatment, *Erbb4*$^{F/F}$ *LysM-Cre*$^{+/-}$ and *ErbB4*$^{+/+}$ *LysM-Cre*$^{+/-}$ mice), with a surgical mortality rate of 5% (Figure 10). Specifically, adult mice were anesthetized with sevoflurane (3% vol/vol; Sevorane®, Abbott Laboratories, Wavre) (5) and ventilated (150–200 strokes/min; 250 µL tidal volume). A partial thoracotomy from the sternum to the second rib was performed. Subsequently, two knots (6.0 silk suture; Fine Science Tools, Germany) were tied around the transverse aorta and a blunt 27-gauge needle. Sham-operated animals underwent the same procedure, except for the constriction (6). Post-operative analgesia was administered (buprenorphine; 0.1 mg.kg^{-1}; intraperitoneal (i.p.)). 3 days to 20 weeks after TAC, mice were euthanized using an overdose of pentobarbital (>150 mg.kg^{-1}, i.p.; Nembutal® CEVA Santé Animale) and hearts were collected (Figure 11).

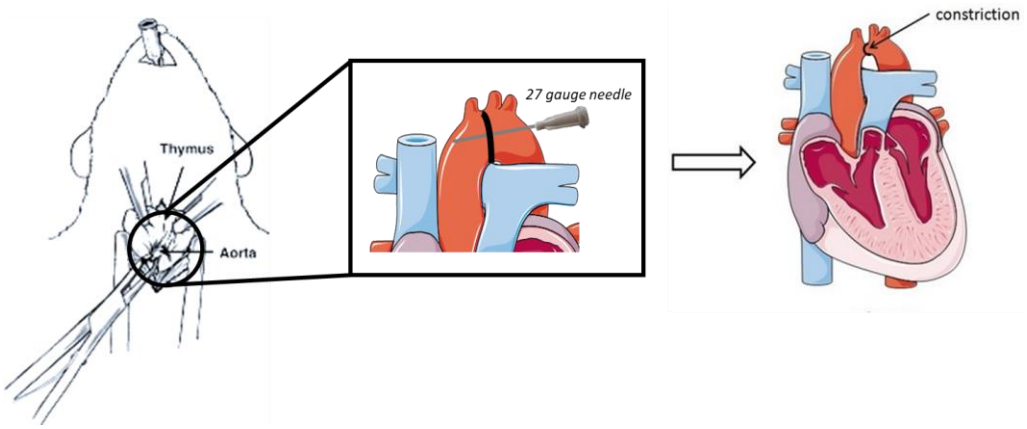


Figure 10: Transverse aortic constriction (TAC): pressure overload-induced HF. Schematic overview of TAC. *TAC was performed in 12-week old mice by placing a suture around the transverse aorta and a blunt 27-gauge needle.*

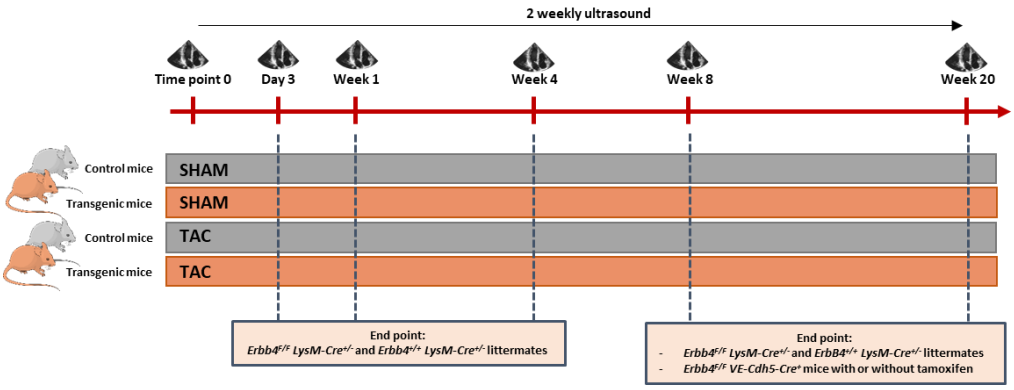


Figure 11: Overview of time course of the TAC—a model of pressure overload–induced cardiac remodeling. *At time point 0, we performed TAC in both control mice (vehicle-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice; Erbb4$^{+/+}$ LysM-Cre$^{+/-}$ mice) and KO mice (tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice; Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice) at the age of 12 weeks. Sham-operated animals underwent the same procedure except for the constriction. Ultrasound measurements were performed at baseline, end point, and every 2 weeks during the experiment. 3 days to 20 weeks after TAC, mice were euthanized and hearts were collected.*

LAD ligation–induced MI

MI was induced in 12-week old mice with endothelial-specific and myeloid-specific deletion of *Erbb4* (n = 8–11 per group; *Erbb4$^{F/F}$ VE-Cdh5-Cre^{+}* mice with or without tamoxifen treatment, *Erbb4$^{F/F}$ LysM-Cre$^{+/-}$* and *Erbb4$^{+/+}$ LysM-Cre$^{+/-}$* mice), with a surgical mortality rate of 5% (Figure 12). Adult mice were anesthetized with sevoflurane (3% vol/vol) and ventilated (150–200 strokes/min; 250 µL tidal volume). A partial thoracotomy between the fourth and fifth rib was performed (5). The LAD, located 1 mm below the left auricle, was ligated (8-0 Prolene suture; Ethicon, Germany). Sham-operated animals underwent the same procedure, except no ligation was performed. Post-operative analgesia was administered as above. 5 days and 4 weeks after MI, mice were euthanized and hearts were collected (Figure 13).

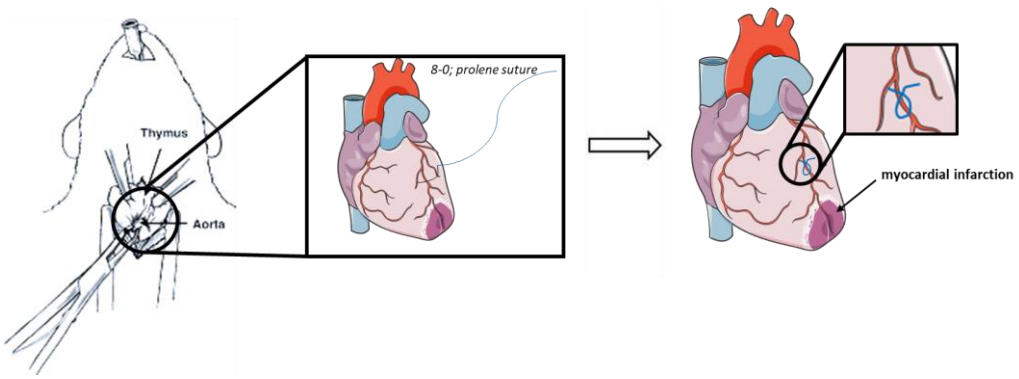


Figure 12: Left anterior descending artery (LAD) ligation–induced myocardial infarction (MI): Schematic overview of MI induction. *In 12-week old mice the LAD was ligated 1 mm below the left auricle to induce a MI with a 8-0 Prolene suture.*

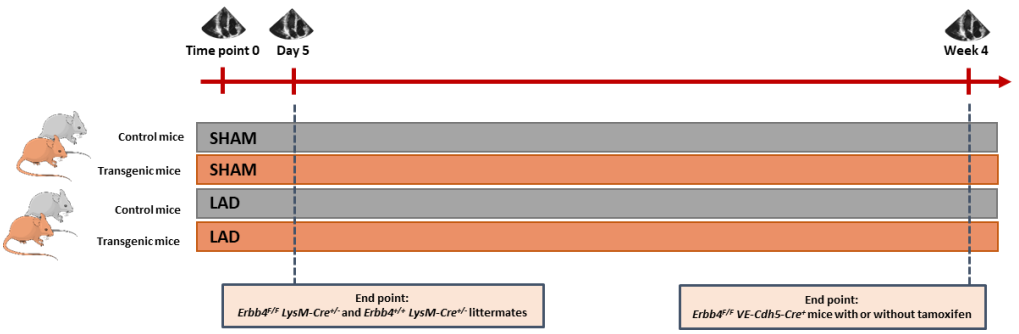


Figure 13: Overview of time course of the LAD–induced MI model. *At time point 0, we performed LAD ligation in both control mice (vehicle-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice; Erbb4$^{+/+}$ LysM-Cre$^{+/-}$ mice) and KO mice (tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice; Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice) at the age of 12 weeks. Sham-operated animals underwent the same procedure except for the ligation. Ultrasound measurements were performed at baseline and end point. 5 days to 4 weeks after MI, mice were euthanized and hearts were collected.*

Ang-II induced myocardial fibrosis

Myocardial fibrosis was induced in 12-week old mice with endothelial-specific deletion of *Erbb4* (n = 9–10 per group; *Erbb4$^{F/F}$ VE-Cdh5-Cre^{+}* mice with or without tamoxifen treatment) by administration of Ang II (1000 ng.kg^{-1}.min^{-1}, Sigma Aldrich) using a micro-osmotic pump (model 1004, Alzet). Mice were anesthetized with sevoflurane (3% vol/vol) and micro-osmotic pumps, filled with Ang II or vehicle, phosphate buffered saline (PBS), were s.c. implanted on the

dorsum, slightly caudal to the scapulae. Post-operative analgesia was administered as above (5, 7). After 4 weeks of Ang II treatment, mice were euthanized and hearts were collected. (Figure 14).

Figure 14: Overview of time course of the Ang II–induced myocardial fibrosis model. *At time point 0, myocardial fibrosis was induced in both control mice (vehicle-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice) and KO mice (tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice) by administration of Ang II using micro-osmotic pumps (1000 ng.kg^{-1}.min^{-1}). Ultrasound measurements were performed at baseline and end point. After 4 weeks of Ang II treatment, mice were euthanized and hearts were collected.*

Echocardiographic evaluation

Transthoracic echocardiography was performed on anesthetized mice (1.5–2.0% vol/vol isoflurane (Forene®, Abbvie)) using a Vevo 2100 Imaging System (VisualSonics) equipped with a MS550D probe. A 2D short-axis view of the mid LV was obtained at chordal level using the B-mode (8). In the TAC experiments, ultrasound measurements were performed every 2 weeks. LV internal diameters (LVID;d, LVID;s), interventricular septal wall thickness (IVS;d, IVS;s), and LV posterior wall thickness (LVPW;d, LVPW;s) were measured in diastole (d) and systole (s), respectively, by a single observer in a blinded fashion on three consecutive M-mode cycles. EF% and FS% were calculated.

Histological analyses

Cardiac tissue was fixed in 4% buffered formalin, dehydrated in 60% isopropanol and embedded in paraffin, cut in sections of 5 µm thickness and dried overnight at 37°C. Subsequently slides were deparaffinized, rehydrated, treated with 3% H_2O_2 to quench peroxidase activity, and blocked with bovine serum albumin (BSA). Heart sections were stained with Masson's trichrome (% total cardiac fibrosis and % perivascular fibrosis) and laminin (CM CSA; Novus Biologicals) according to the manufacturer's instructions or with antibodies specific for MAC3 (macrophage infiltration; PharMingen), biotinylated isolectin B4 (IB4, capillary density/mm^2; Vector Laboratories), or an anti-Cre recombinase antibody (ab190177). Histological images were captured using light microscopy and additional software (Olympus SC50, Olympus Stream Motion Software), and manually analyzed in a blinded fashion (investigator is blinded to the intervention protocol) using ImageJ 1.52i software. Cardiac interstitial fibrosis and perivascular fibrosis were expressed as the ratio of positively stained fibrotic area (blue staining) to the total cardiac area or vascular lumen area, respectively. CM CSA was quantified by averaging the CSA of 20 representative CMs per section per animal (5 sections per animal; at a 20x magnification). Myocardial macrophage content was expressed as percentage macrophage positive area to the total cardiac area. Capillary density/mm^2 was calculated by counting all capillaries present in 5 fields per sample at a 20x magnification.

Vascular function measurements on isolated aortic segments

Thoracic aortae from both control mice and *Erbb4*$^{F/F}$ *VE-Cdh5-Cre*$^{+}$ mice treated with tamoxifen were isolated and cut into 6 segments. Segments 2 to 5 were used in an isometric setting and contractile properties were studied as described by Van Hove *et al*. (9). Briefly, aortic segments were mounted between two parallel tungsten wire hooks in 10 mL organ baths. Isometric force was

measured with a Statham UC2 force transducer (Gould, Cleveland, OH, USA) connected to a data acquisition system (Powerlab 8/30, ADInstruments, Spechbach, Germany), and was reported in mN. Segments were gradually stretched until a stable loading tension of 16 mN was attained. This is the optimal pre-load to attain maximal force development by phenylephrine (PE; 10^{-9} to 10^{-5} M). Endogenous NO release was induced by the cumulative administration of acetylcholine (Ach; 10^{-9} to 10^{-5} M). Relaxation experiments with exogenous NO from Diethylamine NONOate sodium salt hydrate (DEANO; 10^{-9} to 10^{-5} M) were performed in segments in which NO formation was inhibited by 300 µM N^{Ω}-nitro-L-arginine methyl ester (L-NAME) (9).

Cell culture

All cell culture experiments were performed in the laminar flow hood, wearing nitrile lab gloves. All items brought into the hood were sprayed with 70% ethanol before each entry. Fresh, sterile media were warmed to 37°C in a water bath prior to plating the cells. All cells were cultured in a humidified 5% CO_2 incubator at 37°C.

Human umbilical vein ECs

Human umbilical vein ECs (HUVECs; Lonza) were cultured in EC basal medium-2 supplemented with 0.5 mL human endothelial growth factor (hEGF), 0.2 mL hydrocortisone, 0.5 mL gentamicin sulfate/ amphotericin (GA)-1000, 10 mL fetal bovine serum (FBS), 0.5 mL VEGF, 2.0 mL human FGF (hFGF)-B, 0.5 mL insulin-like growth factor-1 (IGF1), 0.5 mL ascorbic acid, 0.5 mL heparin (EGM-2 bulletkit; cc-3162) at 37°C in a humidified 5% CO_2 incubator on collagen (Collagen type I, rat tail, Gibco Life Technologies) coated culture flasks. Cells were used in passage 2 to 10 and 24 h prior to experiments cells were serum-starved (0.1% FBS).

Based on previous observations, expression of the ERBB4 receptor, phosphorylation of AKT, ERK, STAT3 in response to NRG1 stimulation over 1 h and phosphorylation of endothelial NOS (eNOS) in response to NRG1 stimulation over 8 h were determined by Western blotting (WB) ((8, 10)). In addition, HUVECs were grown in collagen-coated 6-well plates and were exposed to a wound healing scratch assay (11). After scratching, cells were placed in the incubator at 37°C for 18 h with NRG1 (50 $ng.mL^{-1}$) or PBS. After incubation, cells were placed under a phase-contrast microscope (Celena™ S Microscope) and images were captured. Scratch area was measured using Image J software and expressed as % closure after 18 h.

Isolated murine cardiac ECs from transgenic mice

Cardiac ECs were isolated from hearts of *Erbb4*$^{F/F}$ *VE-Cdh5-Cre*$^{+}$ mice treated with tamoxifen or vehicle using Cardiac EC Isolation kit according to manufacturer's instructions (Macs Miltenyi Biotec). Briefly, cardiac ECs were labeled with Cardiac EC Isolation Cocktail and magnetic selection over a MACS LS column was performed. Magnetically retained cardiac ECs were eluted as the positively selected cell fraction. Separated cells were used immediately or cultured in ECs basal medium-2 supplemented with 0.5 mL hEGF, 0.2 mL hydrocortisone, 0.5 mL GA-1000, 10 mL FBS, 0.5 mL VEGF, 2.0 mL hFGF-B, 0.5 mL IGF1, 0.5 mL ascorbic acid, 0.5 mL heparin (EGM-2 bulletkit; cc-3162) at 37°C in a humidified 5% CO_2 incubator on collagen coated culture flasks. Purity of ECs was examined by flow cytometry (BD Accuri C6 flow cytometer) using an CD31-APC antibody (murine, 30 $\mu g.mL^{-1}$; Macs Miltenyi Biotec) and expression of the ERBB4 receptor on these isolated ECs was determined by WB.

Primary human cardiac microvascular ECs

Primary human cardiac microvascular ECs (HMVECs; Lonza) were cultured in EC basal medium-2 supplemented with 0.5 mL hEGF, 0.2 mL hydrocortisone, 0.5 mL GA-1000, 25 mL FBS, 0.5 mL VEGF, 2.0 mM hFGF-B, 0.5 mL IGF1, 0.5 mL ascorbic acid (EGM-2 MV bulletkit; cc-3202) at 37°C in a humidified 5% CO_2 incubator on collagen coated culture flasks. HMVECs were used in passage 6 and were serum-starved 24 h prior to experiments. HMVECs were stimulated with NRG1 (50 ng.mL^{-1}) or PBS for 8 or 24 h. After collection of the cells, gene expression was examined by RNA-sequencing.

Aortic Ring Assay

The aorta from *Erbb4$^{F/F}$ VE-Cdh5-Cre^{+}* mice with or without tamoxifen treatment were isolated and prepared for experiment (12). Fat tissue and branching vessels were removed with sterile scissors and forceps. An adult mouse aorta provides up to 20 rings and rings from three separate aortae per condition (with or without tamoxifen) were used. Rings of 0.5 mm in width were incubated with Opti-MEM medium (Gibco, Life Technologies) supplemented with NRG1 (50 ng.mL^{-1}) or PBS. Growth medium was changed on day 3 and 5. After 5 days incubation, rings were placed under a phase-contrast microscope (Celena^{TM} S Microscope), images were captured, and microvessel growth was quantified. Each microvessel emerging from the main ring and each individual branch arising from separate vessels was counted. Data was plotted as mean microvessel numbers per ring.

Western blot analysis

Cell cultures and isolated cells were collected in lysis buffer (950 µL Laemmli buffer; BIO-RAD + 50 µL β-mercaptoethanol; Sigma Aldrich) suitable for WB.

Cell lysates were boiled at 95 °C for 4 min. Subsequently, denatured samples were loaded and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE; 200V, 100 mA, 45–55 min) was used to separate proteins on a NuPAGE™ 4–12% Bis-Tris Gel (Invitrogen). Following, proteins were transferred onto a polyvinylidene fluoride membrane (PVDF), using electroblotting (30V, 170 mA, 1 h). After the transfer, the membrane was blocked for 1 h with 1:5 dilution of Li-Cor blocking buffer (Li-Cor) in PBS at room temperature. After 1 h, the primary antibody was added, using a 1:5 dilution of Li-Cor blocking buffer supplemented with 0.1% Tween (BIO-RAD), and incubated over night at 4°C on a shaker. After washing in PBS-0.1% Tween, the membrane was incubated for 1 h at room temperature with corresponding secondary IRDye-conjugated secondary antibody in 1:5 dilution of Li-Cor blocking buffer supplemented with 1% SDS. The membrane was placed directly on the glass plate of the Odessey imaging system. The 700 nm channel will display the anti-mouse labeled antibody, the 800 nm channel will display the anti-rabbit labeled antibody. WB was analyzed using with the Odyssey Software (LI-COR Biosciences).

Following antibodies were used: ERBB2 (2165, Cell signaling), ERBB3 (SC285, Santa Cruz), ERBB4 (sc283; Santa Cruz), phospho-ERBB2 (sc-81507, Santa Cruz), phospho-ERBB3 (4791, Cell signaling), phospho-ERBB4 (4757, Cell signaling), phospho-STAT3 (P-STAT3; 9131, Cell signaling), STAT3 (9132, Cell signaling), phospho-ERK1/2 (P-ERK1/2; 9101, Cell signaling), ERK1/2 (9102, Cell signaling), phospho-AKT (P-AKT; 4058, Cell signaling), AKT (9272, Cell signaling), eNOS (NOS3; PS1177, BD transductions), inducible NOS (iNOS, 13120, Cell signaling), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 2118 (rabbit) – 97166 (mouse); Cell signaling). Primary antibodies were diluted at 1:500 to 1:1000 according to the manufacturer's data sheets. For detection IRDye-conjugated secondary antibodies were used: IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (926-32211, Li-Cor), IRDye® 680RD Goat anti-

Mouse IgG Secondary Antibody (926-68070, Li-Cor). Secondary antibodies were diluted at 1:20000.

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

mRNA isolation

Prior to RT-qPRC, total mRNA was isolated from tissue or cells by using the Nucleospin RNA® kit (Macherey Nagel). Frozen tissues were homogenized in lysis buffer containing β-mercaptoethanol, to release mRNA and inactivate RNases, by using a precellys24 homogenizer and ceramic mix beads (Bertin Instruments). Total mRNA was extracted from homogenized tissue or cell lysates according to the manufacturer's instructions. Briefly, isopropyl alcohol was added to precipitate mRNA. Next, samples were applied to high-capacity silica column to bind total mRNA. DNA depletion from the RNA sample was performed using DNase treatment. Purified mRNA was eluted in RNase-free water and ready to use in polymerase chain reaction (PCR) applications. Samples were stored at -20°C until further use.

Prior to RNA-sequencing, total mRNA was isolated from primary HMVECs by using the mirVana isolation kit (Ambion® by Life Technologies). Cultured cells were homogenized in lysis/binding solution, to release mRNA and inactivate RNases. Following, total RNA was purified according to the manufacturer's protocol. Total RNA was eluted in nuclease-free water and ready to use for RNA sequencing. Samples were stored at -80°C until further use.

Polymerase chain reaction

mRNA samples were transcribed to cDNA via PCR according to the manufacturer's protocol (TaqMan Reverse Transcription Reagents, Applied

Biosystems, Thermo Fisher Scientific). Briefly, purified total RNA was added to a solution containing oligo $(dT)_{16}$ and reverse transcriptase enzymes. Parameters for PCR were: 10 min at 25°C, 30 min at 48°C, 5 min at 95°C. After PCR, cDNA samples were stored at -20°C until further use.

RT-qPCR

RT-qPCR was performed on cDNA. TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) and TaqMan primers were used according to manufacturer's instructions. Samples were initially incubated for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C (denaturation) and 1 min at 60°C (hybridization and elongation). RT-qPCR was executed with the 7300 Real-Time PCR system and software (Applied Biosystems, Thermo Fisher Scientific). Following TaqMan primers were used for mouse samples (Invitrogen): *Il-6* (Mm00446190_m1), *Il-1β* (Mm00434228_m1), *Tnf-α* (Mm00443258_m1), fibronectin-1 (Mm01256744_m1), Collagen 1a1 (*Col1a1*, Mm00801666_g1), collagen 3a1 (*Col3a1*, Mm01254476_m1), fibroblast-specific protein 1 (*Fsp1* or *s100a4*, Mm00803372_g1), transforming growth factor-β (*Tgf-β*, Mm01178820_m1), Vegf (*Vegfa*, Mm00437306_m1), *Fgf2* (Mm01285715_m1), placental growth factor (*Plgf*, Mm00435613_m1), angiopoietin1 (*Angpt1*, Mm00456503_m1). mRNA expression of the gene of interest was normalized to the expression of β-actin (Mouse; Mm00607939_s1). Relative expression of mRNA concentrations was calculated using the cycle threshold (CT) value (normalized to housekeeping gene; ΔCT) and was expressed as fold change mRNA expression relative to control values.

RNA-sequencing

As previously described, total mRNA was isolated from primary HMVECs by using the mirVana isolation kit prior to RNA-sequencing. RNA purity,

concentration and quality was measured as previously described (13). Sequence libraries were constructed using QuantSeq 3' mRNA-Seq Library Prep kit for Illumina (Lexogen) and amplified using 15 cycles. RNA-sequencing was performed on NextSeq 500 (Illumina) using NextSeq 500/500 High output Kit v2 (150 cycles), pooling barcoded samples per run.

Bioinformatics analysis

Bioinformatics analysis was outsourced and performed as previously described (14). Briefly, the low quality reads were trimmed and the adapter sequences were removed with BBTools (bbduk) using the parameters recommended by Lexogen (GSE150619). The reads were aligned to the human reference genome (ENSEMBL release 98) using STAR (14). Gene expression levels were quantified using featureCounts, assembling the uniquely mapped reads at exon level (15). Subsequently, the differential gene expression analyses were performed using DESeq2 R package (16). Correction for multiple testing was performed using the Benjamini-Hochberg false discovery rate (FDR), adjusted p-values < 0.1 were considered statistically significant.

Data analysis and statistics

All data are presented as mean ± standard error of mean (SEM). Multiple comparisons of means were performed using one-way ANOVA followed by Dunnett's Multiple Comparison test or two-way ANOVA followed by Bonferroni's Multiple Comparison test. Statistical significance of column factor was defined as $p<0.05, $$p<0.01, $$$p<0.001; statistical significance of row factor was defined as #p<0.05, ##p<0.01, ###p<0.001; statistical significance of post hoc test was defined as *p<0.05, **p<0.01, ***p<0.001. All statistical analyses were performed using GraphPad Prism 6 and IBM SPSS Statistics 23.

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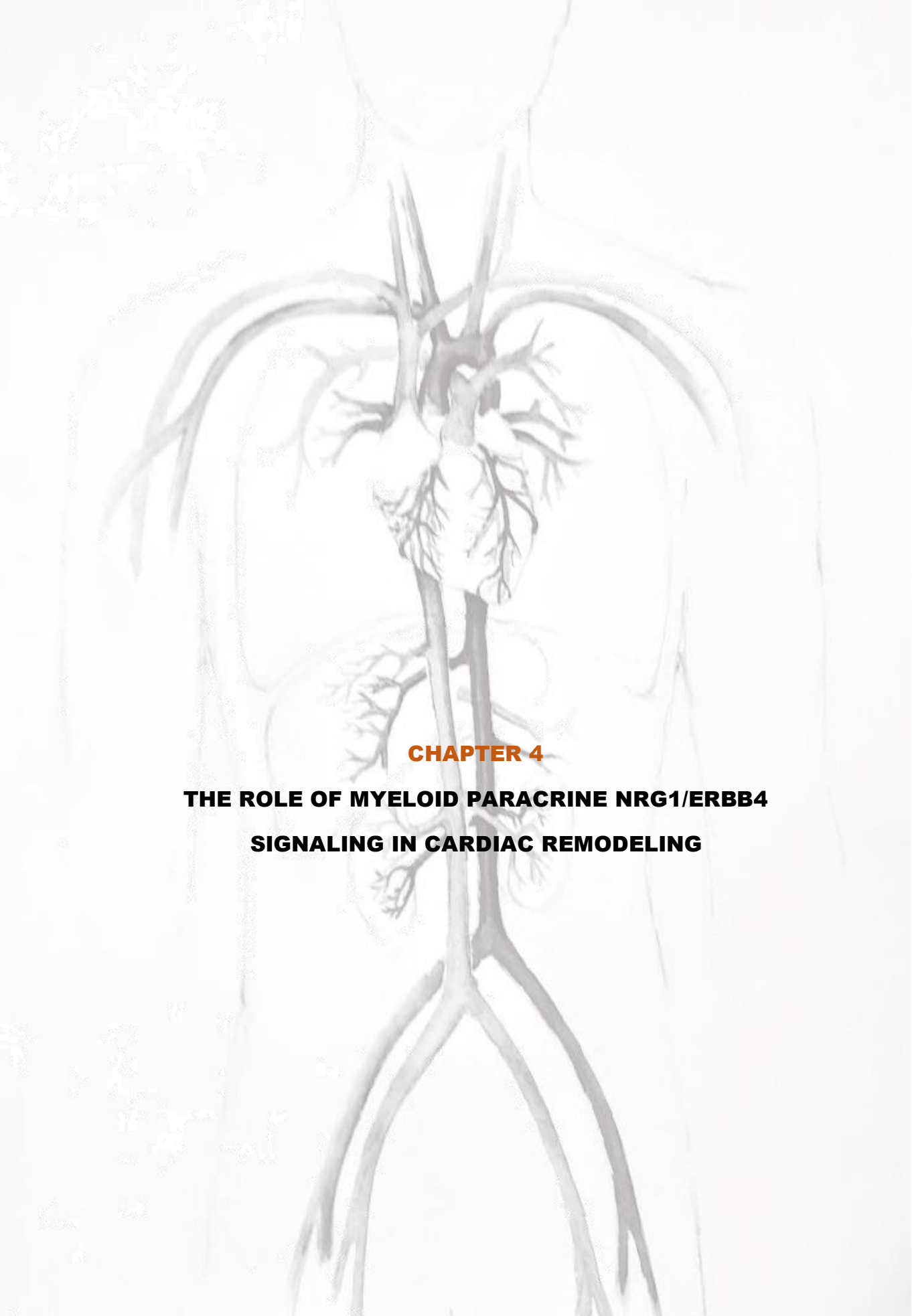

CHAPTER 4

THE ROLE OF MYELOID PARACRINE NRG1/ERBB4 SIGNALING IN CARDIAC REMODELING

ADAPTED FROM

Dugaucquier Lindsey, Feyen Eline, Bruyns Tine, De Keulenaer Gilles W, Segers Vincent FM. Targeting myeloid ERBB4 accentuates macrophage infiltration in Ischemic, but not pressure overload–induced cardiac remodeling. (Manuscript in preparation)

SUMMARY

Cardiac remodeling is characterized by phenotypic changes in both cardiomyocytes and cardiac non-myocytes. In recent years it has become clear that macrophages play an important role in cardiac remodeling. We previously demonstrated that NRG1, a member of the epidermal growth factor family, has direct effects on macrophages through ERBB4 and decreases inflammation and fibrosis in a model of Ang II–induced myocardial fibrosis. Here, we aimed at further investigating the regulatory role of the NRG1/ERBB4 system in myeloid cells on myocardial macrophage density and cardiac remodeling in different models of cardiac injury/overload.

We generated mice with myeloid-specific deletion of *Erbb4* and tested the effects of myeloid-specific *Erbb4* deletion in 2 models of cardiac remodeling—MI and pressure overload. We observed that myeloid-specific *Erbb4* deletion accentuated the early increase of myocardial macrophage density in the viable myocardium after MI, but that subsequent ventricular dilation and dysfunction, CM hypertrophy, or interstitial myocardial fibrosis remained unaffected. Interestingly, myeloid-specific *Erbb4* deletion reduced infarct scar. In the TAC model, pressure overload–induced myocardial inflammation remained absent, and LV remodeling progressed independently of myeloid ERBB4.

Overall, this study shows a modulatory role of NRG1/ERBB4 signaling in myeloid cells during the early inflammatory phase of MI.

Introduction

Cardiac remodeling—the pathophysiological mechanism underlying the progression of chronic heart failure CHF—is characterized by phenotypic changes in CMs and cardiac non-myocytes, including macrophages. Various cardiac stressors induce myocardial inflammation, with increased myocardial macrophage density due to local macrophage proliferation and monocyte recruitment (1). Increased macrophage proliferation and infiltration following cardiac ischemic and hemodynamic stress have been intensively studied in both rodent (2, 3) and non-rodent (4) animal studies. For instance, activated macrophages modulate cardiac responses following ischemic injury by removing necrotic and apoptotic cells, and secreting cytokines, chemokines, and growth factors (5). In addition, increased myocardial macrophage density was observed in mice with diastolic dysfunction. Cardiac macrophages produce interleukin-10 (IL-10), and subsequently stimulate fibroblasts and collagen deposition, leading to advanced diastolic dysfunction (6). In a model of transverse aortic constriction TAC, ventricular pressure overload induced a transient inflammatory reaction, *i.e.* chemokine and cytokine upregulation and recruitment of macrophages, and upregulation of TGF-β involved in the transition from inflammatory to fibrotic state (7). In a model of Ang II–induced arterial hypertension, MCP-1 and the C-C chemokine receptor 2 (CCR2) were involved in myocardial fibrosis (8). Overall, various models of cardiac overload are associated with myocardial inflammation, but the regulatory systems controlling myocardial macrophage density and activity are complex and need further investigation.

Recently, we observed that NRG1, a member of the epidermal growth factor family, was upregulated in the myocardium during Ang II–induced arterial hypertension, acting as a counter-regulatory factor during interstitial myocardial fibrosis (9). Interestingly, specific deletion of *Erbb4* in macrophages exacerbated Ang II–induced myocardial fibrosis, suggesting that NRG1 attenuates myocardial fibrosis by diminishing Ang II–induced myocardial inflammation.

Further studies showed that NRG1 attenuated the release of cytokines from activated macrophages in culture (10, 11). These observations add important knowledge to the mechanisms underlying the cardioprotective properties of NRG1, previously established in numerous animal models (12, 13). The cardiac effects of NRG1 are mostly explained by binding to the ERBB4 tyrosine kinase receptor (cardiac ERBB4 is much more abundant than ERBB3) (13-15). Cardiac ERBB4 receptors are expressed in CMs, but also in ECs, fibroblasts and macrophages (9, 10).

Here, we aimed at further investigating the regulatory role of the NRG1/ERBB4 system in myeloid cells on myocardial macrophage density and cardiac remodeling in different models of cardiac injury/overload. Therefore, we studied early and late cardiac responses induced by MI, in which myocardial macrophage density is abundant, and by TAC, in which myocardial inflammation is less pronounced. The role of NRG1/ERBB4 signaling in macrophages was studied in mice with myeloid-specific deletion of *Erbb4* (*Erbb4*$^{F/F}$ *LysM-Cre*$^{+/-}$ mice). More specific, we analyzed to what extent myeloid-specific *Erbb4* deletion influenced myocardial macrophage density, myocardial hypertrophy, fibrosis and cardiac performance.

Materials and methods

Materials and methods are described in chapter 3.

Results

Effects of myeloid-specific Erbb4 deletion on MI-induced cardiac remodeling. First, we studied the effect of myeloid-specific *Erbb4* deletion in a model of MI. This model is characterized by robust myocardial inflammation and replacement fibrosis. Figure 15A shows increased total myocardial macrophage density in both control and myeloid-specific *Erbb4* KO mice following MI. This increase was significantly higher in myeloid-specific *Erbb4* KO mice than in control mice, and was largely explained by differences in the remote/border zone after MI, less so by differences in the infarcted zone. We next studied whether the differences in myocardial macrophage density between myeloid-specific *Erbb4* KO and control mice were tracked by differences in myocardial mRNA expression of inflammatory factors (IL-6, IL-1β, TNF-α and TGF-β). No significant differences between both groups were observed (Figure 15B).

Five days after MI, we observed a significant increase in heart weight/body weight (HW/BW) in both control mice and myeloid-specific *Erbb4* KO mice, with no differences between these groups. CM CSA after 5 days remained unchanged in both groups (Figure 15C).

We also analyzed myocardial fibrosis 4 weeks after MI. Figure 16A shows that total myocardial fibrosis was increased in both groups, but significantly less in myeloid-specific *Erbb4* KO mice. This difference was explained by a smaller infarct scar, not by reduced interstitial fibrosis in the remote/border zone. Figure 16B shows myocardial mRNA expression of fibrotic markers *fibronectin-1*, *Col1a1*, and *Col3a1* in both control mice and myeloid-specific *Erbb4* KO mice. These markers significantly increased 4 weeks after MI in both control and KO mice, with no differences between these 2 groups.

Four weeks after induction of MI, LVs were significantly dilated in both control and myeloid-specific *Erbb4* KO mice. *Erbb4* deletion in myeloid cells did not change this outcome (Figure 16C). Also decreases in FS% were not influenced by myeloid-specific *Erbb4* deletion (data not shown). Similarly, cardiac

hypertrophic response was not different between myeloid-specific *Erbb4* KO and control mice (Figure 16D).

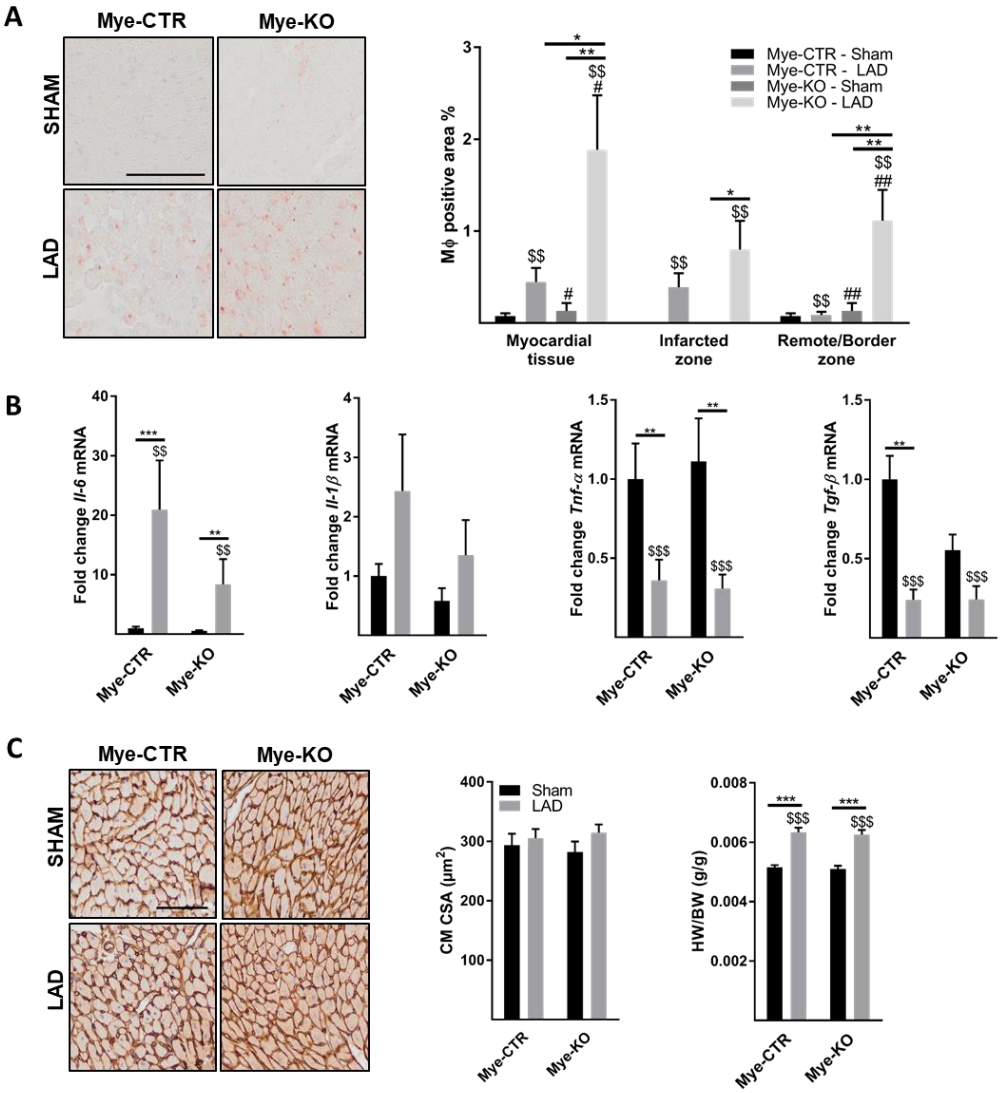

Figure 15: Myeloid-specific Erbb4 deletion did not affect hypertrophy, but alters inflammatory responses 5 days after MI. *A: Representative Mac3 stained heart sections (n=7–11 per group; scale bar=100 µm; → indicates infiltrated macrophages in the remote/border zone of the infarcted myocardium), and quantification of macrophage density in total myocardial tissue, infarcted zone, and remote/border zone (MΦ positive area %) of control mice (Mye-CTR) and Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ (Mye-KO) mice 5 days after MI. B: Fold induction of mRNA expression of Il-6, Il-1β, Tnf-α, and Tgf-β in heart samples of control mice versus Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice 5 days after MI. Results are expressed relative to control (sham). C: Representative laminin stained heart*

*sections (n=7–11 per group; scale bar=100 µm), and quantification of CM CSA (µm^2) and HW/BW of control mice and myeloid-specific Erbb4 KO mice 5 days after MI. Significant effect of two-way ANOVA is defined as $$p<0.01, $$$p<0.001, indicating a significant overall effect of MI relative to sham regardless of myeloid-specific Erbb4 deletion, and as #p<0.05, ##p<0.01, indicating a significant overall effect of KO relative to CTR regardless the procedure; Post hoc test (Bonferroni's Multiple Comparison test): *p<0.05, **p<0.01, ***p<0.001; Results are expressed as mean ± SEM.*

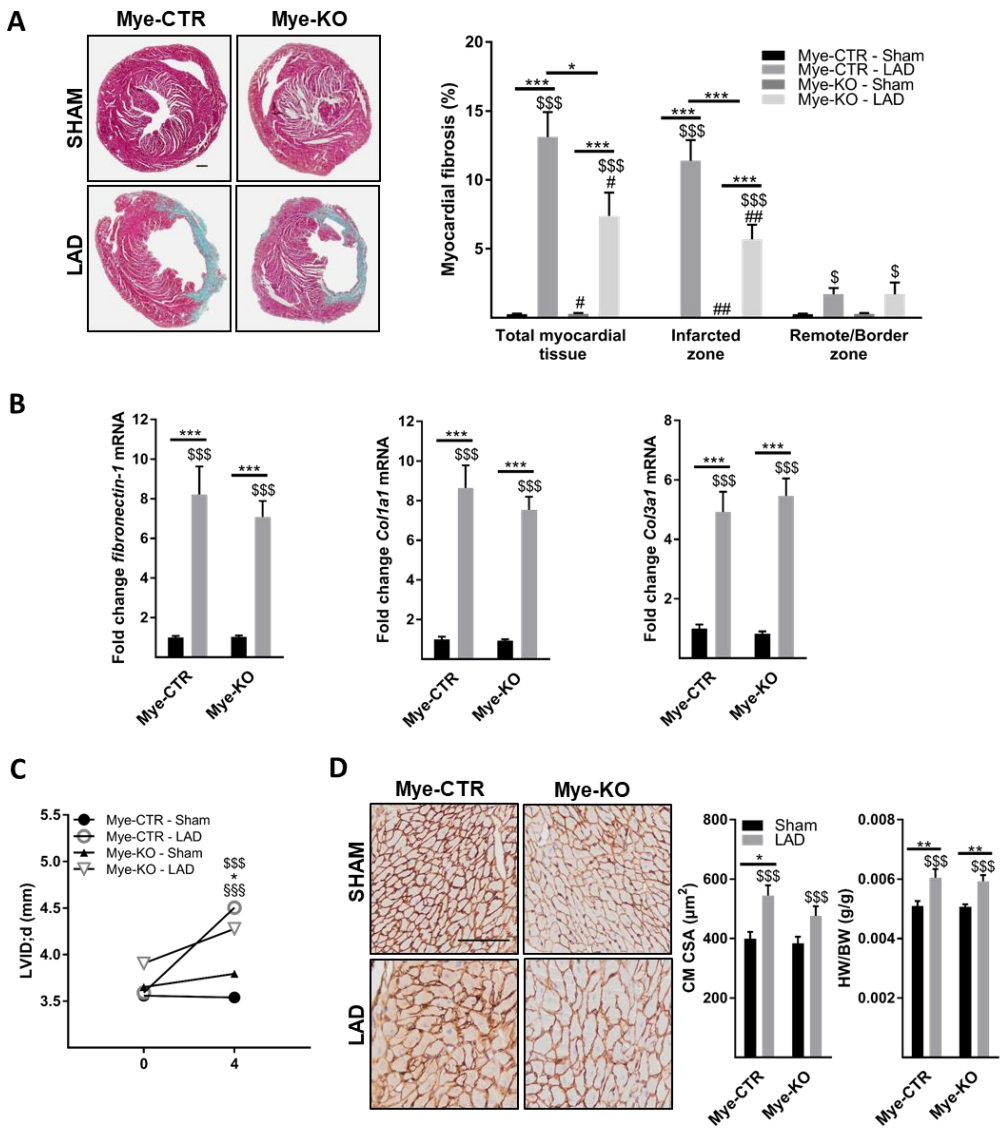

Figure 16: Myeloid-specific Erbb4 deletion did not affect cardiac performance, or hypertrophy, but alters myocardial fibrosis 4 weeks after MI. *A: Representative Masson's Trichrome stained heart sections (n=7–11; scale bar=1000 µm) of control mice (Mye-CTR) versus*

*Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice (Mye-KO) 4 weeks after MI, and quantification of myocardial fibrosis in total myocardial tissue, infarcted zone, and remote/border zone of control mice versus Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice 4 weeks after MI. B: Fold induction of mRNA expression of fibronectin-1, Col1a1, Col3a1 in heart samples of control mice versus Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice 4 weeks after MI. Results are expressed relative to control (sham). C: Echocardiographic measurement of left ventricular internal diameter in diastole (LVID;d) in control mice and Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice with a follow-up of 4 weeks after MI (n=7–11 per group); § indicates a significant difference between Mye-CTR-sham and Mye-CTR-LAD; * indicates a significant difference between Mye-KO-sham and Mye-KO-LAD. D: Representative laminin stained heart sections (n=7–11 per group; scale bar=100 µm), and quantification of CM CSA (µm^2) and HW/BW of control mice and myeloid-specific Erbb4 KO mice 4 weeks after MI. Significant effect of two-way ANOVA is defined as $p<0.05, $$$p<0.001, indicating a significant overall effect of MI relative to sham regardless of myeloid-specific Erbb4 deletion, and as #p<0.05, ##p<0,01, indicating a significant overall effect of KO relative to CTR regardless the procedure; Post hoc test (Bonferroni's Multiple Comparison test): *p<0.05, **p<0.01, ***p<0.001; Results are expressed as mean ± SEM.*

Effects of myeloid-specific Erbb4 deletion in TAC-induced cardiac overload. We next studied the effect of myeloid-specific *Erbb4* deletion on pressure overload–induced cardiac hypertrophy by performing TAC in both control mice and *Erbb4$^{F/F}$ LysM-Cre$^{+/-}$* mice. TAC is a model of extensive fibrotic remodeling in which myocardial inflammation is much less pronounced compared to MI.

First, we evaluated the effects of myeloid-specific *Erbb4* deletion on myocardial macrophage density. Both control mice and myeloid-specific *Erbb4* KO mice showed a small time-dependent and transient increased myocardial macrophage density, following TAC and sham operation. This effect was not influenced by myeloid-specific *Erbb4* deletion (Figure 17A). TAC induced a significant time-dependent increase of myocardial interstitial fibrosis starting 1 week after TAC, with no significant differences between myeloid-specific *Erbb4* KO mice and control mice (Figure 17B).

Figure 17C shows that TAC induced a time-dependent LV dilatation and dysfunction, starting 2 weeks after TAC with increases of both LVID;d and LVID;s (latter not shown). *Erbb4* deletion in myeloid cells did not affect these outcomes. Also increases of hypertrophic parameters (CM CSA (Figure 17D), HW/BW and septal wall thickness (data not shown)) were not influenced by myeloid-specific *Erbb4* deletion.

Finally, TAC induced increased mortality in both control mice and myeloid-specific *Erbb4* KO mice, with no statistically significant difference between groups (Figure 17E).

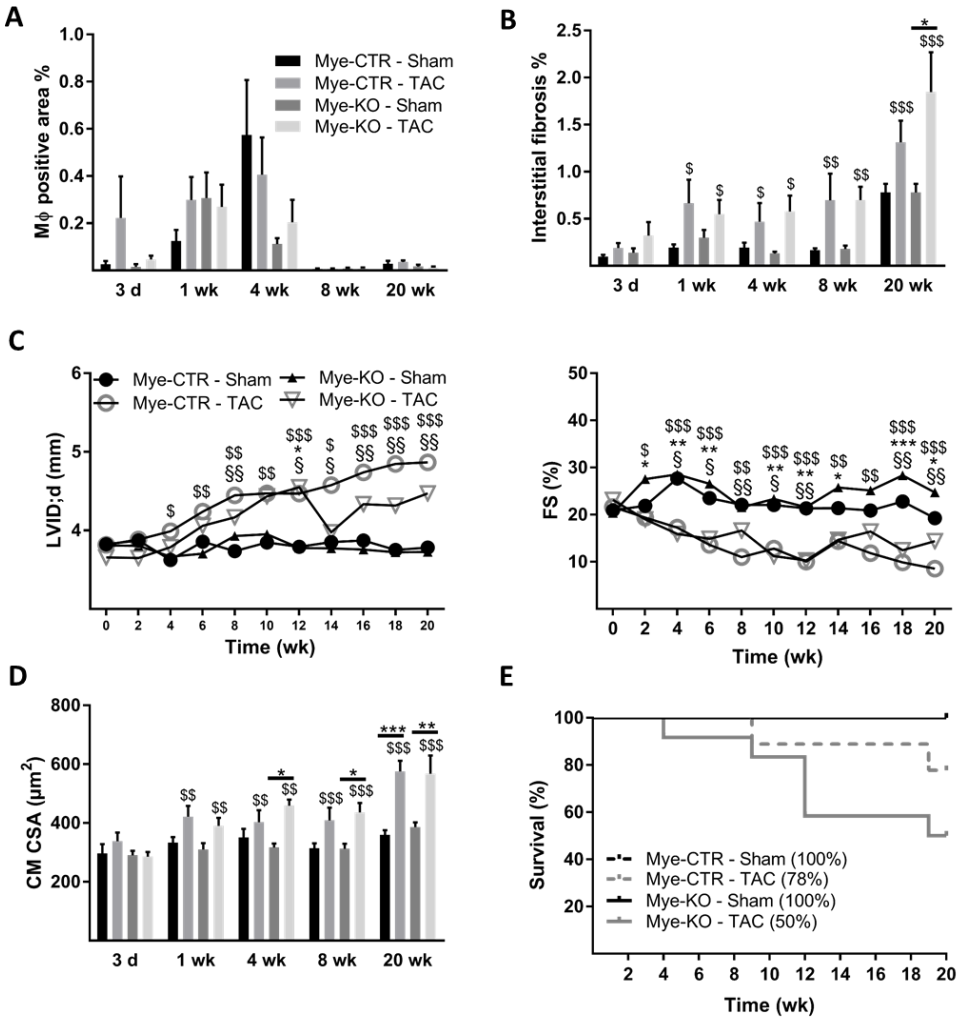

Figure 17: Myeloid-specific Erbb4 deletion did not affect cardiac performance, survival, or hypertrophic responses 3 days to 20 weeks after TAC. *A: Quantification of myocardial macrophage density (MΦ positive area %) of control mice (Mye-CTR) versus Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice (Mye-KO) (n=6–12 per group) 3 days to 20 weeks after TAC. B: Quantification of cardiac interstitial fibrosis of control mice versus Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice (n=6–12 per group) 3 days to 20 weeks after TAC. C: Echocardiographic measurement of fractional shortening (FS%) and left ventricular internal diameter in diastole (LVID;d) in control mice and Erbb4$^{F/F}$ LysM-Cre$^{+/-}$ mice with a follow-up of 20 weeks after TAC (n=9–12 per group); § indicates a significant difference between Mye-CTR-sham and Mye-CTR-TAC; * indicates a significant difference between Mye-KO-sham and Mye-KO-TAC. D: Quantification of CM CSA (µm^2) of control mice and myeloid-*

*specific Erbb4 KO mice (n=6–12 per group) 3 days to 20 weeks after TAC. E: Effects of TAC on survival in control mice and myeloid-specific Erbb4 KO mice (n=9–12 per group) with a follow-up of 20 weeks. Significant effect of two-way ANOVA is defined as $p<0.05, $$p<0.01, $$$p<0.001, indicating a significant overall effect of MI relative to sham regardless of myeloid-specific Erbb4 deletion; Post hoc test (Bonferroni's Multiple Comparison test): §p<0.05, §§p<0.01, §§§p<0.001; *p<0.05, **p<0.01, ***p<0.001; Results are expressed as mean ± SEM.*

Discussion

In this study, we examined the role of NRG1/ERBB4 signaling in myeloid cells during cardiac remodeling using two models of cardiac overload and injury. The study shows that myeloid-specific *Erbb4* deletion accentuated the early increase of myocardial macrophage density in the viable myocardium after MI, but that subsequent ventricular dilation and dysfunction, CM hypertrophy, or interstitial myocardial fibrosis remained unaffected. Interestingly, myeloid-specific *Erbb4* deletion reduced infarct scar. In the TAC model, pressure overload–induced myocardial inflammation remained absent, and LV remodeling progressed independently of myeloid ERBB4.

Several studies have addressed the modulatory role of NRG1 on macrophages, both within and outside the heart. First, Ryzhov *et al*. showed that GGF2, a secreted NRG, reduces tumor necrosis factor-α (TNF-α) expression in peripheral blood mononuclear cells of CHF patients (11), proposing that the NRG/ERBB pathway is a modulator of the immune system in heart failure. These authors related these effects to the activation of the ERBB3 receptor on human monocytes, whereas we studied the effect of ERBB4 in myeloid cells in mice. Second, Vermeulen et al showed that myeloid-specific deletion of ERBB4 accentuated myocardial fibrosis in a model of Ang II–induced hypertension (9), suggesting that NRG1 is a negative regulator of myocardial fibrosis through modulation of myocardial inflammation. Next, Schumacher *et al.* showed that ERBB4 expression was induced in cultured murine and human macrophages by stimulation with LPS and that this upregulation allowed ERBB4 ligands to serve as negative feed-back regulators of inflammation by enhancing macrophage

clearance through the induction of macrophage apoptosis (10). In view of this knowledge, here we discuss how to interpret the observations of this study.

First, since deletion of myeloid *Erbb4* increased post-MI myocardial macrophage density, this study endorses an anti-inflammatory effect of NRG1/ERBB4 signaling in the heart, at least in the setting of ischemic myocardial injury. The underlying mechanisms remain unclear, and further studies should reveal whether effects of NRG1/ERBB4 signaling on macrophage proliferation, infiltration or apoptosis are involved.

On the other hand, this study may also downplay the significance of inflammatory modulation by myeloid NRG1/ERBB4 signaling during cardiac remodeling. In the post-MI setting despite increasing macrophage density, myeloid *Erbb4* deletion did not affect subsequent myocardial hypertrophy, ventricular dilatation and dysfunction or interstitial fibrosis of myocardium in the remote zone of infarction. In addition, in the TAC model, myeloid *Erbb4* deletion—resulting in a blunted negative feedback regulation of NRG1 signaling—did not trigger any myocardial inflammation. These negative observations diverge from previous observations in our laboratory in which myeloid-specific *Erbb4* deletion accentuated myocardial inflammation and interstitial myocardial fibrosis in a model of Ang II–induced myocardial fibrosis. Altogether, these observations argue that the modulatory role of NRG1/ERBB4 signaling on myeloid cells may be circumstance-dependent. However, we observed a 20 to 50% mortality 20 weeks after TAC. In consequence, the effects studied in the TAC model are based on the examination of surviving animals, and are therefore subject to biases that may have limited our analyses.

Finally, a surprising result of this study was that myeloid-specific *Erbb4* deletion attenuated post-MI infarct scar. It is well established that post-MI macrophage infiltration regulates post-MI wound healing or fibrosis (1, 5) and either reduces or increases myocardial fibrotic responses and infarct size. The latter depends on the phenotypic switch of macrophages induced by the microenvironment, which may either be pro-fibrotic or fibrolytic (16). Some cytokines, released

during cardiac inflammation, like IL-6 levels, are associated with anti-inflammatory and anti-fibrotic responses (17). The biological activity of IL-6 is regulated by binding to the IL-6R/gp130 signal transduction complex, and different studies have addressed protective actions of the IL-6-gp130 pathway in the myocardium (17-19). IL-1β and TGF-β, on the other hand, have pro-inflammatory and pro-fibrotic properties (20, 21). Therefore, the observation in this study that myeloid-specific *Erbb4* deletion decreases post-MI infarct scar may suggest that impaired NRG1 signaling in macrophages promotes a fibrolytic phenotype of macrophages in the infarcted myocardium.

To summarize, this study supports a modulatory role of NRG1/ERBB4 signaling in myeloid cells during the early inflammatory phase of MI. Myeloid-specific *Erbb4* deletion in mice accentuates early inflammatory responses post-MI and decreases infarct scar.

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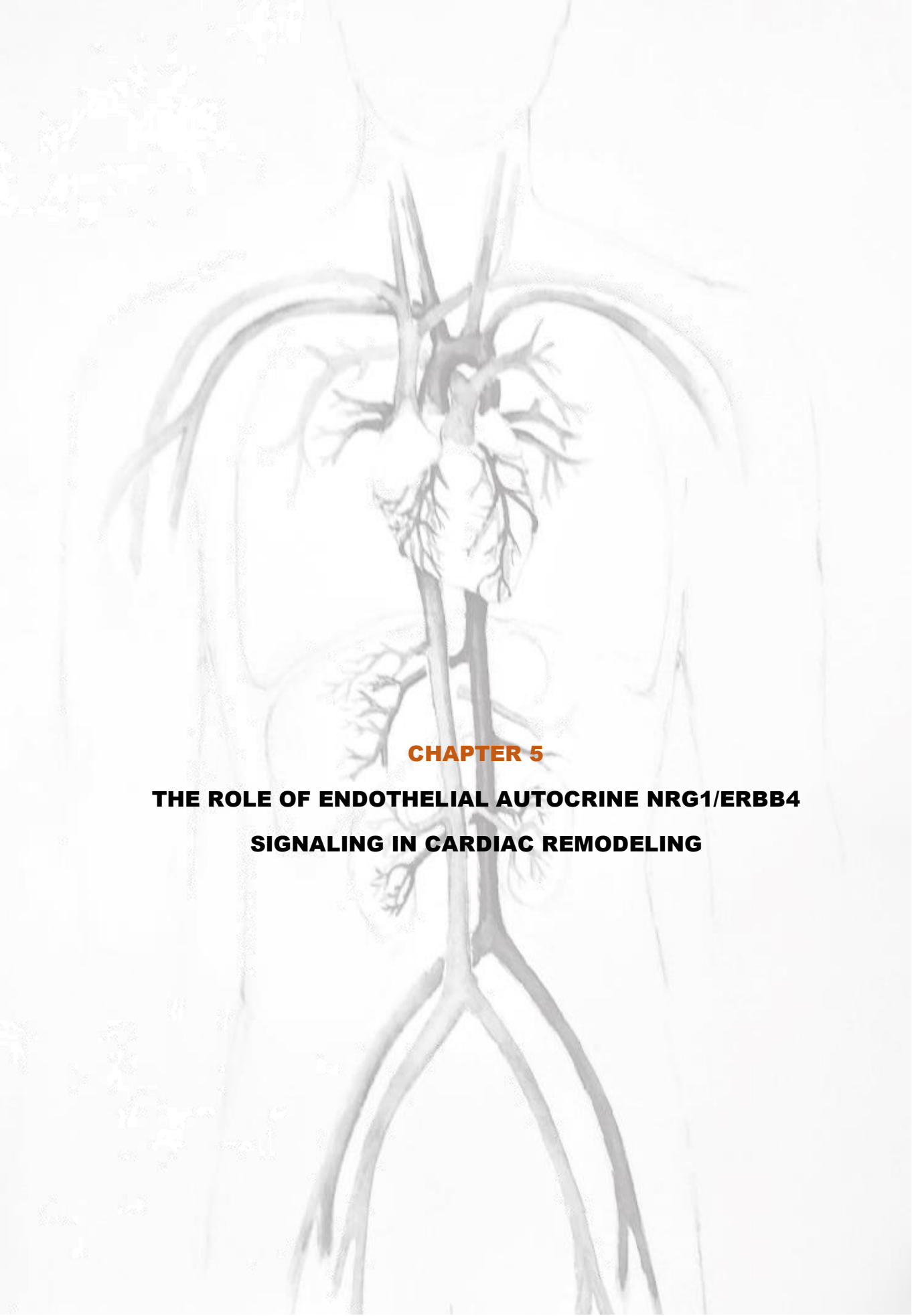

CHAPTER 5

THE ROLE OF ENDOTHELIAL AUTOCRINE NRG1/ERBB4 SIGNALING IN CARDIAC REMODELING

ADAPTED FROM

Dugaucquier Lindsey, Feyen Eline, Mateiu Ligia, Bruyns Tine, De Keulenaer Gilles W, Segers Vincent FM. Role of endothelial autocrine NRG1/ERBB4 signaling in cardiac remodeling. (American Journal of Physiology-Heart and Circulatory Physiology. Published online ahead of print: 2020 Jul 3.)

SUMMARY

NRG1 is a paracrine growth factor, secreted by cardiac ECs in conditions of cardiac overload/injury. The current concept is that the cardiac effects of NRG1 are mediated by activation of ERBB4/ERBB2 receptors on CMs. However, recent studies have shown that paracrine effects of NRG1 on fibroblasts and macrophages are equally important. Here, we hypothesize that NRG1 autocrine signaling plays a role in cardiac remodeling.

We generated EC–specific *Erbb4* KO mice to eliminate endothelial autocrine ERBB4 signaling without affecting paracrine NRG1/ERBB4 signaling in the heart. We first observed no basal cardiac phenotype in these mice up to 32 weeks. We next studied these mice following TAC, exposure to Ang II or MI in terms of cardiac performance, myocardial hypertrophy, myocardial fibrosis and capillary density. In general, no major differences between EC–specific *Erbb4* KO mice and control littermates were observed. However, 8 weeks following TAC both myocardial hypertrophy and fibrosis were attenuated by EC–specific *Erbb4* deletion, albeit these responses were normalized after 20 weeks. Similarly, 4 weeks after Ang II treatment myocardial fibrosis was less pronounced compared to control littermates. These observations were supported by RNA-sequencing experiments on cultured ECs showing that NRG1 controls the expression of various hypertrophic and fibrotic pathways.

Overall, this study shows a role of endothelial autocrine NRG1/ERBB4 signaling in the modulation of hypertrophic and fibrotic responses during *early* cardiac remodeling. This study contributes to understanding the spatio-temporal heterogeneity of myocardial autocrine and paracrine responses following cardiac injury.

Introduction

Cardiac remodeling—a pathophysiological process with changes in cardiac size, structure, and function—often leads to heart failure in advanced stages. Cardiac remodeling comprises cellular changes in different cell types in the heart— ECs, CM, fibroblasts, inflammatory cells, and smooth muscle cells. ECs are the most abundant cell type accounting for 45% of the total cardiac cell population and 65% of the non-myocyte population (1, 2). Cross-talk between ECs and other cardiac cells is enabled by a dense capillary network of 3,000–4,000 capillaries/mm^2 (2-5). CMs depend on ECs not only for oxygenated blood supply but also for paracrine protective signals, mediated through endothelial-derived cardio-active factors. A growing number of these factors has been identified, including NO, ET1, Ang II, angiopoietins, prostaglandins, and growth factors, FGF, VEGF, and NRG1 (4-8). NRG1 is expressed by ECs and binds in a paracrine manner on ERBB4 and/or ERBB3 (9).

NRG1/ERBB signaling is critical during cardiac development (9-12) and in adult physiology and pathophysiology (13). Conditional *Erbb2*- or *Erbb4*-null mice develop cardiomyopathy and are more sensitive to cardiac stressors, for example ischemia and toxic drugs (10, 14-16). The NRG1/ERBB system also allows the heart to cope with physiological stress—for example during pregnancy (17). ERBB signaling activates several signaling cascades, including ERK1/2, MAPK, PI3K/AKT, and NOS, to modulate CM growth and survival (18-20). Increased cardiac expression of NRG1, shedding of NRG1 in the circulation, or activation of cardiac ERBB receptors have been demonstrated in different animal models, including pressure overload–induced LV hypertrophy (7), ischemia-reperfusion injury (10), rapid ventricular pacing (10) and Ang II–induced arterial hypertension (11, 13, 21). Both preclinical and clinical studies indicate that administration of NRG1 attenuates cardiac remodeling and improves cardiac function in different models of heart failure (22-26).

The generally accepted concept is that the cardiac effects of NRG1 are mediated by paracrine activation of ERBB4/ERBB2 receptors on CMs (7, 10). However, recent studies have shown that myeloid-specific deletion of *Erbb4* intensifies myocardial fibrotic responses to Ang II (21). This indicates that paracrine NRG1/ERBB4 signaling in cardiac non-myocytes is equally important. This finding is reinforced by the fact that also fibroblasts express ERBB receptors. Galindo *et al.* showed that GGF2, a secreted neuregulin which binds the ERBB receptors, attenuated myocardial fibrosis, altered ECM structure, and reduced the number of myofibroblasts in a swine model of MI. These findings suggest a direct inhibitory effect of GGF2 on cardiac fibroblasts (27).

The role of ERBB receptors in ECs is less understood. Previous studies showed the expression of different ERBB receptors (ERBB2, ERBB3, ERBB4) on ECs, and stimulation of ECs with NRG1 induces rapid calcium fluxes, receptor tyrosine phosphorylation, and cell proliferation (7). In addition, it has been shown that stimulation of ERBB2, ERBB3, or ERBB4 receptors by NRG1 results in both *in vitro* and *in vivo* activation of several signaling pathways involved in angiogenesis (28-30). This effect has been reported to be both dependent and independent of VEGF (28, 30). Kalinowski et al. showed that cytokine-activated MMP cleavage of NRG1 may play an important role in autocrine activation of EC signaling pathways, contributing to its key biological effects (29). Yen et al., on the other hand, showed a modulatory role of ERBB2/ERBB3 heterodimers in the upregulation of VEGF and subsequent in vivo angiogenesis (31). Moreover, endothelial-specific Nrg1 deletion impairs ischemia-induced angiogenesis and decreases flow recovery after femoral artery ligation. This indicates a potential role of NRG1 in vascular disease, like diabetes and coronary artery disease (32).

Overall, previous studies have established a role of endothelial NRG1/ERBB signaling in the heart. However, the role of ERBB receptors in ECs during cardiac remodeling remains unclear, and knowledge about the underlying mechanism remains limited. Most endothelium-derived substances like NO and ET-1 have both paracrine and autocrine properties. Endothelial dysfunction

results in impairement of these substances and leads to adverse cardiac remodelling and dysfunction (4, 33, 34). Here, we hypothesize that ERBB4-dependent autocrine signaling in ECs plays a role in angiogenesis and cardiac remodeling. Therefore, we generated an EC–specific *Erbb4* KO mouse to specifically eliminate endothelial autocrine ERBB4 signaling without affecting paracrine NRG1/ERBB4 signaling in other cardiac cell types. We tested whether this specific conditional deletion of the endothelial ERBB4 receptor, and thus impaired autocrine ERBB4 signaling, changes cardiac remodeling in three different models of cardiac overload/injury.

Materials and methods

Materials and methods are described in chapter 3.

Results

HMVECs and HUVECs are responsive to NRG1 *in vitro*. ECs are the primary cellular source of NRG1 in the heart, but their responsiveness to NRG1 is incompletely studied (1). Figure 18A demonstrates that HUVECs express ERBB4, ERBB2 and, weakly, ERBB3 receptors. NRG1 (50 $ng.mL^{-1}$) induces phosphorylation of both endothelial ERBB4 and ERBB2 receptors 30 minutes after stimulation; endothelial ERBB3 was not activated. In addition, exposure of HUVECs to NRG1 (50 $ng.mL^{-1}$) showed a transient, and statistically insignificant increase in phosphorylation of AKT and ERK1/2 (Figure 18B; Figure 18C). STAT3 phosphorylation was transiently attenuated (data not shown), whereas total eNOS and iNOS protein levels gradually decreased during 8 h following NRG1 treatment, again these data showed no statistical significance (Figure 18B; Figure 18C). We next treated HMVECs for 8 or 24 h with NRG1 and analyzed gene expression levels with RNA-sequencing (n=4 per group). Table S2 and Table S3 summarize differentially expressed genes by NRG1 in human HMVECs, classified with relation to their function in fibrosis, inflammation, proliferation, growth, differentiation, and angiogenic response. This RNA-sequencing experiment suggests a modulatory role of NRG1 in endothelium-dependent regulation of cardiac hypertrophy, fibrosis, and angiogenesis. Endothelial genes involved in fibrotic responses are mostly upregulated upon NRG1 treatment, whereas inflammatory, hypertrophic and angiogenic genes are both up- and downregulated upon NRG1 treatment. For example, NRG1-induced downregulation in *Igf1* could be involved in cardiac hypertrophy (35) and angiogenesis (36). NRG1-induced upregulation in *Mmp2* may contribute to changes in ECM (35). Based on these results, we hypothesized that abrogating NRG1-induced endothelial signaling would change cardiac remodeling.

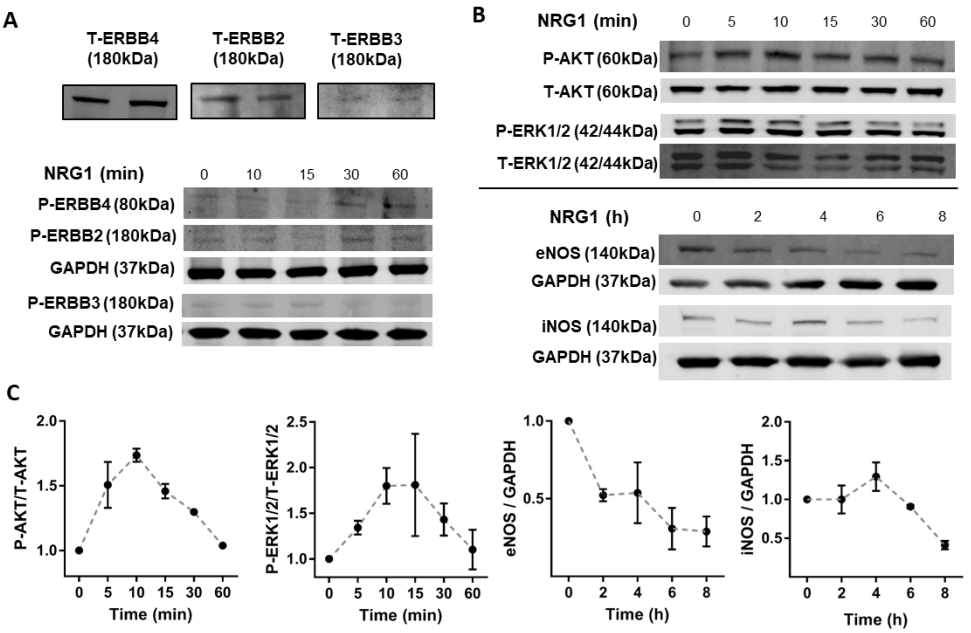

Figure 18: HUVECs are responsive to NRG1 in vitro. *A: Total (in duplo) and phosphorylated protein levels of ERBB4, ERBB2, and ERBB3 receptors in Human Umbilical Vein ECs (HUVECs) were analyzed using Western blot analysis (3 repeats). B: Protein levels of (P-)AKT, (P-)ERK1/2, and total eNOS and iNOS in HUVECs were determined using Western blot analysis after 0 to 60 min (AKT, ERK) and after 0 to 8 h (eNOS, iNOS) stimulation with NRG1. C: Protein levels of (P-)AKT, (P-)ERK1/2, and total eNOS and iNOS in HUVECs were quantified using Odyssey Software (2 repeats). Results are expressed as mean ± SEM.*

Validation of endothelial-specific Erbb4 KO mice. We created Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} transgenic mice in which endothelial Erbb4 can be conditionally deleted by administration of tamoxifen (Figure 19A). We validated the model by isolating cardiac ECs from hearts of Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice treated with tamoxifen or vehicle (EC purity more than 85% in all samples). Western blotting showed that ERBB4 receptor expression was almost completely suppressed by tamoxifen treatment, whereas ERBB2 and the weak ERBB3 expression were not affected (Figure 19B). EC–specific *Erbb4* KO mice showed no overt phenotypic abnormalities, changes in cardiac function or myocardial histology at baseline. In particular, they showed normal body weight, HW/BW, and echocardiographic parameters (

Table S4). Vascular reactivity studies with isolated aortas also showed that EC–specific *Erbb4* deletion induced no changes in vasomotoricity, neither in aortic contractile properties, nor in endothelium-dependent or endothelium-independent aortic dilation (Figure 19C).

NRG1 stimulates EC migration and angiogenesis *in vitro*. A few studies have shown that NRG1 induces an angiogenic response in ECs (24, 28, 32). Consistent with these findings, in wound healing assays in HUVECs, we found that NRG1 significantly increased migration of ECs compared to PBS after 18 h (Figure 19D). In addition, in aortic ring assays NRG1 significantly increased the number of microvessels sprouting from the main aortic ring from vehicle-treated *Erbb4*$^{F/F}$ *VE-Cdh5-Cre*$^{+}$ mice. This effect was ERBB4-dependent, since aortic rings from tamoxifen-treated *Erbb4*$^{F/F}$ *VE-Cdh5-Cre*$^{+}$ mice showed significantly less NRG1-induced microvessel sprouting (Figure 19E). These data confirm that NRG1 induces an angiogenic response in ECs *in vitro* and demonstrate the role of endothelial ERBB4 in this effect. Based on this finding, we analyzed the effect of *Erbb4* deletion on myocardial capillary density *in vivo* in aging mice (from 12 to 32 weeks). Both control and tamoxifen-treated *Erbb4*$^{F/F}$ *VE-Cdh5-Cre*$^{+}$ mice showed a small age-dependent capillary rarefaction, but there was no significant difference between groups (Figure 19F). In addition, myocardial mRNA expression of angiogenic factors, like *Vegf, Fgf2*, *Plgf*, and *Angpt1* did not consistently change by EC–specific *Erbb4* deletion (Figure 19G). In conclusion, NRG1 induces angiogenic responses *in vitro,* but post-natal induced EC–specific *Erbb4*-deletion did not change baseline myocardial capillary density *in vivo.* Next, we studied the effect of EC–specific *Erbb4* deletion in cardiac remodeling.

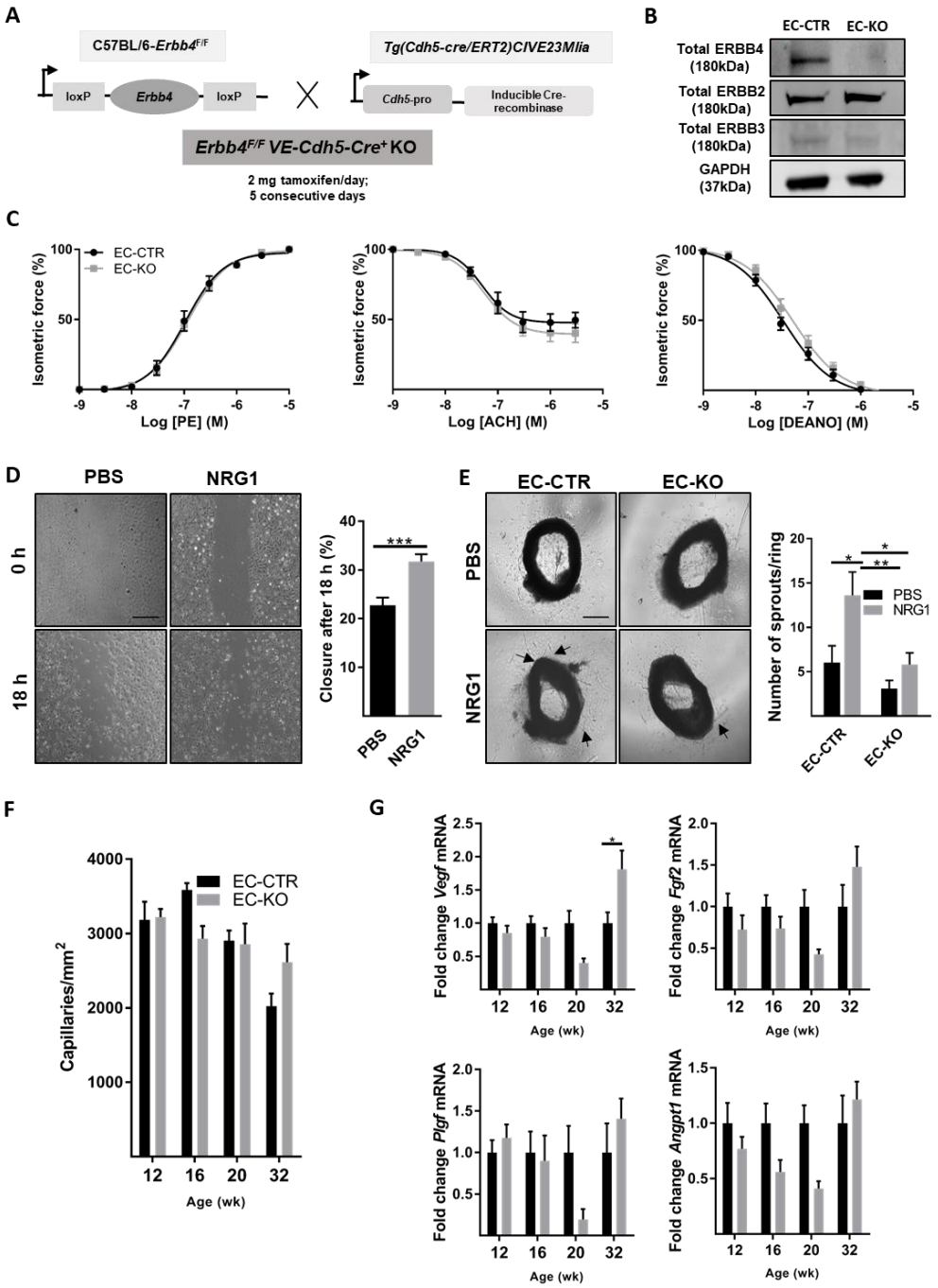

Figure 19: Phenotypic characterization of endothelial-specific Erbb4 KO mice and angiogenic properties of NRG1. *A: Creation of tamoxifen-inducible Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice (EC-KO). B: Western blot analysis of ERBB4, ERBB2, and ERBB3 protein levels in isolated endothelial cells (EC) of Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice treated with tamoxifen (EC-KO) or vehicle*

*(EC-CTR). C: Isometric force (%) of isolated aortic segments of both tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} and control mice after phenylephrine (PE), acetylcholine (Ach) or DEANO administration (n=5 per group). D: Representative bright field images of cultured HUVECs in a scratch wound-healing assay (n=3; 3 repeats; scale bar=100 µm), and quantification of % closure 18 h after scratching with vehicle (PBS) or NRG1 (50 ng.mL^{-1}) treatment. E: Representative bright field images of aortic rings from both control mice and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice (n=20 rings per condition; 3 repeats; scale bar=100 µm) after treatment with PBS or NRG1 (50 ng.mL^{-1}), and quantification of mean number of microvessel sprouts per ring (indicated by →). F: Quantification of capillary density (capillaries/mm^{2}) of control mice and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice at baseline. G: Fold induction of mRNA expression of Vefg, Fgf2, Plgf, Angpt1 in heart samples of control mice versus tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice at baseline over a time course of 32 weeks. Results are expressed relative to control (sham). There was a significant effect of age on capillary density and Vegf, Fgf2, and Angpt1 mRNA (not indicated on the figure). Post hoc test (Dunnett's Multiple Comparison test (D); Bonferroni's Multiple Comparison test (E;F)): *p<0.05, **p<0.01, ***p<0.001. Results are expressed as mean ± SEM.*

Effect of endothelial-specific *Erbb4* deletion in three models of cardiac remodeling. The NRG1/ERBB system is an intrinsic system which is activated by several stimuli including pressure overload, Ang II, or ischemia and attenuates subsequent LV remodeling through NRG1 activity on CMs, inflammatory cells, and fibroblasts (7, 21, 37). Given the fact that NRG1 changes endothelial angiogenic responses (Figure 19D; Figure 19E) and its transcriptome (Table S2; Table S3), we tested whether *Erbb4* deletion in ECs affected cardiac remodeling induced by different triggers, including pressure overload, Ang II, and MI. We systematically analyzed the effect of EC-specific *Erbb4* deletion during remodeling on LV diameters and performance, LV hypertrophy, myocardial capillary density and myocardial interstitial and perivascular fibrosis.

(i) Effect of endothelial-specific Erbb4 deletion in TAC–induced cardiac overload. First, we studied the effects of EC–specific *Erbb4* deletion on pressure overload–induced cardiac hypertrophy by performing TAC in both control mice and tamoxifen-treated *Erbb4$^{F/F}$ VE-Cdh5-Cre^{+}* mice. TAC is a model of LV remodeling with a time-dependent transition from concentric to eccentric LV hypertrophy, and interstitial and perivascular fibrosis.

Figure 20A shows that both control mice and EC–specific *Erbb4* KO mice showed a time-dependent decrease of FS%, and an increase of LVID;d and LVID;s (Table S5; Table S6). Control mice showed significantly increased CM CSA and increased HW/BW both at 8 and 20 weeks (Figure 20B; Figure 20C). This hypertrophic response was also seen in EC–specific *Erbb4* KO mice at 20 weeks (Figure 20C), but remained statistically insignificant at 8 weeks (Figure 20B), suggesting a delay in hypertrophy by EC–specific *Erbb4* deletion.

Next, we evaluated the effects of *Erbb4* deletion in ECs on capillary density. No significant effects of TAC on capillary density were observed after 8 weeks (Figure 21A) and 20 weeks (Figure 21B), neither in control mice, nor in EC–specific *Erbb4* KO mice.

With regard to myocardial fibrosis, control mice showed significant increases of interstitial (Figure 21C; Figure 21D) and perivascular fibrosis (Figure 21E; Figure 21F) both at 8 and 20 weeks after TAC. These fibrotic responses were also seen in EC–specific *Erbb4* KO mice at 20 weeks (Figure 21D; Figure 21F). At 8 weeks, however, interstitial fibrosis was statistically milder than control mice (Figure 21C), suggesting that EC–specific *Erbb4* deletion causes a delay in myocardial fibrosis after TAC. In relation to the delayed fibrotic response, we did not observe altered macrophage density, neither in control mice, nor in EC–specific *Erbb4* KO mice 8 weeks after TAC (Figure S24A and B).

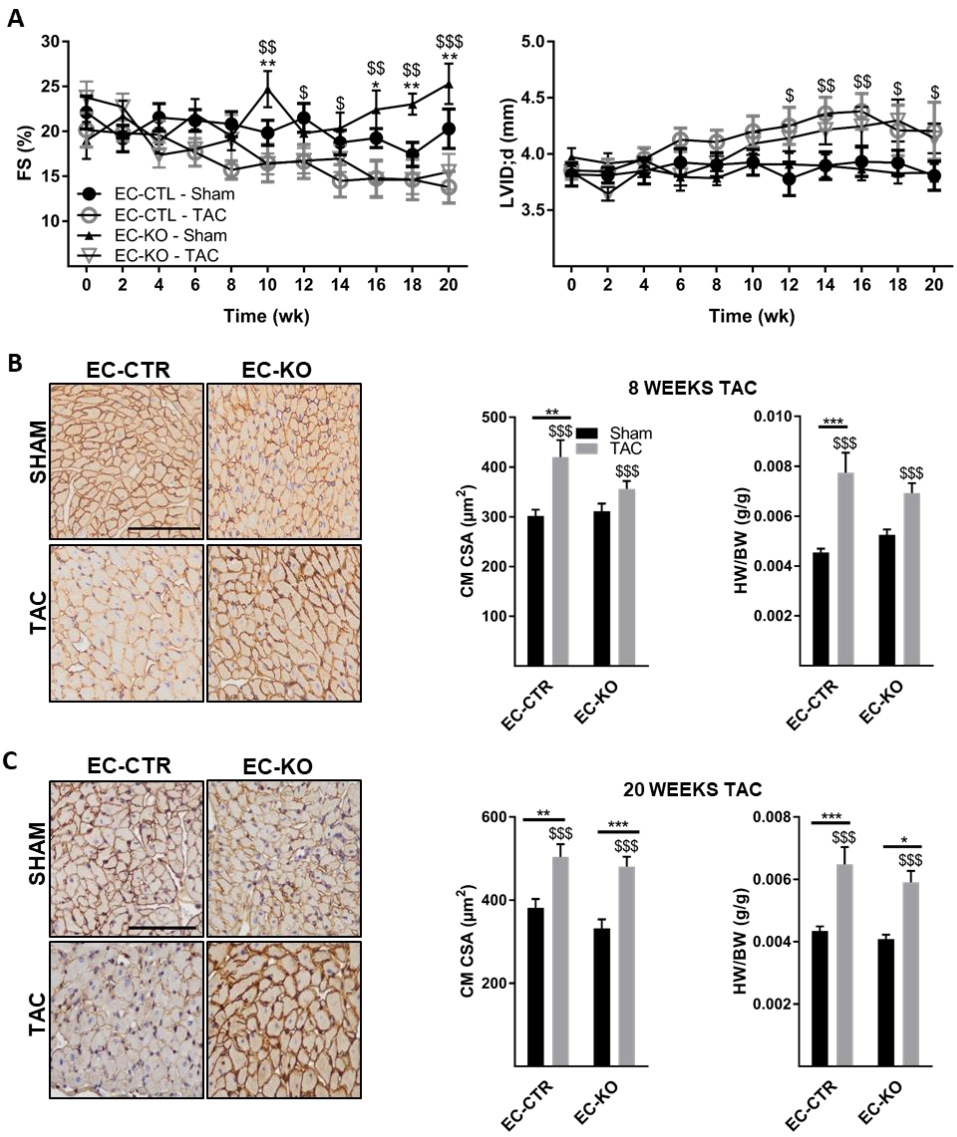

Figure 20: Endothelial-specific Erbb4 deletion did not affect cardiac performance and a delayed hypertrophic response was observed 8 to 20 weeks after TAC. *A: Echocardiographic measurement of fractional shortening (FS%) and left ventricular internal diameter in diastole (LVID;d) in control mice (EC-CTR) and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice (EC-KO) with a follow-up of 20 weeks after TAC (n=9–15 per group); * indicates a significant difference between EC-KO–sham and EC-KO–TAC (post hoc test). B: Representative laminin stained heart sections (n=9–15 per group; scale bar=100 µm), and quantification of CM CSA (µm²) and HW/BW (g/g) of control mice and EC–specific Erbb4 KO mice 8 weeks after TAC. C: Representative laminin stained heart sections (n=9–15 per group; scale bar=100 µm), and quantification of CM CSA (µm²) and HW/BW (g/g) of control mice and EC–specific Erbb4 KO mice 20 weeks after TAC. Statistical significance of two-way ANOVA is defined as $p<0.05, $$p<0.01, $$$p<0.001,*

*indicating a significant overall effect of TAC relative to sham regardless of EC–specific Erbb4 deletion; Post hoc test (Bonferroni's Multiple Comparison test): *p<0.05, **p<0.01, ***p<0.001; Results are expressed as mean ± SEM.*

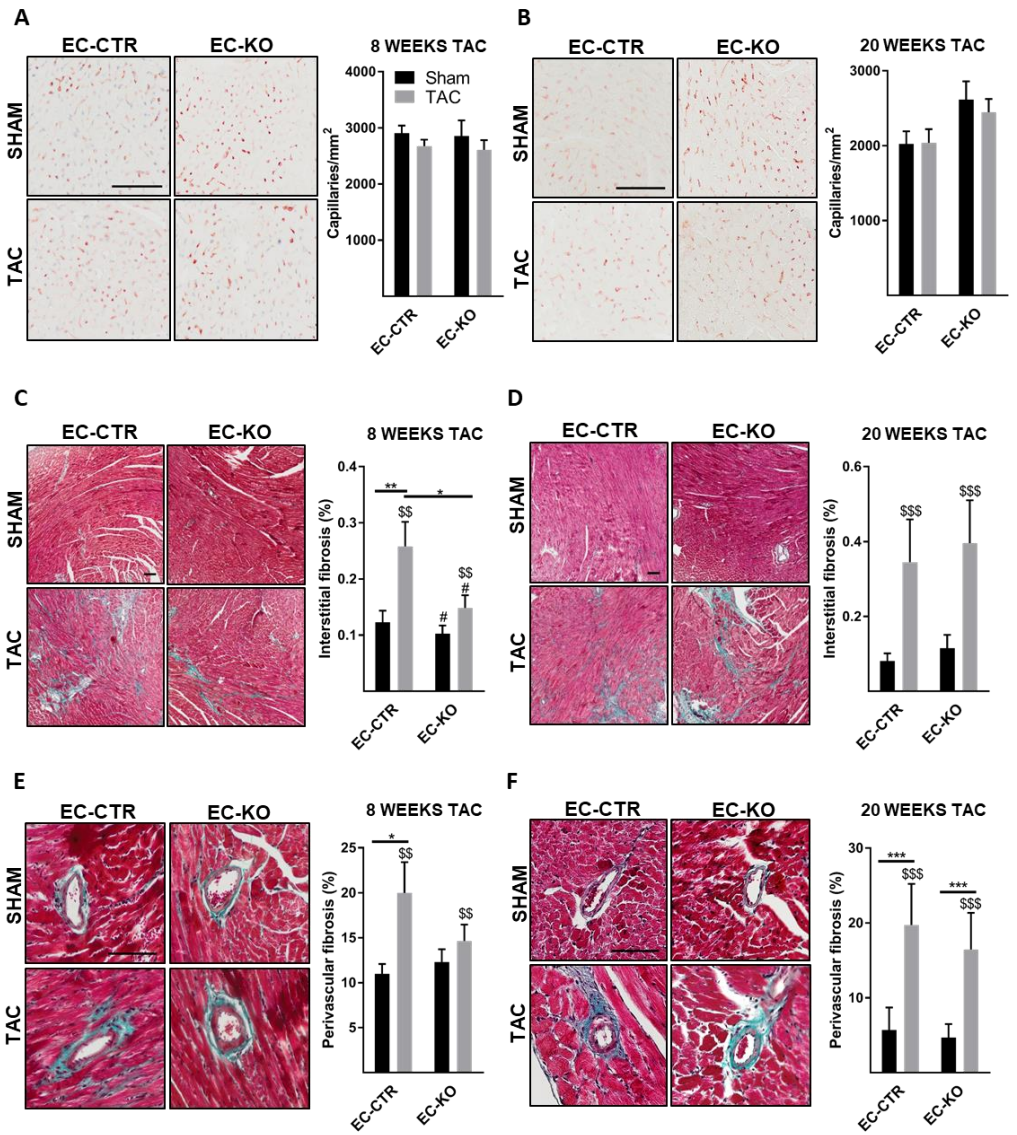

Figure 21: Endothelial-specific Erbb4 deletion did not affect capillary density, but alters fibrotic responses 8 to 20 weeks after TAC. *A, B: Representative IB4 stained heart sections (n=9–15; scale bar=100 µm), and quantification of capillary density (capillaries/mm^2) of control mice (EC-CTR) and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre$^+$ mice (EC-KO) 8 weeks after TAC (A) and 20 weeks after TAC (B). C, D: Representative Masson's Trichrome stained heart sections (n=9–15; scale bar=100 µm), and quantification of cardiac interstitial fibrosis of control mice versus tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre$^+$ mice 8 weeks after TAC (C) and 20 weeks after TAC*

*(D). E, F: Representative Masson's Trichrome stained heart sections (n=9–15; scale bar=100 µm), and quantification of perivascular fibrosis of control mice versus tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice 8 weeks after TAC (E) and 20 weeks after TAC (F). Statistical significance of two-way ANOVA is defined as $$p<0.01, $$$p<0.001, indicating a significant overall effect of TAC relative to sham regardless of EC–specific Erbb4 deletion, and as #p<0.05, indicating a significant overall effect of KO relative to CTR regardless the procedure; Post hoc test (Bonferroni's Multiple Comparison test): *p<0.05, **p<0.01, ***p<0.001; Results are expressed as mean ± SEM.*

(ii) Effect of endothelial-specific Erbb4 deletion in Ang II–induced cardiac remodeling. Next, effects of EC–specific *Erbb4* deletion were examined in a model of Ang II–induced cardiac remodeling. This model is typified by pronounced interstitial and perivascular fibrosis. Both control mice and EC–specific *Erbb4* KO mice were treated with Ang II for 4 weeks.

First, despite a significant increase in diastolic septal wall thickness from 0.87±0.02 mm to 1.04±0.05 mm ($p<0.05$), CM CSA (Figure 22A) and HW/BW were only mildly and not significantly increased in control mice (from $5.1*10^{-3}±0.3*10^{-3}$ to $5.5*10^{-3}±0.2*10^{-3}$; $p>0.05$). EC–specific *Erbb4* deletion did not affect this outcome (increase from $4.9*10^{-3}±0.2*10^{-3}$ to $5.3*10^{-3}±0.2*10^{-3}$; $p>0.05$) (Table S7).

As expected, myocardial capillary density was not changed by Ang II treatment neither in control mice nor in EC–specific *Erbb4* KO mice, with no statistically significant differences between groups (Figure 22B).

Next, Figure 22C and Figure 22D show the effects of Ang II treatment on cardiac fibrosis. Both interstitial and perivascular fibrosis were significantly increased by Ang II in control and EC–specific *Erbb4* KO mice. However, this fibrotic response was less pronounced in EC–specific *Erbb4* KO mice, and with regard to perivascular fibrosis, significantly less when compared to control mice (Figure 22D). To further explore this, we analyzed myocardial expression of fibrotic mediators. Figure 22E shows that myocardial *Fsp1* mRNA expression levels were lower in EC–*Erbb4* KO mice compared to control mice, but this remained unaffected by Ang II administration. Also *Tgf-β* mRNA levels were not altered by Ang II treatment. In addition, macrophage density was not altered, neither in

control mice, nor in EC–specific *Erbb4* KO mice after 4 weeks Ang II (Figure S24C and D).

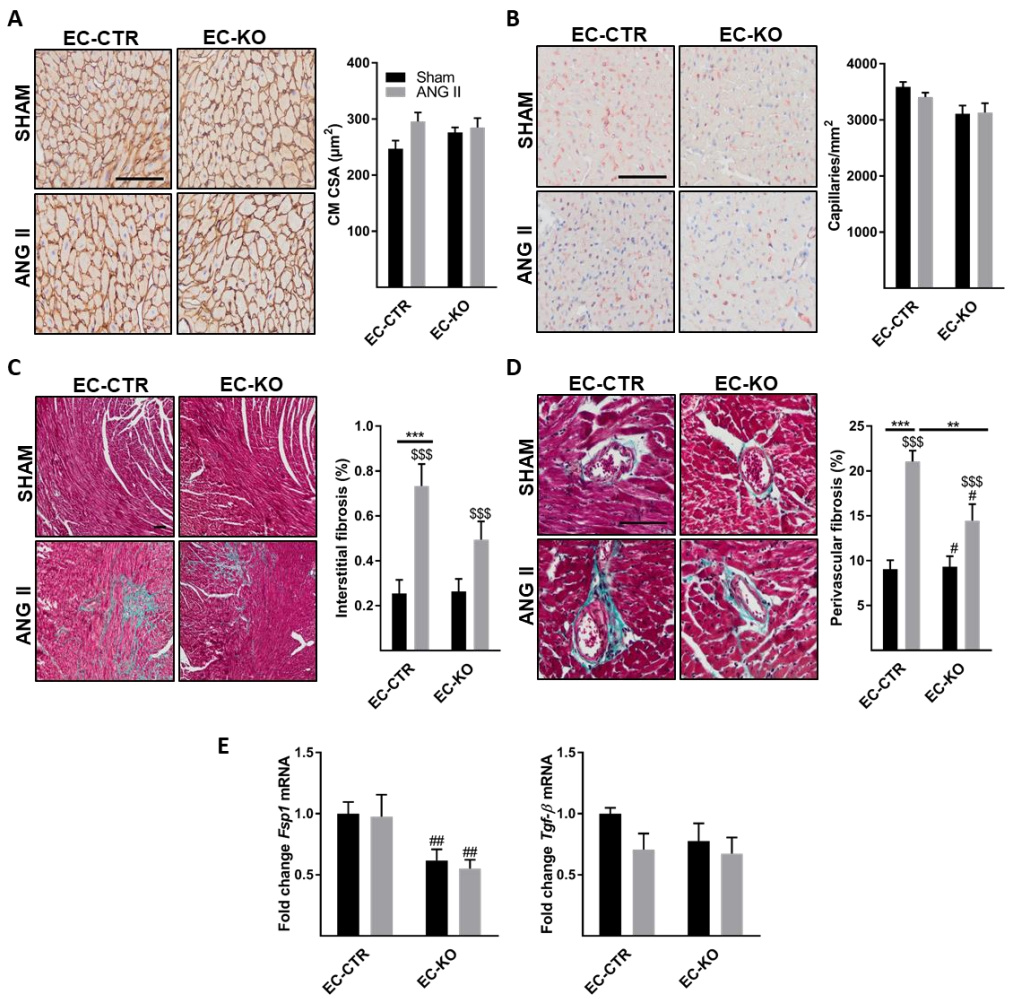

Figure 22: Endothelial-specific Erbb4 deletion did not affect hypertrophic responses or capillary density, but attenuated cardiac fibrosis after 4 weeks of Ang II treatment. *A: Representative laminin stained heart sections (n=9–10 per group; scale bar=100 µm), and quantification of CM CSA (µm²) of control mice (EC-CTR) and EC–specific Erbb4 KO mice (EC-KO) after 4 weeks of Ang II treatment. B: Representative IB4 stained heart sections (n=9–10; scale bar=100 µm), and quantification of capillary density (capillaries/mm²) of control mice and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice after 4 weeks of Ang II treatment. C: Representative Masson's Trichrome stained heart sections (n=9–10 per group; scale bar=100 µm), and quantification of cardiac interstitial fibrosis in hearts of control mice versus tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice after 4 weeks of Ang II treatment. D: Representative Masson's Trichrome stained heart sections (n=9–10 per group; scale bar=100 µm), and*

*quantification of perivascular fibrosis in hearts of control mice versus tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice after 4 weeks of Ang II treatment. E: Fold induction of mRNA expression of Fsp1, and Tgf-β, in heart samples of control mice versus tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice after 4 weeks of Ang II treatment. Results are expressed relative to control (sham). Statistical significance of two-way ANOVA is defined as $$$p<0.001, indicating a significant overall effect of Ang II relative to sham regardless of EC–specific Erbb4 deletion, and as #p<0.05, ##p<0.01, indicating a significant overall effect of KO relative to CTR regardless the procedure; Post hoc test (Bonferroni's Multiple Comparison test): **p<0.01, ***p<0.001; Results are expressed as mean ± SEM.*

(iii) Effect of endothelial-specific Erbb4 deletion MI–induced cardiac remodeling. Finally, we studied the effect of EC–specific *Erbb4* deletion in a model of MI. This model is characterized by LV dilation, decreased myocardial capillary density in the infarcted myocardium and its border zone, and by replacement fibrosis.

First, we observed a significant increase in LVID;d after MI in both control mice and EC–specific *Erbb4* KO mice, and this change was not different between both groups (Figure 23A). Also changes in FS% were not influenced by EC–specific *Erbb4* deletion (Table S8). Figure 23B shows that MI induced a significant increase in HW/BW in both control and EC–*Erbb4* KO mice, and also this effect was not different in both groups. CM CSA in the remote zone of infarction after 4 weeks remained unchanged in both groups (Figure 23A).

As expected, myocardial capillary density was significantly reduced in both the infarcted myocardium and its border zone. Again, these changes were not affected by EC–specific *Erbb4* deletion. In the remote zone, no changes in capillary density were observed, neither in control mice, nor in EC–specific *Erbb4* KO mice (Figure 23C).

Finally, with regard to myocardial fibrosis after MI, Figure 23D shows that both control mice and EC–specific *Erbb4* KO mice developed increased total myocardial fibrosis. At 4 weeks, there were no statistically significant differences between groups. Of note, at 4 weeks after MI, we did not observe increased interstitial myocardial fibrosis in the remote zone of infarction, neither in control mice, nor in EC–specific *Erbb4* KO mice (data not shown). 4 weeks after MI,

macrophage density was similar in EC-specific *Erbb4* mice and their control littermates (Figure S24E and F).

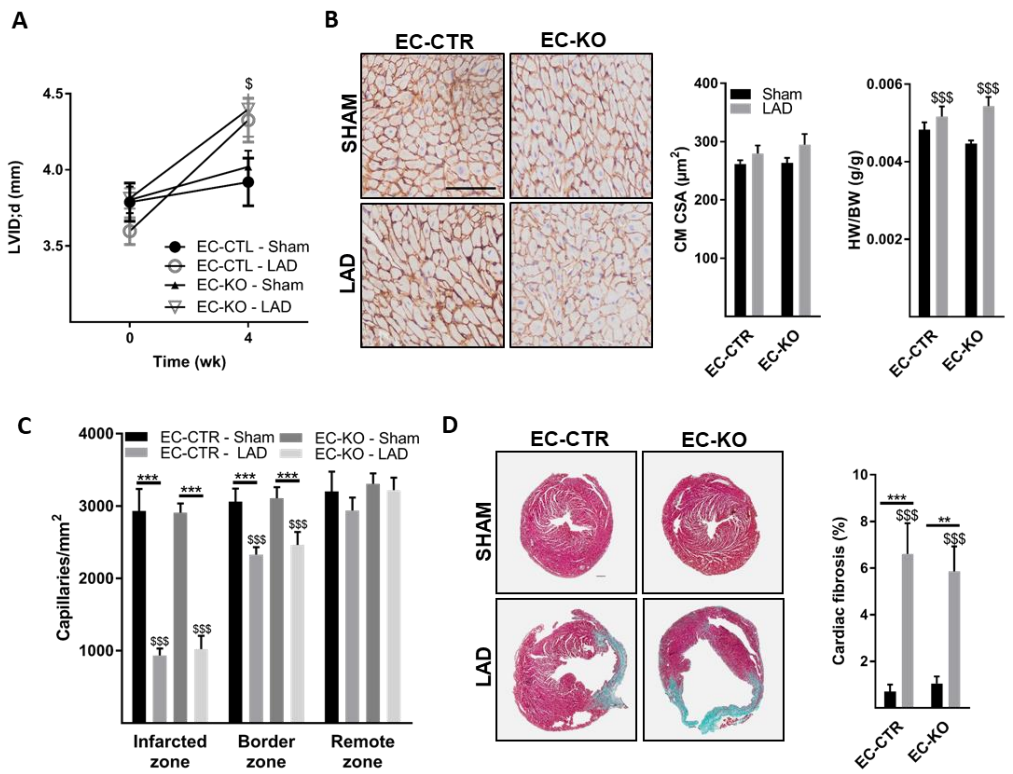

Figure 23: Endothelial-specific Erbb4 deletion did not affect cardiac performance, hypertrophy, capillary density, or fibrosis in an in vivo MI model. *A: Echocardiographic measurements of control mice (EC-CTR) and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice (EC-KO) with a follow-up of 4 weeks after MI (n=8–11 per group). Left ventricular internal diameter in diastole (LVID;d) is shown. B: Representative laminin stained heart sections (n=8–11 per group; scale bar=100 μm), and quantification of CM CSA (μm^2) and HW/BW (g/g) of control mice and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice 4 weeks after MI. C: Quantification of IB4 stained heart sections (n=8–11), capillary density (capillaries/mm^2) of both control mice and tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice was analyzed 4 weeks after MI in the infarcted zone, border zone, and remote zone. D: Representative Masson's Trichrome stained heart sections (n=8–11 per group; scale bar=1000 μm), and quantification of cardiac fibrosis of control mice versus tamoxifen-treated Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} mice 4 weeks after MI. Statistical significance of two-way ANOVA is defined as $p<0.05, $$$p<0.001, indicating a significant overall effect of LAD relative to sham regardless of EC–specific Erbb4 deletion; Post hoc test (Bonferroni's Multiple Comparison test): **p<0.01, ***p<0.001; Results are as mean ± SEM.*

Discussion

The major findings of this study are that increased NRG1/ERBB4 signaling in ECs *in vitro* induces angiogenic responses and reprograms several endothelial functions at the genomic level, but that selectively impeding the NRG1/ERBB4 autocrine loop *in vivo* only mildly affects cardiac remodeling following diverse types of myocardial injury.

Paracrine communication in the heart is crucial for normal cardiac function, but also plays an important role in cardiac remodeling (7, 10). NRG1 is a paracrine endothelial protein, which binds to ERBB4 on CMs, but also other cell types such as macrophages and fibroblasts (13, 21, 27). Here, we examined the role of endothelial autocrine NRG1/ERBB4 receptor signaling during cardiac remodeling. By specifically deleting the ERBB4 receptor on ECs, we selectively eliminated endothelial autocrine ERBB4 signaling without affecting paracrine NRG1/ERBB signaling in other cardiac cell types. Previous studies showed a modulatory role of ERBB2/ERBB3 signaling in angiogenesis *in vivo* (31). Here, we confirmed that NRG1 promotes endothelial angiogenic activity *in vitro*, and demonstrated the role of endothelial ERBB4 in this effect. Furthermore, we showed that NRG1 affects a broad range of functional pathways at the genomic level.

However, EC–specific *Erbb4* KO mice did not exhibit phenotypic abnormalities at baseline, including cardiac performance or capillary density in aging mice. Also, EC–specific *Erbb4* deletion did not impair *in vitro* vascular vasomotoricity or impair endothelium-dependent relaxation, which was coherent with our observation that NRG1 downregulated endothelial eNOS protein expression in cultured ECs. Although we did not observe a baseline phenotype in mice with EC-specific *Erbb4* deletion, EC-specific *Erbb4* deletion in other organs through the body should be acknowledged and carefully examined in future experiments. By applying three different models of cardiac overload/injury (pressure overload, Ang II, and MI) we observed that the NRG1/ERBB4 autocrine loop has no overt

role during cardiac remodeling. For example, EC–specific *Erbb4* deletion had no influence on cardiac performance or capillary density in either of these three models. However, in conditions of pressure overload LV remodeling was delayed by EC–specific *Erbb4* deletion, albeit returning to levels of control mice at 20 weeks after TAC, both in terms of CM hypertrophy and interstitial and perivascular fibrosis. Similar observations were made in mice exposed to 4 weeks of Ang II treatment, although in this model longer time points were not studied.

Attenuated cardiac hypertrophy and fibrosis by EC–specific deletion of *Erbb4*, albeit temporarily, is a surprising finding because it is in contrast with a previous study in which we showed that specific deletion of *Erbb4* in macrophages increased fibrosis (21). It is also surprising since NRG1/ERBB4 signaling is considered to be a cardioprotective system, and hypertrophy and fibrosis are viewed as maladaptive myocardial responses. However, our data suggest that EC–specific deletion of *Erbb4* only attenuates *early* remodeling. Early myocardial responses following injury are essential to cope with injury and overload and are not maladaptive *per se* (38). It is interesting to notice that these changes are diminished by EC–specific deletion of the ERBB4 receptor, hence that autocrine NRG1/ERBB4 signaling promotes these changes. Therefore, these observations are thought provoking in the understanding of myocardial cell-cell communication during remodeling, and performing future controlled time course experiments could contribute to our understanding of the role of autocrine NRG1/ERBB4 signaling during early cardiac remodeling, and its effects on late cardiac remodeling. Clearly, along with our previous study on specific *Erbb4* deletion in macrophages, this study shows the complexity of intercellular communication during cardiac remodeling, comprising spatio-temporal heterogeneity of myocardial autocrine and paracrine signaling following cardiac overload/injury. Moreover, our study suggests that endothelial NRG1/ERBB4 signaling could contribute to the protective effects of NRG1 during early cardiac remodeling in patients with CHF, but not during late cardiac remodeling. This

indicates that the previous observations regarding paracrine anti-fibrotic effects of NRG1 remain unaffected (21, 27).

What could be the underlying molecular mechanism of this autocrine endothelial modulation of early cardiac remodeling? RNA-sequencing data on altered gene expression in HMVECs, showed that NRG1 upregulated *Skil*, *Pmepa1*, *Thbs1*, *Mmp2*, *Chst11*, *Egr1*, *Egr2*, *Pdgfa*, and *Htra1*. *Skil*, *Pmepa1*, *Thbs1*, *Mmp2*, *Htra1* are positively correlated with mRNA *Tgf-β* levels and associated with fibrotic processes (39-41) like wound healing (42) and synthesis of ECM (43). A recent study showed that treatment of vascular smooth muscle cells with TGF-β increases *Chst11* mRNA concentrations (44). *Egr1*, a Smad-independent mediator of TGF-β signaling, induces fibrotic processes and is linked to different animal models of fibrosis (45, 46). *Egr2*, on the other hand, could stimulate collagen gene expression and myofibroblast differentiation (47). Gallini *et al.* showed the association of *Pdgfa* with cardiac fibrosis in transgenic mice with overexpression of *Pdgfa* (48). Therefore, NRG1 could activate early cardiac fibrotic responses by activation of these endothelial factors, but further experimental proof for these pathways is currently missing.

This study does not address the dimerization partner of ERBB4. ERBB2 is known to be the preferred dimerization partner of ligand-activated ERBB4. However, NRG1 may also induce the formation of ERBB4 homodimers, or ERBB4/ERBB3 heterodimers. However, our *in vitro* and *in vivo* results indicate that expression—and hence activation—of ERBB3 in ECs is low. Currently, it is unknown to what extend EC-specific homotypic (ERBB4/ERBB4) and heterotypic signaling (ERBB2/ERBB4) contribute to the cardioprotective effects of NRG1. Experiments with EC-specific deletion of *Erbb2* could be helpful in answering this question.

To summarize, this study shows direct effects of NRG1 on human ECs *in vitro*. EC–specific *Erbb4* deletion in mice attenuated early cardiac responses following pressure overload and Ang II activation, as shown by a delayed hypertrophic and fibrotic response. EC–specific *Erbb4* deletion did not influence capillary

density or change cardiac performance *in vivo*. These data contribute to our understanding of myocardial cell-cell communication during cardiac remodeling and to the role of autocrine NRG1/ERBB4 signaling specifically, and provides new avenues for future research.

Supplementary data

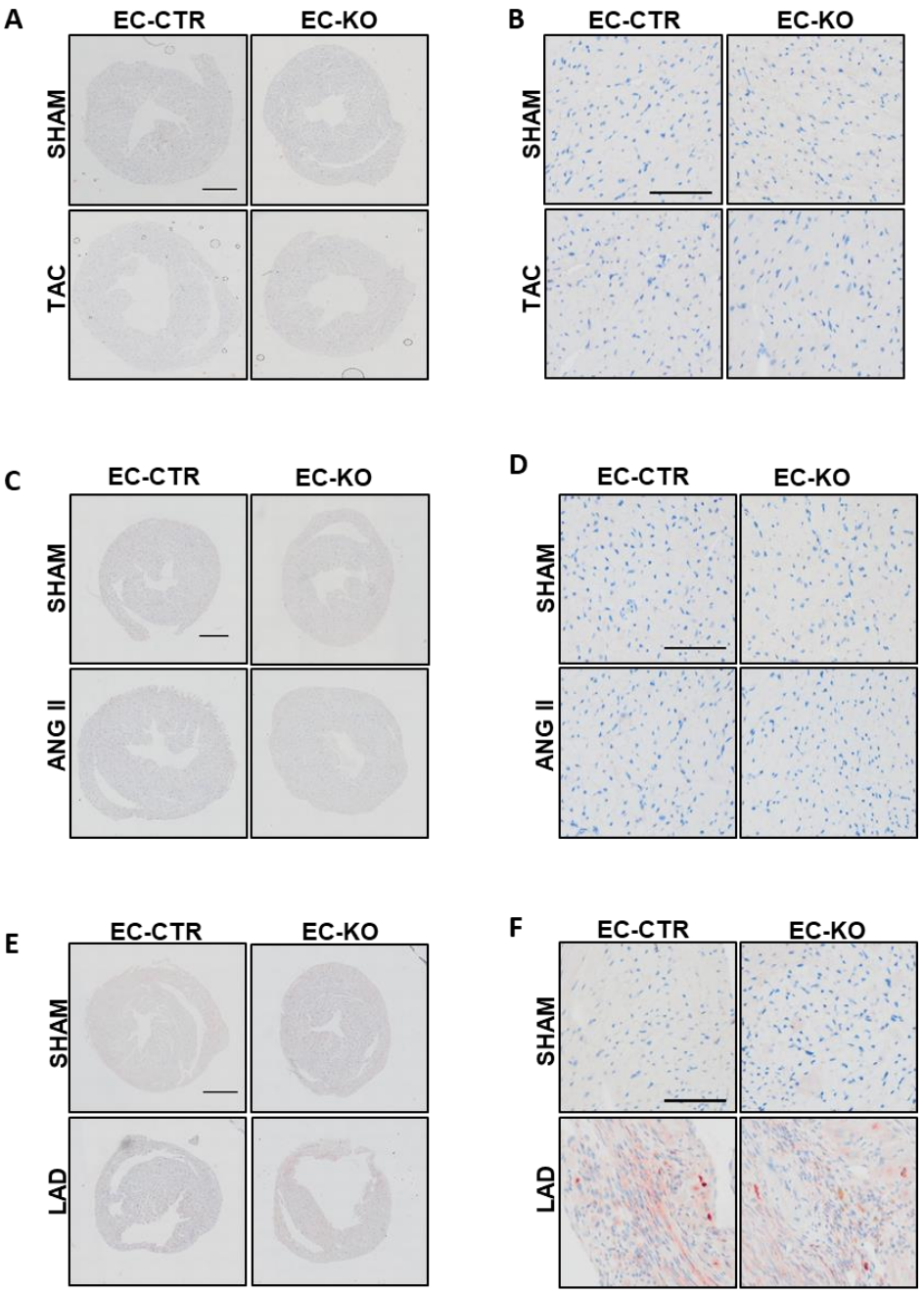

Figure S24: Endothelial-specific Erbb4 deletion did not affect macrophage density following TAC, Ang II treatment, or MI. *A, B, C, D, E, F: Representative MAC3 stained heart*

sections (n=7–15 per group; scale bar=1000 µm (A, C), scale bar=100 µm (B, D)) of control mice (EC-CTR) and EC–specific Erbb4 KO mice (EC-KO) 8 weeks after TAC (A, B),after 4 weeks of Ang II treatment (C, D), and 4 weeks after MI (E, F).

Table S2: Comparison of the transcriptome of HMVECs with and without NRG1 treatment. *Upregulated genes in human microvascular endothelial cells (HMVECs) upon 8 and 24 h NRG1 treatment were analyzed using RNA-sequencing. Data are ranked according linkage to different biological processes; fold change (FC) and adjusted p-values (padj) are shown relative to control samples; n=4 per group.*

Upregulated genes in HMVECs upon NRG1 treatment

	NRG1 (h)	Gene	Gene name	FC	padj
Fibrosis/ ECM	8	NR4A1	nuclear receptor subfamily 4 group A member 1	4.7	$5.2*10^{-5}$
		PMEPA1	prostate transmembrane protein, androgen induced 1	1.6	$6.3*10^{-6}$
		CHST11	carbohydrate sulfotransferase 11	1.4	$4.2*10^{-2}$
		SYNM	synemin	1.4	$5.0*10^{-2}$
		SKIL	SKI like proto-oncogene	1.3	$5.3*10^{-4}$
		MMP2	matrix metallopeptidase 2	1.3	$1.3*10^{-2}$
		HTRA1	HtrA serine peptidase 1	1.2	$7.1*10^{-2}$
		PDGFA	platelet derived growth factor subunit A	1.2	$6.2*10^{-3}$
		FNDC3B	fibronectin type III domain containing 3B	1.1	$6.1*10^{-2}$
		THBS1	thrombospondin 1	1.1	$8.1*10^{-3}$
	24	EGR2	early growth response 2	15.1	$1.6*10^{-2}$
		NR4A1	nuclear receptor subfamily 4 group A member 1	6.1	$2.0*10^{-7}$
		EGR1	early growth response 1	2.2	$4.6*10^{-7}$
		THBS1	thrombospondin 1	1.1	$1.1*10^{-2}$
Inflammation	8	TNFRSF10D	TNF receptor superfamily member 10d	1.6	$6.3*10^{-6}$
		IL6ST	interleukin 6 signal transducer	1.2	$6.7*10^{-2}$
	24	CSF3	colony stimulating factor 3	4.7	$9.1*10^{-2}$
		CXCL2	C-X-C motif chemokine ligand 2	2.0	$1.2*10^{-8}$

		CEBPD	CCAAT enhancer binding protein delta	1.8	$4.2*10^{-2}$
		TNFAIP 2	TNF alpha induced protein 2	1.6	$2.3*10^{-5}$
		PTGS2	prostaglandin-endoperoxide synthase 2	1.6	$5.7*10^{-6}$
		CXCL1	C-X-C motif chemokine ligand 1	1.5	$9.6*10^{-3}$
		CXCL3	C-X-C motif chemokine ligand 3	1.5	$2.5*10^{-2}$
		ANXA5	annexin A5	1.2	$2.4*10^{-2}$
Proliferation/ Growth/ Differentiation	8	ZNF365	zinc finger protein 365	2.4	$6.2*10^{-2}$
		ADRA1B	adrenoceptor alpha 1B	1.7	$8.1*10^{-3}$
		INHBA	inhibin subunit beta A	1.5	$5.7*10^{-2}$
		SPRY1	sprouty RTK signaling antagonist 1	1.5	$3.9*10^{-2}$
		CLN8	CLN8, transmembrane ER and ERGIC protein	1.4	$8.6*10^{-2}$
		STK38L	serine/threonine kinase 38 like	1.2	$8.4*10^{-2}$
		HSPB1	heat shock protein family B (small) member 1	1.2	$9.3*10^{-2}$
		ALPK3	alpha kinase 3	1.2	$8.6*10^{-2}$
		DAAM1	dishevelled associated activator of morphogenesis 1	1.2	$4.4*10^{-2}$
	24	FOS	Fos proto-oncogene, AP-1 transcription factor subunit	1.8	$4.7*10^{-9}$
		DUSP5	dual specificity phosphatase 5	1.6	$1.9*10^{-3}$
		DUSP1	dual specificity phosphatase 1	1.3	$1.1*10^{-}$
		ANXA5	annexin A5	1.2	$2.4*10^{-2}$
Immune system	8	SLC7A2	solute carrier family 7 member 2	1.4	$4.9*10^{-2}$
		SPSB1	splA/ryanodine receptor domain and SOCS box containing 1	1.4	$7.1*10^{-2}$
		IL4R	interleukin 4 receptor	1.2	$4.7*10^{-2}$
		HRH1	histamine receptor H1	1.2	$5.0*10^{-2}$

	24	TIFA	TRAF interacting protein with forkhead associated domain	1.6	$4.1*10^{-2}$
Mitosis	8	SPDL1	spindle apparatus coiled-coil protein 1	1.3	$8.6*10^{-2}$
	24	EGR1	early growth response 1	2.2	$4.6*10^{-7}$
		PTGS2	prostaglandin-endoperoxide synthase 2	1.6	$5.7*10^{-6}$
Angiogenesis	8	VASH1	vasohibin 1	1.3	$3.9*10^{-5}$
		ESM1	endothelial cell specific molecule 1	1.2	$1.6*10^{-2}$
		PDGFA	platelet derived growth factor subunit A	1.2	$6.2*10^{-3}$
Apoptosis	8	GULP1	GULP PTB domain containing engulfment adaptor 1	1.3	$3.9*10^{-2}$
		PPP1R10	protein phosphatase 1 regulatory subunit 10	1.3	$4.5*10^{-2}$
		NEDD9	neural precursor cell expressed, developmentally down-regulated 9	1.2	$1.1*10^{-2}$
		FAM129B	family with sequence similarity 129 member B	1.2	$8.4*10^{-2}$
		MT2A	metallothionein 2A	1.2	$2.9*10^{-2}$
	24	C3orf38	chromosome 3 open reading frame 38	1.5	$6.5*10^{-2}$
		PPP1R15A	protein phosphatase 1 regulatory subunit 15A	1.3	$6.5*10^{-4}$
		RAPGEF1	Rap guanine nucleotide exchange factor 1	1.3	$6.4*10^{-2}$
Transcription/ Translation	8	FIZ1	FLT3 interacting zinc finger 1	2.0	$7.1*10^{-2}$
		CSRNP1	cysteine and serine rich nuclear protein 1	1.4	$5.7*10^{-2}$
		ZNF264	zinc finger protein 264	1.3	$9.3*10^{-2}$
		RGL1	ral guanine nucleotide dissociation stimulator like 1	1.3	$3.6*10^{-2}$
		MAFK	MAF bZIP transcription factor K	1.3	$9.7*10^{-2}$
		TULP4	tubby like protein 4	1.3	$5.0*10^{-2}$

		RPS19BP1	ribosomal protein S19 binding protein 1	1.2	$9.7*10^{-2}$
	24	ATF3	activating transcription factor 3	2.0	$4.1*10^{-10}$
		CSRNP1	cysteine and serine rich nuclear protein 1	1.5	$6.3*10^{-4}$
		RASD1	ras related dexamethasone induced 1	1.5	$1.8*10^{-3}$
		JUNB	JunB proto-oncogene, AP-1 transcription factor subunit	1.5	$1.2*10^{-4}$
		IER2	immediate early response 2	1.4	$8.4*10^{-9}$
		HES1	hes family bHLH transcription factor 1	1.4	$3.4*10^{-3}$
		TCEAL3	transcription elongation factor A like 3	1.2	$7.6*10^{-2}$
		YBX3	Y-box binding protein 3	1.3	$1.6*10^{-3}$
Metabolism	8	CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	1.6	$9.3*10^{-2}$
		PDXK	pyridoxal kinase	1.3	$2.8*10^{-3}$
		STC2	stanniocalcin 2	1.2	$6.4*10^{-2}$
	24	SOD2	superoxide dismutase 2	1.4	$7.8*10^{-2}$
		SELENOF	selenoprotein F	1.2	$7.81*10^{-2}$
		SAT1	spermidine/spermine N1-acetyltransferase 1	1.2	$5.4*10^{-3}$
Ion Channel	8	CHRNA5	cholinergic receptor nicotinic alpha 5 subunit	2.8	$3.7*10^{-2}$
		GJA5	gap junction protein alpha 5	2.2	$2.3*10^{-8}$
		KCMF1	potassium channel modulatory factor 1	1.3	$2.2*10^{-2}$
Ribosomal factors	8	RRBP1	ribosome binding protein 1	1.2	$5.3*10^{-2}$
	24	RPL23A	ribosomal protein L23a	1.2	$4.6*10^{-2}$
		RPS25	ribosomal protein S25	1.2	$2.3*10^{-2}$
Transmembrane proteins	8	PMEPA1	prostate transmembrane protein, androgen induced 1	1.6	$6.3*10^{-6}$

		JAG2	jagged 2	1.4	$3.5*10^{-2}$
		CRIM1	cysteine rich transmembrane BMP regulator 1	1.2	$5.0*10^{-2}$
	24	GNG11	G protein subunit gamma 11	1.2	$4.7*10^{-3}$
Calcium ion binding	8	C2CD4B	C2 calcium dependent domain containing 4B	1.3	$5.7*10^{-2}$
	24	C2CD4B	C2 calcium dependent domain containing 4B	1.8	$4.4*10^{-10}$

Table S3: Comparison of the transcriptome of HMVECs with and without NRG1 treatment. *Downregulated genes in human microvascular endothelial cells (HMVECs) upon 8 and 24 h NRG1 treatment were analyzed using RNA-sequencing. Data are ranked according linkage to different biological processes; fold change (FC) and adjusted p-values (padj) are shown relative to control samples; n=4 per group.*

Downregulated genes in HMVECs upon NRG1 treatment

	NRG1 (h)	Gene	Gene name	FC	padj
Fibrosis	8	MMP1	matrix metallopeptidase 1	0.8	$7.4*10^{-2}$
Inflammation	8	NFKBIA	NFKB inhibitor alpha	0.8	$1.1*10^{-3}$
		ANXA1	annexin A1	0.8	$3.6*10^{-3}$
		PLCB2	phospholipase C beta 2	0.7	$5.0*10^{-2}$
		CXCL2	C-X-C motif chemokine ligand 2	0.7	$9.3*10^{-2}$
		SAMHD1	SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1	0.7	$1.5*10^{-2}$
		CXCL8	C-X-C motif chemokine ligand 8	0.6	$2.8*10^{-5}$
		CDCP1	CUB domain containing protein 1	0.5	$7.! *10^{-5}$
		MILR1	mast cell immunoglobulin like receptor 1	0.5	$9.3*10^{-2}$
		FES	FES proto-oncogene, tyrosine kinase	0.5	$1.3*10^{-2}$
		CXCL3	C-X-C motif chemokine ligand 3	0.3	$1.6*10^{-20}$
Immune system	8	WSB1	WD repeat and SOCS box containing 1	0.8	$1.8*10^{-4}$
		NHLRC3	NHL repeat containing 3	0.7	$9.3*10^{-2}$
		TARBP1	TAR (HIV-1) RNA binding protein 1	0.6	$2.6*10^{-2}$
	24	WSB1	WD repeat and SOCS box containing 1	0.9	$3.4*10^{-2}$
		PSMB1	proteasome subunit beta 1	0.9	$4.2*10^{-2}$
		ARL8A	ADP ribosylation factor like GTPase 8A	0.8	$2.4*10^{-2}$
	8	SHTN1	shootin 1	0.8	0.1

Proliferation/ Growth/ Differentiation		PHLDA1	pleckstrin homology like domain family A member 1	0.8	$1.3*10^{-2}$
		FAM107A	family with sequence similarity 107 member A	0.8	$9.7*10^{-3}$
		GADD45A	growth arrest and DNA damage inducible alpha	0.8	$2.8*10^{-2}$
		CLDN11	claudin 11	0.7	$4.3*10^{-2}$
		IRF1	interferon regulatory factor 1	0.7	$4.9*10^{-2}$
		IGF1	insulin like growth factor 1	0.7	$4.9*10^{-2}$
		TXNIP	thioredoxin interacting protein	0.6	$1.2*10^{-12}$
		TXNIP	thioredoxin interacting protein	0.6	$1.2*10^{-12}$
		FES	FES proto-oncogene, tyrosine kinase	0.5	$1.3*10^{-2}$
		FAM72A	family with sequence similarity 72 member A	0.2	$1.5*10^{-2}$
	24	CD63	CD63 molecule	0.9	$3.2*10^{-3}$
		ZBTB20	zinc finger and BTB domain containing 20	0.8	$6.7*10^{-4}$
		AKT2	AKT serine/threonine kinase 2	0.7	$2.9*10^{-2}$
Transcription/ Translation	8	CD63	CD63 molecule	0.9	3.2
		ZFP36L1	ZFP36 ring finger protein like 1	0.9	$3.5*10^{-2}$
		CD34	CD34 molecule	0.8	$9.8*10^{-2}$
		SOX7	SRY-box 7	0.8	$1.1*10^{-2}$
		DACH1	dachshund family transcription factor 1	0.7	$2.2*10^{-2}$
		MDFIC	MyoD family inhibitor domain containing	0.7	$3.8*10^{-2}$
	24	DDB1	damage specific DNA binding protein 1	0.9	$1.0*10^{-2}$
		SUGP2	SURP and G-patch domain containing 2	0.8	$4.2*10^{-2}$
		RBM6	RNA binding motif protein 6	0.8	$3.2*10^{-3}$
		DIP2A	disco interacting protein 2 homolog A	0.8	$1.6*10^{-3}$

		GLIS3	GLIS family zinc finger 3	0.7	$5.7*10^{-2}$
Angiogenesis	8	CAV1	caveolin 1	0.9	$4.4*10^{-2}$
		ZFP36L1	ZFP36 ring finger protein like 1	0.9	$3.5*10^{-2}$
		JUN	Jun proto-oncogene, AP-1 transcription factor subunit	0.8	$4.9*10^{-2}$
		ANGPT2	angiopoietin 2	0.7	$5.8*10^{-4}$
		IGF1	insulin like growth factor 1	0.7	$4.9*10^{-2}$
		ADM	adrenomedullin	0.7	$4.6*10^{-11}$
	24	DPYSL3	dihydropyrimidinase like 3	0.9	$2.3*10^{-2}$
Metabolism	8	SAT1	spermidine/spermine N1-acetyltransferase 1	0.9	$5.4*10^{-2}$
		ND6	NADH dehydrogenase, subunit 6 (complex I)	0.8	$3.4*10^{-2}$
		TXNIP	thioredoxin interacting protein	0.6	$1.2*10^{-12}$
		TIMM8A	translocase of inner mitochondrial membrane 8A	0.6	$6.2*10^{-2}$
	24	ATP5MG	ATP synthase membrane subunit g	0.9	$7.1*10^{-2}$
		SLC20A1	solute carrier family 20 member 1	0.8	$3.4*10^{-3}$
		RABL2B	RAB, member of RAS oncogene family like 2B	0.8	$9.1*10^{-2}$
Mitosis	24	ZNF207	zinc finger protein 207	0.8	$4.2*10^{-2}$
Autophagy	8	ATG14	autophagy related 14	0.8	$3.7*10^{-2}$
Apoptosis	8	PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	0.8	$1.7*10^{-5}$
Migration/ Motility	8	AFAP1L1	actin filament associated protein 1 like 1	0.8	$5.0*10^{-2}$
		FILIP1	filamin A interacting protein 1	0.7	$5.0*10^{-2}$
		APOL2	apolipoprotein L2	0.7	$4.4*10^{-3}$
		CCDC40	coiled-coil domain containing 40	0.2	$1.8*10^{-2}$
	24	TUBA1B	tubulin alpha 1b	0.8	$6.0*10^{-3}$

		TUBA1A	tubulin alpha 1a	0.8	$3.2*10^{-3}$
Transmembrane proteins	8	TMEM217	transmembrane protein 217	0.8	$4.6*10^{-2}$
	24	TMEM186	transmembrane protein 186	0.7	$1.0*10^{-2}$
		TMEM144	transmembrane protein 144	0.6	$9.0*10^{-3}$

Table S4: Body weight, heart weight/body weight (HW/BW), and echocardiographic measurements of Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} + vehicle versus Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} + tamoxifen at 12 weeks of age. *Results are expressed as mean ± SEM.*

Parameter	Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} + vehicle	Erbb4$^{F/F}$ VE-Cdh5-Cre^{+} + tamoxifen
BW, g	28.0 ± 2.2	29.6 ± 2.2
HW/BW	4.6 ± 0.2*10^{-3}	4.5 ± 0.2*10^{-3}
IVS;d, mm	1.0 ± 0.1	0.9 ± 0.0
IVS;s, mm	1.3 ± 0.1	1.3 ± 0.0
LVPWd, mm	0.9 ± 0.1	0.9 ± 0.1
LVPWs, mm	1.1 ± 0.1	1.2 ± 0.1
LVIDd, mm	3.9 ± 0.1	4.1 ± 0.1
LVIDs, mm	2.9 ± 0.1	3.1 ± 0.1
EF%	51 ± 3	51 ± 3
FS%	26 ± 2	26 ± 2

Table S5: Body weight, heart weight/body weight (HW/BW), and echocardiographic measurements of control mice versus EC-specific Erbb4 KO mice 8 weeks after TAC. *Statistical significance of two-way ANOVA is defined as $p<0.05, $$$p<0.001, indicating a significant overall effect of TAC relative to sham regardless of EC–specific Erbb4 deletion; Post hoc test: §§p<0.01, §§§p<0.001 (EC-WT-sham versus EC-WT-TAC); **p<0.01, ***p<0,001 (EC-KO-sham versus EC-KO-TAC). Results are expressed as mean ± SEM.*

Parameter	EC–WT sham	EC–WT 8 wk TAC	EC–KO sham	EC–KO 8 wk TAC
BW, g	27.4 ± 1.9	27.6 ± 1.7	25.4 ± 2.1	23.1 ± 1.4
HW/BW ($$$)	4.5 ± 0.2*10^{-3}	7.8 ± 0.8*10^{-3} (***)	5.3 ± 0.2*10^{-3}	6.9 ± 0.4*10^{-3}
IVS;d, mm ($$$)	0.9 ± 0.0	1.2 ± 0.1 (**)	1.0 ± 0.0	1.2 ± 0.1
IVS;s, mm	1.3 ± 0.0	1.5 ± 0.1	1.3 ± 0.0	1.5 ± 0.1
LVPWd, mm ($)	0.9 ± 0.0	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
LVPWs, mm	1.1 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
LVIDd, mm	3.9 ± 0.1	4.1 ± 0.1	3.8 ± 0.1	3.9 ± 0.1
LVIDs, mm	3.1 ± 0.1	3.5 ± 0.1	3.1 ± 0.1	3.2 ± 0.1
EF%	43 ± 2	33 ± 2	41 ± 3	39 ± 5
FS%	21 ± 1	16 ± 1	19 ± 2	19 ± 3

Table S6: Body weight, heart weight/body weight, and echocardiographic measurements of control mice versus EC-specific Erbb4 KO mice 20 weeks after TAC. *Statistical significance of two-way ANOVA is defined as $p<0.05, $$p<0.01, $$$p<0.001, indicating a significant overall effect of TAC relative to sham regardless of EC–specific Erbb4 deletion; Post hoc test: §p<0.05, §§§p<0.001 (EC-WT-sham versus EC-WT-TAC); *p<0.05, **p<0.01 (EC-KO-sham versus EC-KO-TAC). Results are expressed as mean ± SEM.*

Parameter	EC–WT sham	EC–WT 20 wk TAC	EC–KO sham	EC–KO 20 wk TAC
BW, g	28.1 ± 1.5	27.8 ± 1.2	29.1 ± 1.4	29.2 ± 1.6
HW/BW ($$$)	4.4 ± $0.2*10^{-3}$	6.5 ± $0.6*10^{-3}$ (§§§)	4.1 ± $0.1*10^{-3}$	5.9 ± $0.4*10^{-3}$ (*)
IVS;d, mm ($$$)	1.1 ± 0.0	1.2 ± 0.0 (§)	1.0 ± 0.0	1.2 ± 0.0 (**)
IVS;s, mm	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.0	1.5 ± 0.1
LVPWd, mm ($$$)	1.0 ± 0.0	1.2 ± 0.1 (§)	0.9 ± 0.0	1.1 ± 0.1 (*)
LVPWs, mm	1.2 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
LVIDd, mm ($)	3.8 ± 0.1	4.2 ± 0.3	3.8 ± 0.1	4.1 ± 0.1
LVIDs, mm ($$)	3.1 ± 0.2	3.7 ± 0.3	2.9 ± 0.1	3.5 ± 0.2
EF% ($$$)	41 ± 4	29 ± 3	50 ± 3	33 ± 4 (*)
FS% ($$$)	20 ± 2	14 ± 2	25 ± 2	16 ± 2 (**)

Table S7: Body weight, heart weight/body weight, and echocardiographic measurements of control mice versus EC-specific Erbb4 KO mice after 4 weeks Ang II treatment. *Statistical significance of two-way ANOVA is defined as $$p<0.01, indicating a significant overall effect of Ang II relative to sham regardless of EC–specific Erbb4 deletion; Post hoc test: §§p<0.01 (EC-WT-sham versus EC-WT-TAC). Results are expressed as mean ± SEM.*

Parameter	EC–WT sham	EC–WT Ang II	EC–KO sham	EC–KO Ang II
BW, g	22.9 ± 0.7	24.6 ± 0.9	24.9 ± 1.1	24.2 ± 1.2
HW/BW	$5.1 \pm 0.3*10^{-3}$	$5.5 \pm 0.2*10^{-3}$	$4.9 \pm 0.2*10^{-3}$	$5.3 \pm 0.3*10^{-3}$
IVS;d, mm ($$)	0.9 ± 0.0	1.1 ± 0.1 (§§)	0.9 ± 0.0	1.0 ± 0.0
IVS;s, mm ($$)	1.1 ± 0.1	1.4 ± 0.1 (§§)	1.2 ± 0.0	1.3 ± 0.1
LVPWd, mm ($$)	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.1
LVPWs, mm	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
LVIDd, mm	3.8 ± 0.1	3.7 ± 0.1	4.0 ± 0.1	3.7 ± 0.1
LVIDs, mm	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.1	3.0 ± 0.1
EF%	37 ± 4	38 ± 2	42 ± 3	38 ± 4
FS%	18 ± 2	18 ± 1	20 ± 2	18 ± 2

Table S8: Body weight, heart weight/body weight, and echocardiographic measurements of control mice versus EC-specific Erbb4 KO mice 4 weeks after MI. *Statistical significance of two-way ANOVA is defined as $p<0.05, $$p<0.01, indicating a significant overall effect of TAC relative to sham regardless of EC–specific Erbb4 deletion; Post hoc test: **p<0.01 (EC-KO-sham versus EC-KO-TAC). Results are expressed as mean ± SEM.*

Parameter	EC–WT sham	EC–WT LAD	EC–KO sham	EC–KO LAD
BW, g	23.1 ± 1.5	23.8 ± 1.0	25.4 ± 1.3	24.5 ± 1.1
HW/BW ($$)	$4.8 \pm 0.2*10^{-3}$	$5.2 \pm 0.3*10^{-3}$	$4.5 \pm 0.1*10^{-3}$	$5.4 \pm 0.2*10^{-3}$ (**)
IVS;d, mm ($)	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
IVS;s, mm ($)	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
LVPWd, mm	0.7 ± 0.0	0.8 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
LVPWs, mm	0.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.1
LVIDd, mm ($)	3.9 ± 0.2	4.3 ± 0.1	4.0 ± 0.1	4.4 ± 0.2
LVIDs, mm	3.2 ± 0.2	3.3 ± 0.1	3.1 ± 0.1	3.6 ± 0.2
EF%	37 ± 4	47 ± 3	46 ± 3	37 ± 3
FS%	18 ± 2	24 ± 1	23 ± 2	18 ± 2

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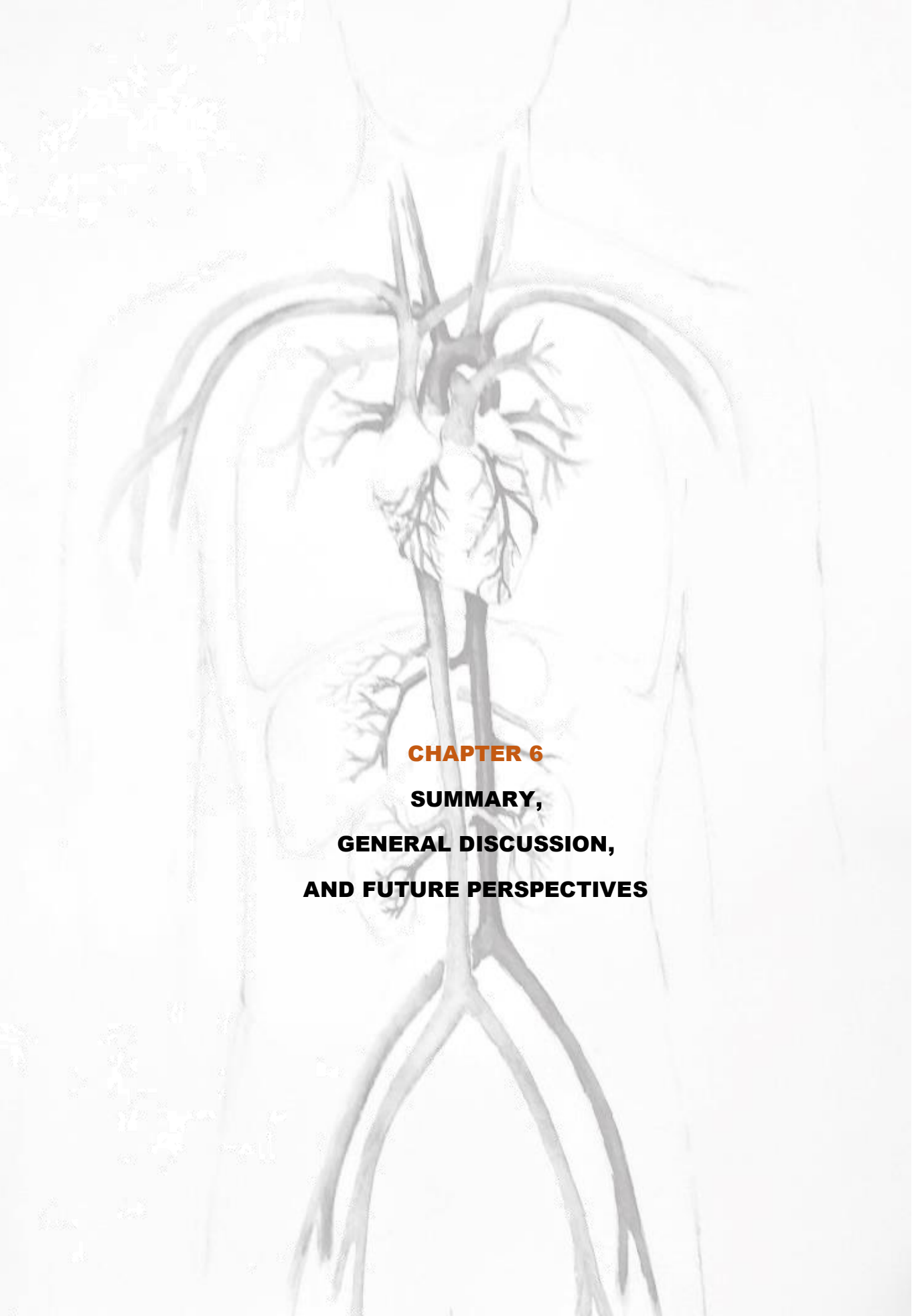

CHAPTER 6

SUMMARY, GENERAL DISCUSSION, AND FUTURE PERSPECTIVES

Background and hypotheses

The heart is a pluricellular organ, in which cardiac remodeling comprises cellular changes in different cell types in the heart—ECs, CMs, fibroblasts, inflammatory cells, and VSMC. NRG1/ERBB signaling is critical during cardiac remodeling (1), it allows the heart to cope with physiological stress (2). It has been shown in both preclinical and clinical studies that administration of NRG1 attenuates cardiac remodeling and improves cardiac function in different models of HF (3-7). Our laboratory has been studying the NRG1/ERBB pathway in the pathophysiology of HF for almost 2 decades. During exploration of ERBB tyrosine kinase signaling in the heart, the central role of ERBB2 signaling in CMs has become evident. Activation of ERBB2 in CMs through ERBB4 or ERBB3 contributes to the cardioprotective effects of NRG1, but it is unclear to what extent this scenario explains the full picture. ERBB2, ERBB3 and ERBB4 receptors are also expressed by non-myocyte cardiac cells, including inflammatory cells, fibroblasts and ECs. Moreover, different studies indicate that NRG1/ERBB4 signaling in cardiac non-myocytes is equally important (8, 9). We previously demonstrated that NRG1, a member of the epidermal growth factor family, has direct effects on macrophages through ERBB4 and decreases inflammation and fibrosis in a model of Ang II–induced myocardial fibrosis (8).

In this thesis, we aimed to study ERBB4 signaling in non-myocytes, specifically in ECs and inflammatory cells, and challenge the current concept that the cardiac effects of NRG1 are mediated by paracrine activation of ERBB4/ERBB2 receptors on CMs (10, 11). We studied the effect of impaired NRG1/ERBB4 signaling in both ECs and myeloid cells, hence how NRG1/ERBB4 signaling in ECs or inflammatory cells could contribute to the beneficial effects of NRG1 during cardiac remodeling.

The central hypothesis of this thesis is that NRG1/ERBB4 receptor signaling in non-myocytes contributes to the beneficial effects of NRG1 during cardiac remodeling:

- ***(i)*** ***<u>ERBB4-dependent paracrine NRG1 signaling in myeloid cells</u>*** *plays a role during cardiac remodeling.*
- ***(ii)*** ***<u>ERBB4-dependent autocrine NRG1 signaling in ECs</u>*** *plays a role during cardiac remodeling.*

Overview of key findings and general conclusions

In ***<u>Chapter 4</u>***, we generated mice with myeloid-specific deletion of *Erbb4* and tested the effects of myeloid-specific *Erbb4* deletion in 2 models of cardiac remodeling—MI and pressure overload. We observed that myeloid-specific *Erbb4* deletion accentuated the early increase of myocardial macrophage density in the viable myocardium after MI, but that subsequent ventricular dilation and dysfunction, CM hypertrophy, or interstitial myocardial fibrosis remained unaffected. Interestingly, myeloid-specific *Erbb4* deletion reduced infarct scar. In the TAC model, pressure overload–induced myocardial inflammation remained absent, and LV remodeling progressed independently of myeloid ERBB4. Overall, this study shows a modulatory role of NRG1/ERBB4 signaling in myeloid cells during the early inflammatory phase of MI.

In conclusion, NRG1/ERBB4 signaling in myeloid cells has a modulatory role during the early inflammatory phase of MI. Myeloid-specific Erbb4 deletion in mice accentuates early inflammatory responses post-MI and decreases infarct scar.

In ***<u>Chapter 5</u>***, we generated EC–specific *Erbb4* KO mice to specifically eliminate endothelial autocrine ERBB4 signaling without affecting paracrine

NRG1/ERBB4 signaling in the heart. First, we observed no basal cardiac phenotype in these mice up to 32 weeks. We next studied these mice following TAC, exposure to Ang II or MI in terms of cardiac performance, myocardial hypertrophy, myocardial fibrosis and capillary density. In general, no major differences between EC–specific *Erbb4* KO mice and control littermates were observed. However, 8 weeks following TAC both myocardial hypertrophy and fibrosis were attenuated by EC–specific *Erbb4* deletion, albeit these responses were normalized after 20 weeks. Similarly, 4 weeks after Ang II treatment myocardial fibrosis was less pronounced compared to control littermates. These observations were supported by RNA-sequencing experiments on cultured ECs showing that NRG1 controls the expression of various hypertrophic and fibrotic pathways. Overall, these data contribute to our understanding of myocardial cell-cell communication during cardiac remodeling, and to the role of autocrine NRG1/ERBB4 signaling specifically.

In conclusion, NRG1 has direct effects on human ECs in vitro, and endothelial autocrine NRG1/ERBB4 signaling plays a role in the modulation of hypertrophic and fibrotic responses during early cardiac remodeling.

The importance of non-myocyte paracrine and autocrine NRG1/ERBB4 signaling in the heart

This thesis challenges the currently accepted concept that the cardiac effects of NRG1 are mediated by paracrine activation of ERBB4/ERBB2 receptors on CMs (10, 11). We showed that NRG1/ERBB4 signaling in myeloid cells has a modulatory role during the early inflammatory phase of MI, and demonstrated direct effects of NRG1 on human ECs in vitro and a modulatory role of

endothelial autocrine NRG1/ERBB4 signaling in hypertrophic and fibrotic responses during early cardiac remodeling (Figure 25).

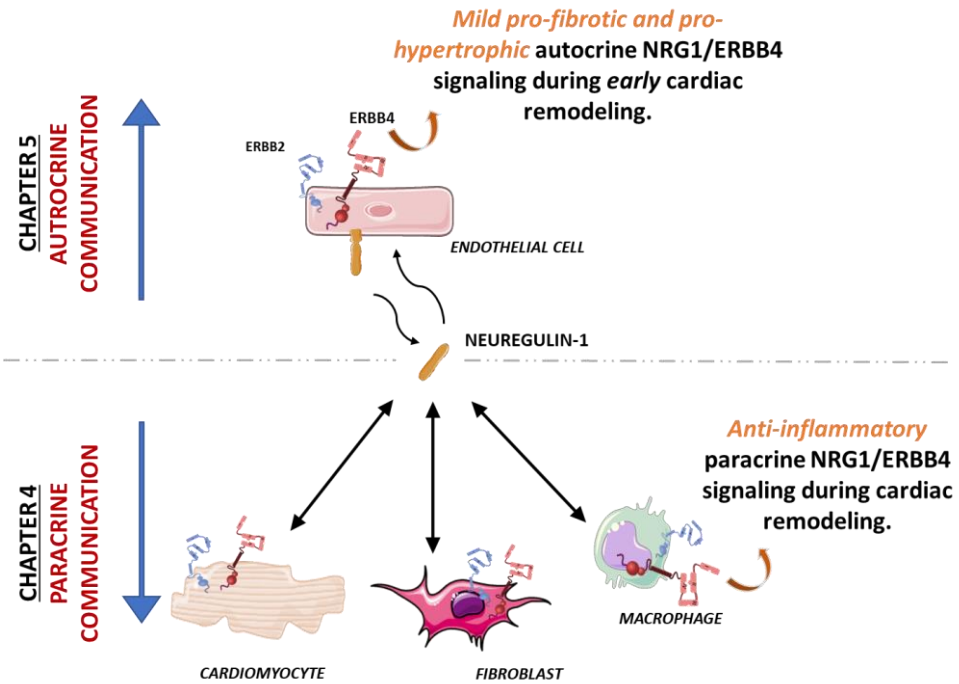

Figure 25: Importance of non-myocyte paracrine and autocrine NRG1/ERBB4 signaling in the heart. *Endothelial autocrine NRG1/ERBB4 signaling plays a role in the modulation of hypertrophic and fibrotic responses during early cardiac remodeling. NRG1/ERBB4 signaling in myeloid cells has a modulatory role during the early inflammatory phase of MI.*

Paracrine communication in the heart is crucial for normal cardiac function, but also plays an important role in cardiac remodeling (10, 11). NRG1 is a paracrine endothelial protein, which binds to ERBB4 on CMs, but also other cell types such as macrophages and ECs (1, 8, 12). We examined the role of endothelial autocrine NRG1/ERBB4 receptor signaling and myeloid paracrine NRG1/ERBB4 receptor signaling during cardiac remodeling by specifically deleting the ERBB4 receptor on ECs and macrophages, respectively. This approach contributes to the understanding of the complex myocardial cell-cell communication during cardiac remodeling, and to the role of both paracrine and

autocrine non-myocyte NRG1/ERBB4 signaling following cardiac overload/injury.

By endothelial *Erbb4* deletion we selectively eliminated endothelial autocrine ERBB4 signaling without affecting paracrine NRG1/ERBB signaling in other cardiac cell types. We confirmed that NRG1 promotes endothelial angiogenic activity in vitro, and demonstrated the role of endothelial ERBB4 in this effect. Furthermore, we showed that endothelial autocrine NRG1/ERBB4 signaling plays a role in the modulation of hypertrophic and fibrotic responses during early cardiac remodeling. On the other hand, previous studies showed the expression of different ERBB receptors (ERBB2, ERBB3, ERBB4) on ECs, and demonstrated a modulatory role of ERBB2/ERBB3 signaling in *in vivo* angiogenesis (13, 14). Therefore, we cannot exclude a modulatory role of NRG1/ERBB3 in ECs during cardiac remodeling, and future studies with EC–specific *Erbb3* deletion could further elucidate the complexity of endothelial NRG1/ERBB signaling during cardiac remodeling.
By deletion of *Erbb4* on myeloid cells, we showed that NRG1/ERBB4 signaling in myeloid cells has a modulatory role during the early inflammatory phase of MI. Accordingly, a previous study showed the expression of ERBB3 on macrophages (8). Therefore, these findings do not allow us to exclude paracrine myeloid NRG1/ERBB3 signaling during cardiac remodeling. Again, future experiments with myeloid-specific *Erbb3* deletion could clarify the role of NRG1/ERBB3 signaling in myeloid cells during cardiac remodeling.

Furthermore, early attenuated cardiac hypertrophy and fibrosis by EC–specific deletion of Erbb4, is a surprising finding because it is in contrast with a previous study in which we showed that specific deletion of Erbb4 in macrophages increased fibrosis (8). On the other hand, NRG1/ERBB4 signaling is considered to be a cardioprotective system, and hypertrophy and fibrosis are viewed as maladaptive myocardial responses. However, early myocardial responses

following injury are essential to cope with injury and overload and are not maladaptive per se (15). Performing future controlled time course experiments could contribute to our understanding of the role of autocrine NRG1/ERBB4 signaling during early cardiac remodeling, and its effects on late cardiac remodeling. Clearly, along with our study on specific *Erbb4* deletion in macrophages, this study shows the complexity of intercellular communication during cardiac remodeling, comprising spatio-temporal heterogeneity of myocardial autocrine and paracrine signaling following cardiac overload/injury. Moreover, these studies suggest that both endothelial and myeloid NRG1/ERBB4 signaling could contribute to the protective effects of NRG1 during early cardiac remodeling in patients with CHF.

In addition, both studies do not address the dimerization partner of ERBB4. ERBB2 is known to be the preferred dimerization partner of ligand-activated ERBB4. However, NRG1 may also induce the formation of ERBB4 homodimers, or ERBB4/ERBB3 heterodimers. Currently, it is unknown to what extend EC–specific or myeloid-specific homotypic (ERBB4/ERBB4) and heterotypic signaling (ERBB2/ERBB4, ERBB3/ERBB4) contribute to the cardioprotective effects of NRG1. For instance, experiments with deletion of *Erbb2* or *Erbb3* in both ECs and myeloid cells could be helpful in answering this question.

As previously shown, LysM-Cre$^{+/-}$ is not organ specific. Vermeulen et al. showed anti-inflammatory and antifibrotic effects of NRG1 outside the heart, in skin and lung. These and our findings add important knowledge regarding the translational potential of NRG1/ERBB4 signaling in different inflammatory and fibrotic diseases. Similarly, VE-Cdh5-Cre^{+} generates EC–specific Erbb4 deletion not only on the heart, but also in other organs. Although we did not observe a baseline phenotype in mice with EC–specific *Erbb4* deletion, EC–specific *Erbb4* deletion in other organs through the body should be acknowledged and carefully examined in future experiments.

To summarize, this thesis demonstrates the complexity of intercellular communication during cardiac remodeling, and argues that the modulatory role of NRG1/ERBB4 signaling in non-myocytes may be circumstance-dependent. Endothelial autocrine NRG1/ERBB4 signaling has modulatory actions on hypertrophic and fibrotic responses during early cardiac remodeling, while paracrine NRG1/ERBB4 signaling in myeloid cells has modulatory actions on early inflammatory responses and infarct scar formation post-MI. This research contributes to our understanding of the spatio-temporal heterogeneity of myocardial autocrine and paracrine NRG1/ERBB4 signaling following cardiac injury, and therefore, to the translation of NRG1/ERBB pathways in CHF patients.

NRG1/ERBB4 signaling in cardiac non-myocytes plays a role in the pathophysiology of cardiac remodeling. Moreover, this thesis emphasizes a substantial role of autocrine and paracrine NRG1/ERBB4 signaling during early cardiac remodeling.

Future perspectives

In this thesis we studied the role of NRG1/ERRB4 signaling in non-myocytes, like ECs and macrophages, to create new opportunities for the treatment of cardiac disease. We showed direct effects of NRG1 on human ECs *in vitro*, and demonstrated that EC–specific *Erbb4* deletion in mice attenuated early cardiac hypertrophic and fibrotic responses following pressure overload and Ang II activation. In addition, we confirmed previous observations by Vermeulen et al. by showing a modulatory role of NRG1/ERBB4 signaling in myeloid cells during the early inflammatory phase of MI. These data contribute to our understanding of myocardial cell-cell communication during cardiac remodeling, and to the role of paracrine and autocrine NRG1/ERBB4 signaling. Still, several research

questions need to be answered contributing to the fundamental understanding of the cardiovascular physiology of NRG1/ERBB signaling and to translational progress and drug development.

In the heart, activation of the NRG1/ERBB system is an adaptive physiological response to cope with overload and injury, and could induce cardiac regeneration and repair (11, 16, 17). The central role of ERBB2 in the protective actions of NRG1 on CMs has been shown through extensive research. However, ERBB2 lacks a ligand-binding domain, hence becomes activated through dimerization with its family members ErbB3 or ErbB4. The current concept is that the cardiac effects of NRG1 are mediated by paracrine activation of ERBB4/ERBB2 receptors on CMs (10, 11). However, it is uncertain to what extent this concept explains the full picture, and future studies should further challenge this hypothesis, and establish the non-myocyte mechanisms of ERBB signaling during CHF:

(i) Different clinical trials have already confirmed the protective actions of rNRG1 in patients with CHF. However, rNRG1 is a protein requiring i.v. administration. According to current clinical protocols, daily administration of 6 to 8 h is necessary, which is an important disadvantage and which is unpleasant for prolonged treatment of patients with CHF. Therefore, it would be interesting to develop small chemical molecules, suitable for oral administration, mimicking the protective actions of rNRG1. Currently, no small molecule agonists of ERBB4 are known. However, at our laboratory, we are currently performing high throughput screening experiments and optimizing assays for this purpose.

(ii) This thesis investigates the role of NRG1/ERBB4 signaling in the heart. However, NRG1 activates both ERBB4 and ERBB3 receptors, and it is still uncertain through which receptor NRG1 exerts its effects. In our study,

endothelial ERBB2 and ERBB3 receptor expression was below detection levels. Therefore, these data only indicate a role of NRG1/ERBB4 signaling in early cardiac hypertrophy and fibrosis. However, different studies showed the expression of ERBB2, ERBB3, and ERBB4 on ECs (14). Therefore, we cannot exclude a role of endothelial NRG1/ERBB3 signaling during cardiac remodeling. Furthermore, different ERBB receptors are also expressed on macrophages. Again, our findings only indicate a modulatory role of NRG1/ERBB4 signaling in macrophages, and cannot exclude a role of NRG1/ERBB3 signaling in inflammatory cells during cardiac remodeling. We could extend this research by generating mice with EC–specific and myeloid-specific *Erbb3* deletion, and by exposing these mice to different models of cardiac remodeling as described in this thesis. This research is important for the future development of small chemical molecules to replace rNRG1, because targeting ERBB3 or ERBB4 could have different physiological effects.

(iii) Above findings indicate a modulatory role of NRG1/ERBB4 signaling in both ECs and myeloid cells during early cardiac remodeling. However, these data do not indicate whether NRG1 exerts its actions through heterodimerization (ERBB4/ERBB2 or ERBB4/ERBB3), or through homodimerization (ERBB4/ERBB4). Therefore, we could implement future experiments using a multiplex microbead immunoassay (18). Briefly, microbeads coated with ERBB monoclonal antibodies are mixed and incubated with lysates of ECs or myeloid cells after treatment with NRG1. Receptor dimerization is detected using ERBB detection antibodies. This approach is a robust and efficient technique used for determination of expression, phosphorylation, and identification of ERBB heterodimerization or homodimerization. This method is considered to be high throughput and quantitative, and is performed using a Luminex platform, recently available at our laboratory. Analysis of ERBB receptor interactions could be useful to address a critical

mechanism of NRG1. Another approach is to conduct experiments with EC–specific or myeloid-specific deletion of *Erbb2* or *Erbb3,* as they *could* be helpful in studying the underlying mechanism of the protective effects of NRG1 signaling.

(iv) In this thesis we did not include the treatment of transgenic mice with rNRG1. rNRG1 treatment in mice with EC–specific and myeloid-specific deletion of *Erbb4*, and with extension in mice with EC–specific and myeloid-specific deletion of *Erbb3*, could extend our knowledge about cellular responsiveness to rNRG1 and the importance of EC–specific or myeloid-specific NRG1/ERBB4 signaling, or NRG1/ERBB3 signaling during cardiac remodeling. This knowledge could contribute to the translational significance of above findings, and would facilitate the development of small chemical ERBB4 or ERBB3 agonists.

(v) As described in Chapter 5, NRG1 could activate early cardiac fibrotic responses by activation of different endothelial factors, but further experimental proof for a specific pathway is currently missing. Further interpretation of RNA-sequencing data by pathway analysis could contribute to unraveling the underlying mechanism. Differentially expressed genes are intersected with sets of genes that could be associated with a particular biological function or pathway. Examining these gene-expression dataset, and identifying upstream biological functions and linked downstream cellular changes could provide important biological insights. However, the amount of genes with significantly altered expression was very low and could hamper these future functional analysis.

In addition, we confirmed a modulatory role of NRG1/ERBB4 signaling in myeloid cells during early myocardial remodeling after MI in Chapter 4. In line with the experiments in chapter 5, we could study the effect of NRG1 treatment on human macrophages by analyzing changes in macrophage

signaling pathways and its transcriptome using RNA-sequencing and functional analysis. These data could connect sets of genes, and therefore could contribute to the understanding of underlying biological mechanisms. Moreover, isolation of ECs and macrophages from diseased human or murine hearts, followed by RNA-sequencing and functional analysis could provide more detailed mechanistic insights.

Overall, these experiments are important for future research, as this gap in knowledge hampers the translational potential of these findings and the development of small chemical molecules to replace NRG1 for the treatment of CHF.

(vi) In Chapter 5, we implemented a 4 week Ang II model. Unlike the TAC-model, longer time points were not studied. However, implementing a Ang II model at a longer time point could be of additional value. Moreover, performing controlled time course experiments for both the Ang II and TAC model could contribute to our understanding of the role of autocrine NRG1/ERBB4 signaling during early and late cardiac remodeling.

This future research could contribute to drug discovery projects aiming at translating the HF mitigating effects of NRG1/ERBB signaling. It is of great importance to determine the effects of NRG1/ERBB activation in different cardiac cell types, as the heart is a pluricellular organ in which cell-cell communication plays a critical role during cardiac physiology and pathophysiology. Overall, filling these gaps in knowledge could address a critical mechanism of NRG1, could contribute to the translational significance of the above findings, and could facilitate the development of a small chemical molecule to replace rNRG-1 and to predict their actions in HF.

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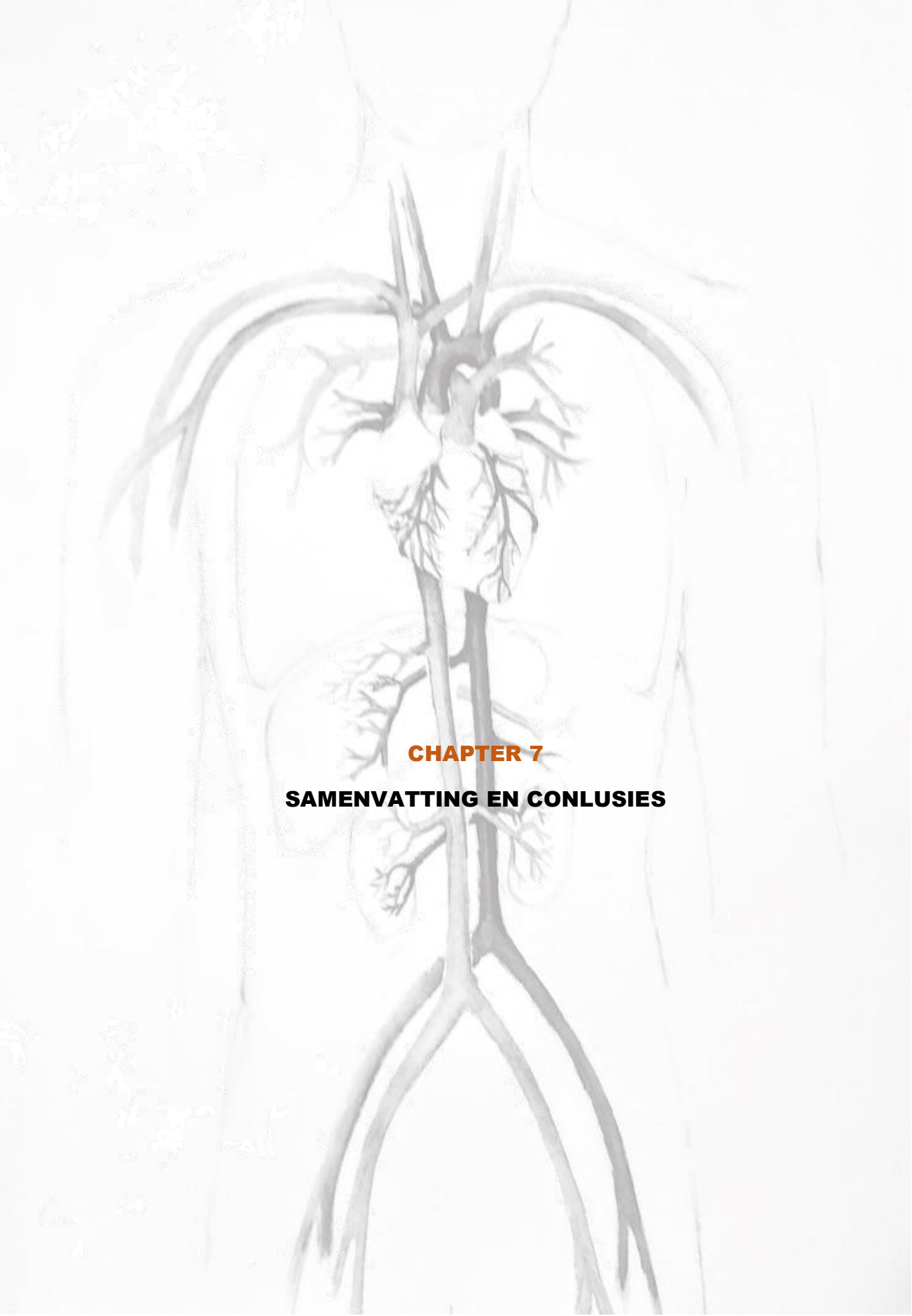


CHAPTER 7

SAMENVATTING EN CONLUSIES

INTRODUCTIE

Sinds de jaren negentig is de behandeling van patiënten met chronisch hartfalen (CHF) positief geëvolueerd, maar de incidentie van CHF blijft hoog en de prognose is slecht. CHF is een van de belangrijkste gezondheidsproblemen, met een prevalentie van 26 miljoen mensen wereldwijd en meer dan 200 000 mensen in België. CHF heeft een gemiddelde sterfte van 26% binnen het jaar na diagnose, voornamelijk bij patiënten ouder dan 65 jaar (1, 2). De huidige generatie geneesmiddelen interfereert met het renine-angiotensine-aldosteronsysteem en het adrenerge zenuwstelsel. Ziekenhuisopnames en heropnames van HF-patiënten blijven echter hoog en er is geen curatieve behandeling beschikbaar (1, 3-5). Een nieuwe generatie geneesmiddelen, waaronder neureguline-1 (NRG1), is daarom volop in ontwikkeling.

NRG1/ERBB signalisatie in het hart

Het menselijk hart is een sterk gestructureerd pluricellulair orgaan dat bestaat uit cardiale endotheelcellen (EC), cardiomyocyten (CM), fibroblasten, ontstekingscellen en gladde spiercellen. Cel-cel communicatie in het hart, en specifiek tussen EC en CM, is essentieel om de hartfunctie te bewaren. CM zijn niet alleen afhankelijk van EC voor de aanvoer van zuurstofrijk bloed, maar ook voor de productie van lokale protectieve factoren. Communicatie tussen deze verschillende celtypen wordt gemedieerd door endotheliale cardio-actieve factoren, zoals NRG1 (1, 6-9). Zo'n 30 jaar geleden werden NRGs voor het eerst beschreven. Deze proteïnes werden ontdekt in de hersenen en worden gecodeerd door *Nrg1–4*. NRGs zijn signaaleiwitten, behorend tot de epidermale groeifactor (EGF)-familie, met een belangrijke rol in celproliferatie, -differentiatie en -overleving in hart, longen, nieren, skeletspieren, zenuwstelsel en borstweefsel (10-12). NRG1 wordt gesecreteerd door het endotheel en kan op paracriene wijze binden aan naburige cellen via *erythroblastic leukemia viral*

oncogene homolog (ERBB)4 en ERBB3 receptoren (13). De fysiologische activiteit van NRG1 werd eerder toevallig ontdekt tijdens de ontwikkeling van een therapie voor borstkanker, gericht tegen ERBB2. Na behandeling van borstkankerpatiënten met trastuzumab (Herceptin®), een humaan monoclonaal antilichaam tegen ERBB2, werd cardiotoxiciteit vastgesteld, voornamelijk wanneer gegeven in combinatie met anthracyclines (14-18).

NRG1 komt sterk tot expressie in het hart. Verschillende studies beschrijven de belangrijke rol van NRG1/ERBB-signalisatie tijdens cardiale ontwikkeling, fysiologie en pathofysiologie (13). Behandeling van verschillende diermodellen met recombinant NRG1 (rNRG1) toonde het therapeutische potentieel van NRG1. rNRG1 kan verschillende aspecten van HF verbeteren, waaronder remodeling van de linkerventrikel (LV), ventriculaire fibrose, hartfunctie en algemene overleving. De veelbelovende preklinische studies hebben geleid tot verschillende klinische studies, waarin werd aangetoond dat toediening van rNRG1 veilig is en de LV-functie verbetert bij patiënten met HF met verminderde ejectiefractie (HFrEF) (15, 19, 20).

NRG1/ERBB signalisatie is essentieel voor de cardiale ontwikkeling

Het NRG1/ERBB systeem is essentieel gedurende de ontwikkeling van het hart. Binding van NRG1 aan ERBB4 stimuleert dimerisatie met ERBB2, resulterend in een signaalcascade die de proliferatie en differentiatie van CM in het ontwikkelende hart bevordert. Specifieke deletie van *Nrg1*, *Erbb2*, *Erbb3* of *Erbb4* bij transgene muizen leidt tot een falen van de hartontwikkeling; meer specifiek tot het falen van ventriculaire trabeculatie, onvoldoende klepvorming en defecten in de ontwikkeling van cardiale pacemakercellen (10, 11, 13, 15, 21).

De role van NRG1/ERBB signalisatie in het adulte hart

Het NRG1/ERBB systeem is cruciaal in het postnatale en adulte hart. Zoals eerder vermeld, werd het belang van NRG1/ERBB signalisatie in het adulte hart voor het eerst aangetoond door een onvoorziene bijwerking tijdens de ontwikkeling van anti-ERBB2-therapieën voor kanker, namelijk reversibele cardiomyopathie (11, 15-17, 22). Verder leidt specifieke deletie van *Erbb2* en *Erbb4* bij transgene muizen tot gedilateerde cardiomyopathie en hogere gevoeligheid voor cardiale stress. Daarnaast werden ook stoornissen in het geleidingssysteem en verminderde contractiliteit waargenomen (15, 23, 24).

De fysiologische rol van het NRG1/ERBB systeem in het hart wordt ook aangetoond door Lemmens *et al.*: toediening van een ERBB1/ERBB2-tyrosinekinaseremmer lapatinib resulteerde in de remming van NRG1/ERBB activatie, LV dilatatie, verminderde LV fractionele verkorting (FS%) en vroegtijdige sterfte tijdens de zwangerschap. Deze studie toont aan dat het NRG1/ERBB systeem cardioprotectieve eigenschappen heeft die worden geactiveerd in omstandigheden van fysiologische stress (25). Deze waarneming ondersteunt het belang van NRG1 in cardiale homeostase (7, 11).

Naast de proliferatieve rol in het ontwikkelende hart en de rol in overleving van CM, heeft het NRG1/ERBB systeem ook een functie in de proliferatie en regeneratie van adulte CM . Bersell *et al.* suggereerden dat NRG1 de proliferatie van mononucleaire CM kan induceren door activatie van ERBB4 receptor en de fosfoinositide-3-kinase (PI3K) signaalroute (26). In contrast met het voorgaande, geeft een studie van Reuter *et al.* aan dat rNRG1 de DNA-synthese van CM in gezonde muizen of muizen met een myocardinfarct (MI) niet verhoogt, en dat het beschermend effect van NRG1 op de hartstructuur en -functie onafhankelijk van de regeneratie van CM optreedt (27). Polizzotti *et al.* toonden daarentegen aan dat rNRG1 geïnduceerde regeneratie van CM het meest effectief is tijdens

de neonatale periode. Dit gegeven vernauwt het therapeutische venster en kan gevolgen hebben voor toekomstige klinische onderzoeken (28). Verder werd in een recente studie van D'Uva *et al.* aangetoond dat ERBB2 noodzakelijk is voor de NRG1 geïnduceerde proliferatie en regeneratie van CM (29).

Belang van het NRG1/ERBB systeem in cardiale pathofysiologie en zijn comorbiditeiten

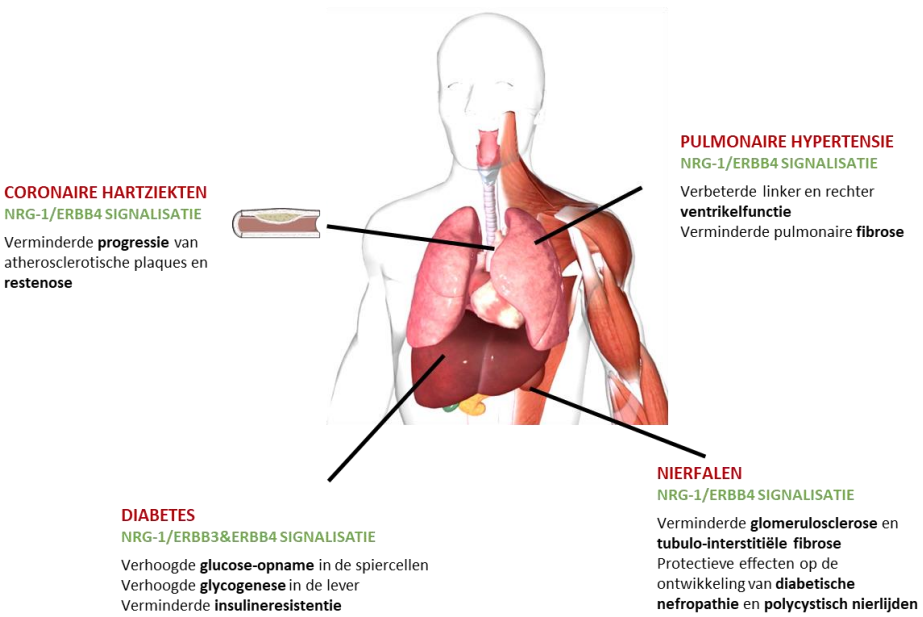
Tabel 1: Klinische studies voor behandeling van cardiovasculaire ziektes met rNRG1

Clinical study	Study number	Last Updated	Patients	Administration	Effect of NRG-1
Fase I, Gerandomiseerd, Dubbelblind, Placebo gecontroleerd, Single dosis studie	NCT0125 8387	Juli 2014 (Afgerond: Maart 2013)	40 patiënten met LV systolische disfunctie en symptomatisch HF	Eenmalig intraveneuze toediening van GGF2	- Goed getolereerd - Dosis-afhankelijke verbetering van LVEF na 28 dagen en tot 90 dagen na behandeling
Fase II, Gerandomiseerd, Dubbelblind, Placebo Parallel gecontroleerde studie	NCT0125 1406	Augustus 2014 (Afgerond: Maart 2014)	120 CHF patiënten	Subcutane toediening van rNRG1 gedurende 10 dagen	- Bewezen veiligheid en efficaciteit
Fase II, Gerandomiseerd, Dubbelblind, Multicenter, Placebo Parallel gecontroleerde studie	ChiCTR-TRC-09000414	Mei 2015	44 CHF patiënten	Intraveneuze toediening van rNRG gedurende 10 dagen	- Significante stijging in LVEF - Gereduceerd ESV en EDV tot 90 dagen na de behandeling - Verminderde remodeling
Fase II, Niet-gerandomiseerd, Open, Single groep studie	ACTRN12 60700033 0448	Januari 2019	15 CHF patiënten	Intraveneuze toediening van rNRG gedurende 11 dagen	- Verbeterde acute en chronische hemodynamische parameters
Fase III, Gerandomiseerd, Dubbelblind, Multicenter, Placebo Parallel gecontroleerde studie	NCT0338 8593	November 2018	1600 CHF patiënten	Intraveneuze toediening van rNRG gedurende 10 dagen, gevolgd door 10 min bolus per week gedurende 23 weken (startend in week 3)	- Geen resultaten gekend

Het belang van NRG1 in het postnatale hart wordt verder aangetoond in verschillende diermodellen die belangrijke veranderingen hebben aangetoond in cardiale NRG1/ERBB activiteit tijdens de progressie van CHF. Transverse aorta constrictie (TAC) in muizen induceert een verhoogde expressie van *Erbb2/Erbb4* mRNA in de LV tijdens compensatoire cardiale hypertrofie. Tijdens de overgang naar CHF zijn deze mRNA levels echter verlaagd (10, 30). In diabetische modellen worden eveneens verlaagde myocardiale NRG1 expressie en *Erbb2/Erbb4* mRNA expressie waargenomen (31). Verder zijn verhoogde

NRG1-spiegels en verlaagde ERBB2/ERBB4 expressie gecorreleerd met de ernst van de hartziekte (32-34). Deze waarnemingen suggereren een belangrijke rol van het NRG1/ERBB systeem bij HF en identificeert NRG1 als een biomarker met potentiële klinische toepassingen. De veelbelovende preklinische studies hebben geleid tot lopende klinische studies, waarin de effecten en veiligheid van NRG1 bij mensen werden onderzocht. Tot op heden worden 5 klinische studies (fase I, II en III) teruggevonden op ClinicalTrials.gov, die de toediening van rNRG1 onderzoeken voor de behandeling van hart- en vaatziekten, deze worden samengevat in Tabel 1.

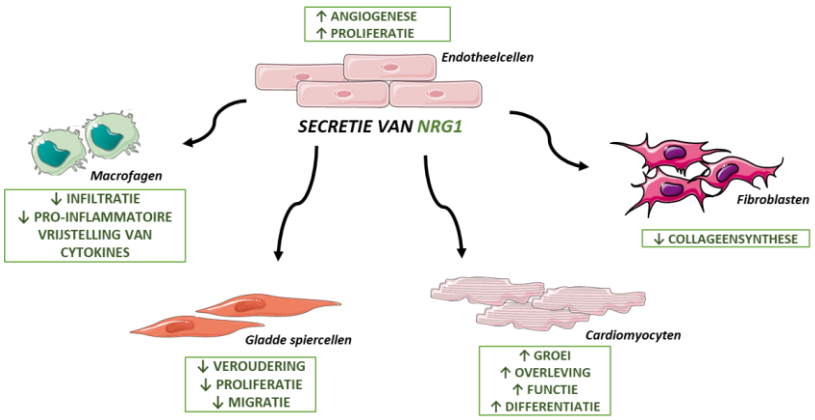
Daarnaast toonden recente studies ook beschermende effecten van NRG1 aan bij verschillende comorbiditeiten van HF, deze staan samengevat in Figuur 1 en benadrukken de pleiotrope effecten van NRG1 (31, 35-40).



Figuur 1: Pleiotrope effecten van NRG1/ERBB signalisatie. *HF wordt geassocieerd met verschillende comorbiditeiten, waarbij het NRG1/ERBB systeem een belangrijke rol kan spelen.*

Paracriene en autocriene NRG1/ERBB signalisatie in cardiale myocyte en niet-myocyte cellen

Het hart is, zoals eerder beschreven, een pluricellulair orgaan bestaande uit CM en niet-myocyte cellen zoals EC, fibroblasten, ontstekingscellen en gladde spiercellen. Zoals samengevat in Figuur 2, bevordert endogeen NRG1 de hartfunctie door in te werken op deze verschillende celtypes (11, 41). NRG1/ERBB signalisatie is van cruciaal belang bij cardiale remodeling (41), het stelt het hart in staat om te gaan met fysiologische stress (25).



Figuur 2: Effecten van NRG1 op cardiale myocyte en niet-myocyte cellen. *Gesecreteerd NRG1 oefent verscheidene effecten uit op CM, maar ook op EC, fibroblasten, gladde spiercellen en macrofagen.*

Het algemeen aanvaarde concept is dat de cardiale effecten van NRG1 worden gemedieerd door paracriene activatie van ERBB4/ERBB2 receptoren op CM (7, 15). Recente studies hebben echter aangetoond dat myeloïd-specifieke deletie van *Erbb4* myocardiale fibrose versterkt in een Ang II model (42). Dit geeft aan dat NRG1/ERBB4 signalisatie in cardiale niet-myocyte cellen minstens even belangrijk is. Dit gegeven wordt versterkt door het feit dat ook fibroblasten ERBB receptoren tot expressie brengen. Galindo *et al.* hebben aangetoond dat *glial*

growth factor 2 (GGF2), een gesecreteerd NRG, myocardiale fibrose verminderd in een porcien infarctmodel, en suggereren een direct remmend effect van GGF2 op cardiale fibroblasten (43). ERBB2, ERBB3 en ERBB4 receptoren worden eveneens tot expressie gebracht op EC (44), hetgeen autocriene NRG1 signalisatie mogelijk maakt. De rol van NRG1/ERBB signalisatie in EC gedurende cardiale remodeling is echter minder gekend. In deze thesis zullen we de effecten van endotheel-specifieke en myeloïd-specifieke *Erbb4* deletie in verschillende modellen van cardiale remodeling bestuderen, opdat onze kennis over NRG1/ERBB signalisatie in niet-myocyte cellen verder uitgebreid kan worden.

DOELSTELLING

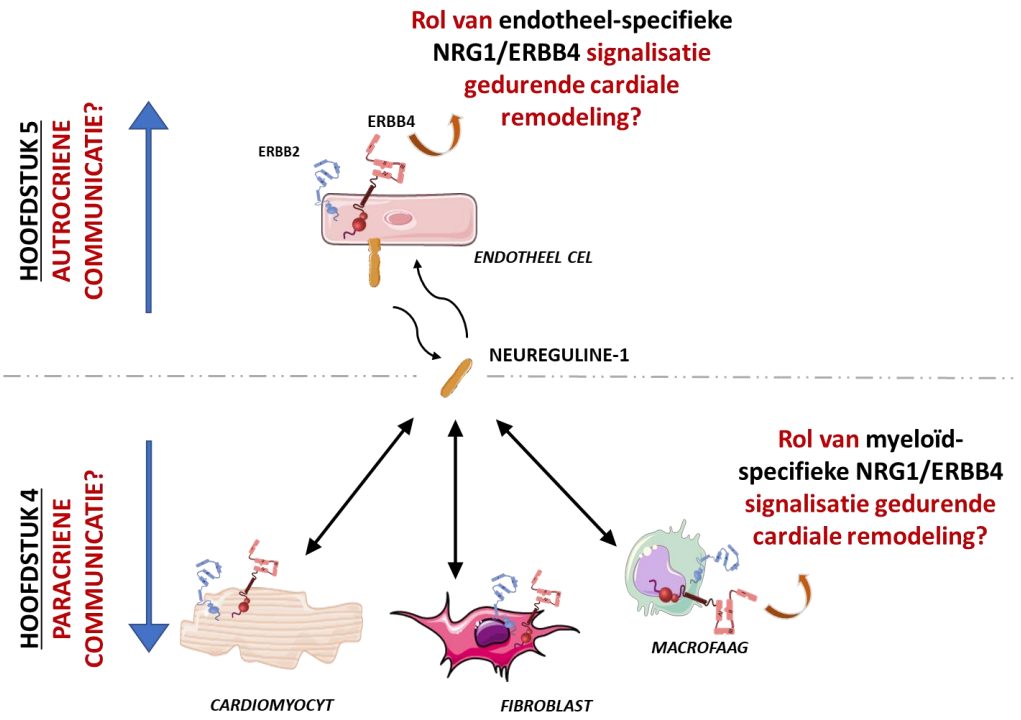
Ons laboratorium bestudeert al bijna 2 decennia het NRG1/ERBB systeem bij de pathofysiologie van HF. Activatie van ERBB2 in CM via ERBB3 of ERBB4 draagt bij tot de cardioprotectieve effecten van NRG1, maar het is onduidelijk in hoeverre dit scenario het volledige werkingsmechanisme van NRG1 verklaart. ERBB2, ERBB3 en ERBB4 receptoren worden ook tot expressie gebracht door cardiale niet-myocyte cellen zijn, waaronder ontstekingscellen, fibroblasten en EC. Daarnaast heeft recent onderzoek in ons laboratorium door Vermeulen *et al.* aangetoond dat myeloïde-specifieke deletie van *Erbb4* de myocardiale fibrotische reacties in een Ang II model versterkt (42). Dit geeft aan dat paracriene NRG1/ERBB4 signalisatie in cardiale niet-myocyte cellen even belangrijk is. In deze thesis zullen we het huidige concept, dat de cardiale effecten van NRG1 worden gemedieerd door paracriene activatie van ERBB4/ERBB2 receptoren op CM, kritisch benaderen en onderzoeken we de rol van NRG1/ERBB4 signalisatie in niet-myocyte cellen, en meer specifiek in EC en macrofagen. **De algemene hypothese is dat NRG1/ERBB4 receptorsignalisatie in niet-myocyte cellen bijdraagt tot de protectieve effecten van NRG1 gedurende cardiale remodeling** (Figuur 3). Deze algemene hypothese zal opgedeeld worden in twee afzonderlijke hypotheses:

(i) Hypothese 1: **Paracriene NRG1/ERBB4 signalisatie in myeloïde cellen speelt een modulerende rol gedurende cardiale remodeling**. Deze hypothese wordt getest in muizen met myeloïd-specifieke *Erbb4* deletie, na blootstelling aan een TAC-model en een MI-model. (i) TAC of sham operaties werden uitgevoerd en muizen werden geëuthanaseerd na 3 dagen tot 20 weken (n=35-45 per time point), (ii) MI werd geïnduceerd door middel van *left anterior descending coronary artery* (LAD) ligatie met een follow-up van 5 dagen (n=35) en 4 weken (n=38), met als doel vroegtijdige en laattijdige cardiale responsen na MI te bestuderen. Het natuurlijke

verloop van de ziekte in transgene muizen werd vergeleken met controledieren. Muizen werden gefenotypeerd door middel van echocardiografie en immunohistologie voor CM dwarsdoorsnede, infiltratie van macrofagen en fibrose.

(ii) Hypothese 2: **Autocriene NRG1/ERBB4 signalisatie in EC speelt een modulerende rol gedurende cardiale remodeling.** Veranderingen in het endotheliaal transcriptoom werden geanalyseerd met RNA-sequencing en *in vitro* angiogenetische effecten van NRG1 werden bestudeerd. Daarnaast werden EC–specifieke *Erbb4* knock-out (KO) muizen gegenereerd, opdat endotheliale autocriene ERBB4 signalisatie specifiek geëlimineerd werd, zonder paracriene NRG1/ERBB4 signalisatie in andere hartcellen te beïnvloeden. Deze muizen werden blootgesteld aan verschillende modellen van cardiale remodeling (TAC, Ang II, MI), en het effect van EC–specifieke *Erbb4* deletie werd onderzocht. (i) TAC of sham operaties werden uitgevoerd en muizen werden geëuthanaseerd na 8 (n=45) of 20 weken (n=45), (ii) Ang II werd toegediend via een micro-osmotische pomp gedurende 4 weken (1000 ng.kg-1.min-1; n=40), (iii) MI werd geïnduceerd door middel van LAD-ligatie met een follow-up 4 van weken (n=40). Opnieuw werd het natuurlijke verloop van de ziekte in transgene muizen vergeleken met controledieren. Muizen werden gefenotypeerd door middel van echocardiografie en immunohistologie voor CM dwarsdoorsnede, fibrose en dichtheid van cardiale capillairen.

Deze hypotheses hebben een grote impact op de translatie van NRG1/ERBB signalisatie bij CHF. Omwille van de complexiteit van HF, worden deze hypotheses bij voorkeur *in vivo* bestudeerd in muizen met cel-specifieke deletie van *Erbb4*. Op deze manier kan de modulerende rol van NRG1/ERBB4 signalisatie bij HF in een specifiek celtype geanalyseerd worden.



Figuur 3: Algemene hypothese: NRG1/ERBB4 receptor signalisatie in niet-myocyte cellen zal bijdragen tot de protectieve effecten van NRG1 gedurende cardiale remodeling.

RESULTATEN EN CONCLUSIES

In ***Hoofdstuk 4*** werden muizen met myeloïd-specifieke deletie van *Erbb4* gegenereerd en werden de effecten van myeloïd-specifieke *Erbb4* deletie in 2 modellen van cardiale remodeling—MI en TAC—bestudeerd. Er werd aangetoond dat myeloïd-specifieke *Erbb4* deletie aanleiding gaf tot toename van vroege macrofaaginfiltratie in het myocardium na MI, maar dat daaropvolgende ventriculaire dilatatie en disfunctie, CM-hypertrofie of interstitiële myocardiale fibrose niet werden beïnvloed. Interessant is dat myeloïd-specifieke *Erbb4* deletie de infarctgrootte verminderde. In het TAC-model werd geen inductie van inflammatie waargenomen, en de LV remodeling verliep onafhankelijk van myeloïd ERBB4. Algemeen toont deze studie een modulerende rol van NRG1/ERBB4 signalisatie in myeloïde cellen tijdens de vroege inflammatoire fase van MI.

Conclusie: NRG1/ERBB4 signalisatie in myeloïde cellen heeft een modulerende rol gedurende de vroege inflammatoire fase van MI. Myeloïd-specifieke Erbb4 deletie in muizen verhoogt de vroege inflammatoire responsen na MI en verminderd de infarctgrootte.

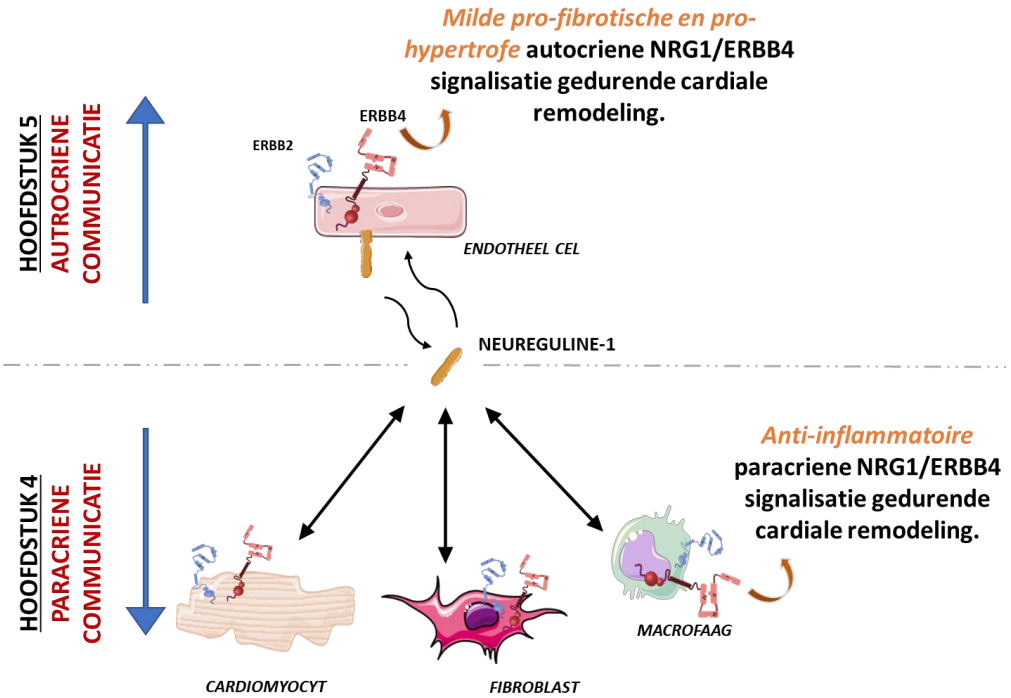
In ***Hoofdstuk 5*** werden EC–specifieke *Erbb4* KO muizen gegenereerd, opdat endotheliale autocriene ERBB4 signalisatie specifiek geëlimineerd werd, zonder paracriene NRG1/ERBB4 signalisatie in het hart te beïnvloeden. Ten eerste werd bij transgene muizen tot 32 weken geen basaal cardiaal fenotype waargenomen. Vervolgens werden deze muizen blootgesteld aan TAC, Ang II of MI, en werden hartfunctie, myocardiale hypertrofie, myocardiale fibrose en de dichtheid van myocardiale capillairen geanalyseerd. Algemeen werden er geen significante verschillen tussen EC–specifieke *Erbb4* KO muizen en controle muizen waargenomen. Echter, 8 weken na TAC werden verminderde

myocardiale hypertrofie en fibrose gezien in harten van muizen met EC–specifieke *Erbb4* deletie. Deze parameters namen normale waarden aan 20 weken na TAC in vergelijking met controle muizen. Gelijkaardige effecten werden gezien 4 weken na Ang II–behandeling: myocardiale fibrose in EC–specifieke *Erbb4* KO muizen was minder uitgesproken in vergelijking met controle muizen. Deze waarnemingen werden ondersteund door RNA-sequencing experimenten in humane EC, hier werd aangetoond dat NRG1 de expressie van verschillende hypertrofe en fibrotische signaalroutes moduleert. Over het algemeen draagt dit onderzoek bij tot onze kennis over myocardiale cel-cel communicatie tijdens cardiale remodeling, en specifiek over de rol van autocriene NRG1/ERBB4 signalisatie in het hart.

Conclusie: NRG1 heeft directe effecten op humane EC in vitro, en endotheliale autocriene NRG1/ERBB4 signalisatie heeft een modulerende functie bij hypertrofe en fibrotische reacties gedurende de vroege fase van cardiale remodeling.

Samenvattend toont dit onderzoek de complexiteit van intercellulaire communicatie tijdens cardiale remodeling. Endotheliale autocriene NRG1/ERBB4 signalisatie heeft modulerende effecten op hypertrofische en fibrotische responsen tijdens vroege cardiale remodeling, terwijl paracriene NRG1/ERBB4 signalisatie in myeloïde cellen modulerende effecten heeft op vroege inflammatoire responsen en infarctgrootte na MI (Figuur 4). Dit onderzoek draagt bij tot onze kennis over de spatio-temporele heterogeniteit van myocardiale autocriene en paracriene NRG1/ERBB4 signalisatie na hartletsel, en tot de translatie van NRG1/ERBB signalisatie in CHF.

NRG1/ERBB4 signalisatie in cardiale niet-myocyte cellen speelt een rol in de pathofysiologie van cardiale remodeling. Bovendien benadrukt dit onderzoek een substantiële rol van autocriene en paracriene NRG1/ERBB4 signalisatie tijdens vroege cardiale remodeling.



Figuur 4: Belang van niet-myocyte paracriene en autocriene NRG1/ERBB4 signalisatie in het hart. *Endotheliale autocriene NRG1/ERBB4 signalisatie heeft een modulerende functie bij hypertrofe en fibrotische reacties gedurende de vroege fase van cardiale remodeling. NRG1/ERBB4 signalisatie in myeloïde cellen heeft een modulerende rol gedurende de vroege inflammatoire fase van MI.*

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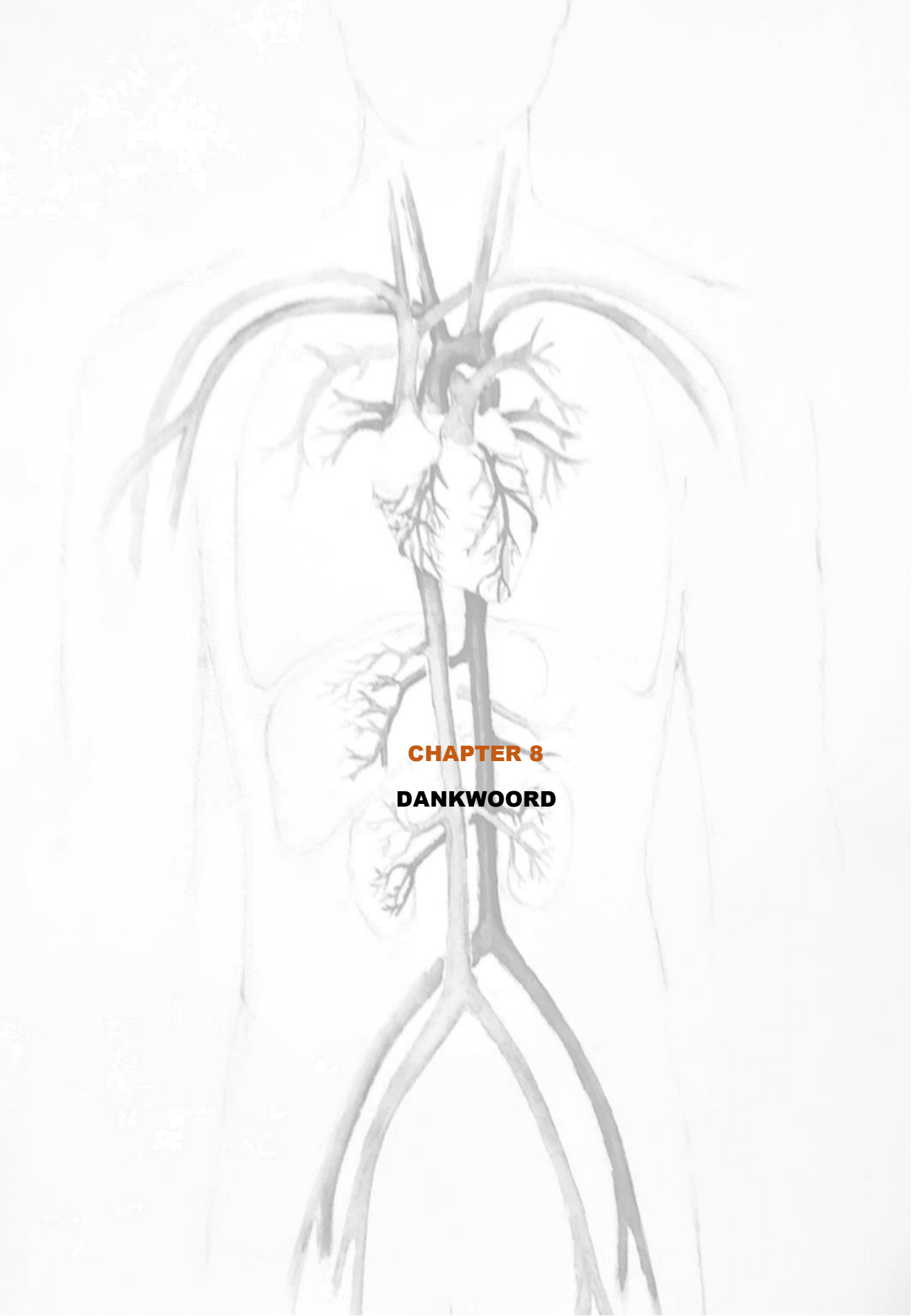

CHAPTER 8

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