Signaling mechanisms in the regulation of cardiomyocyte cohesion in arrhythmogenic cardiomyopathy

Dissertation der Fakultät für Biologie
der Ludwigs-Maximilians-Universität München
zur Erlangung des Doktorgrades

vorgelegt von:
Maria Shoykhet
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Erstgutachter/in: Prof. Heinrich Leonhardt
Zweitgutachter/in: Prof. Anja Horn-Bochtler

Declaration/Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 01.03.2023

Maria Shoykhet

Erklärung

Hiermit erkläre ich, Maria Shoykhet,

(x) dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

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München, den 01.03.2023

Maria Shoykhet

Table of Contents

Abbreviations

abbreveation	meaning
AC	arrhythmogenic cardiomyopathy
ACh	acetylcholine
AC-IgG	IgG isolated from blood sera of AC patients
ACTN2	Z-disc protein alpha actinin
ADAM	A Disintegrin And Metalloprotease
AJ	adherens junction
AMPK	AMP-activated protein kinase
Ang II	angiotensin II
ANKB	ankyrin B
Atp2a2	gene coding for SERCA2
AV node	atrioventricular node
CAMKII	calcium/calmodulin-dependent protein kinase II
CD	Crohn's disease
Cdh2	gene coding for N-Cadherin
cKO	conditional knockout
CRP	C reactive protein
CTNNA3	α-T-catenin
Ctnnb1	gene coding for β-catenin
Cx43	connexin 43
CXCL	chemokine (C-X-C motif) ligand
DAG	diacylglycerol
DCM	dilated cardiomyopathy
DD	Darier disease
DES	desmin
DP	desmoplakin
DSC	desmocollin
DSG	desmoglein
***Dsg2*$^{E4-5}$**	*Dsg2* gene lacking exons 4 and 5
Dsp	gene coding for DP
E-C coupling	excitation-contraction coupling
E-CAD	E-Cadherin
EGFR	epidermal growth factor receptor
ERK1/2	extracellular signal regulated kinase 1/2
F/R	forskolin/rolipram (increases cAMP levels)
FLN-C	filamin-C
GAP19	Cx43 inhibitor
GJ	gap junction
Gja1	gene coding for Cx43
GPCR	G-protein roupled receptor
GSK3β	glycogen synthase 3β
HB-EGF	heparin-bound EGF
IBD	inflammatory bowel disease

ICD	intercalated disc
IF	intermediate filament
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IL6R	interleukin 6 receptor
IP_3	inositol 1,4,5 trisphosphate kinase
iPSC	induced pluripotent stem cell
Jup	junctional plakoglobin (gene coding for plakoglobin)
LDB3	lim-domain binding 3 protein
LMNA/C	lamin A/C
LP	linking peptide
MAPK	mitogen associated protein kinase
MCP1	monocyte chemoattractant protein 1
mc-PV	mucocutaneous pemphigus vulgaris
MEK1/2	mitogen activated protein kinase kinase 1/2
MI	myocardial infarction
MIP1β	macrophage inflammatory protein 1β
miR	micro RNA
MLC2	myosin light chain 2
MMP	matrix metalloproteinase
m-PV	mucosal pemphigus vulgaris
nAChR	nicotinic acetylcholine receptor
$Na_v1.5$	sodium voltage-gated channel alpha subunit 5
N-CAD	N-Cadherin
NFκB	nuclear factor κB
NRVM	neonatal rat ventricular myocyte
p38MAPK	p38 mitogen associated protein kinase
PDE	phosphodiesterase
PF	pemphigus foliaceus
PG	plakoglobin
PI3K	phosphatidylinositol-3 kinase
PI4K	phosphatidylinositol-4 kinase
PIK3IP1	PI3K interacting protein 1
PKA	protein kinase A
PKC	protein kinase C
PKP	plakophilin
PLC	phospholipase C
PLN	phospholamban
PMA	phorbol 12-myristate 13-acetate (PKC activator)
PP2	SRC inhibitor
PTP	protein tyrosin phosphatase
PV	pemphigus vulgaris
PV-IgG	IgG isolated from blood sera of PV patients
ROCK	Rho-associated kinase

RyR2	human ryoanodine receptor 2
SB20	SB2102190 (p38MAPK inhibitor)
SB21	SB216763 (GSK3β inhibitor)
SCD	sudden cardiac death
Scn5a	gene coding for sodium voltage-gated channel alpha subunit 5
SERCA	sarcoplasmic Ca^{2+} ATPase
shRNA	short hairpin RNA
SORBS2	SH3 domain-containing protein 2
TACE	TNFα converting enzyme, a.k.a. ADAM17
TAPI-1	ADAM17 inhibitor
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGFB3	transforming growth factor β3
TGFβ	transforming growth factor β
TIMP3	tissue inhibitor of metalloproteinases 3
Tjp1	tight junction protein 1 gene (coding for ZO-1)
TMEM43	transmembrane protein 43
TNFα	tumor necrosis factor α
TT	titin
TWEAK	TNF-like weak inducer of apoptosis
ZO-1	zonula occludens 1

List of Publications

First author publications:

- Cardiomyocyte adhesion and hyperadhesion differentially require ERK1/2 and plakoglobin (2020, JCI Insight)
- Cardiomyocyte cohesion is increased after inhibition of ADAM17 (2023, Frontiers in Cell and Developmental Biology)
- EGFR inhibition leads to enhanced desmosome assembly and cardiomyocyte cohesion via ROCK activation (2023, JCI Insight)

Publications as co-author:

- Catalytic autoantibodies in arrhythmogenic cardiomyopathy patients cleave desmoglein 2 and N-cadherin and impair cardiomyocyte cohesion (2023, CMLS)

Declarations of contribution as a co-author

Cardiomyocyte adhesion and hyperadhesion differentially require ERK1/2 and plakoglobin (2020, JCI Insight)

I performed all experiments apart from the dispase-based dissociation assays in murine cardiac slices cultures under basal conditions (Figure 1E left panel, performed by Ellen Kempf), the immunohistochemistry for collagen, the H&E and Picrosirius red stainings (Figures 5D and 5E, performed by Tatjana Williams) and the dissociation assays with propranolol and bisoprolol (Supplementary Figure 2, performed by Sunil Yeruva). The dispase-based dissociation assays in HL-1 cardiomyocytes shown in Figures 1C and Figure 3A, as well as the dispase-based dissociation assays with EGTA in murine cardiac slice cultures were performed together with Sebastian Trenz. I analyzed the data, prepared all figures and wrote the manuscript. Sunil Yeruva and Jens Waschke made critical revisions to the manuscript, handled supervision and designed the study.

Cardiomyocyte cohesion is increased after inhibition of ADAM17 (2023, Frontiers in Cell and Developmental Biology)

I performed all experiments apart from the dispase-based dissociation assay shown in Figure 2B (performed by Sunil Yeruva). I analyzed the data, prepared all figures and wrote the manuscript. Sunil Yeruva, Jens Waschke and I designed the study. Sunil Yeruva and Jens Waschke made critical revisions to the manuscript and handled supervision.

EGFR inhibition leads to enhanced desmosome assembly and cardiomyocyte cohesion via ROCK activation (2023, JCI Insight)

I performed all experiments apart from immunostainings in murine cardiac slice cultures (performed by Philipp Menauer and Orsela Dervishi) and the PamGene Kinase assay (sample analysis was performed by Colin Osterloh, data analysis was performed by me). RhoA G-LISA was performed together with Sina Moztarzadeh. I analyzed the data, prepared all figures and wrote the manuscript. Sunil Yeruva, Jens Waschke and I designed the study. Sunil Yeruva and Jens Waschke made critical revisions to the manuscript and handled supervision.

Catalytic autoantibodies in arrhythmogenic cardiomyopathy patients cleave desmoglein 2 and N-cadherin and impair cardiomyocyte cohesion (2023, CMLS)

I performed the dispase-based dissociation assay shown in Figure 5E, analyzed the corresponding data and proof-read the manuscript.

Summary

Arrhythmogenic cardiomyopathy (AC) is a genetic disease, leading to fibro-fatty replacement of the myocardium, which untreated can lead to sudden cardiac death. One of the underlying mechanisms of AC is loss of cardiomyocyte cohesion due to mutations in genes coding for proteins of the intercalated disc (ICD), such as plakoglobin (PG), desmoplakin (DP), plakophilin 2 (PKP2) or desmoglein 2 (DSG2). In this work, several, in part interdependent mechanisms that stabilize cardiomyocyte cohesion were investigated. The *Jup*$^{-/-}$ murine AC model (*Jup* being the gene coding for PG), which shows arrhythmia and fibrosis was used in this work. *Jup*$^{-/-}$ mice had increased epidermal growth factor receptor (EGFR) levels and p38 mitogen activated protein kinase (p38MAPK) activation. EGFR levels were also increased in heart lysates obtained from *Pkp2*$^{-/-}$ mice, representing another AC model. Furthermore, EGFR levels were increased in AC patients' hearts compared to dilated cardiomyopathy hearts, and EGFR localization at the ICD was found in AC patients' hearts.

Adrenergic signaling, protein kinase C (PKC) activation, p38MAPK, EGFR, SRC or A Disintegrin and Metalloprotease 17 (ADAM17) inhibition led to positive adhesiotropy in HL-1 cardiomyocytes and wildtype mice, and (apart from adrenergic signaling) also in *Jup*$^{-/-}$ mice.

Positive adhesiotropy in HL-1 cardiomyocytes upon adrenergic signaling, PKC activation or p38MAPK inhibition was extracellular signal regulated kinase 1/2 (ERK1/2)-dependent under basal conditions but not upon hyperadhesion. In all cases but PKC activation, positive adhesiotropy in HL-1 cardiomyocytes was paralleled by an ERK1/2-dependent enhanced DSG2 localization at the membrane. DP localization at the membrane was enhanced upon inhibition of EGFR, SRC or ADAM17. In wildtype hearts, enhanced DSG2 and DP staining width at the ICD upon EGFR or SRC inhibition was observed, whereas in *Jup*$^{-/-}$ hearts, only DSG2 was enhanced at the ICD upon EGFR or SRC inhibition. Adrenergic signaling, PKC activation or p38MAPK inhibition-mediated positive adhesiotropy was dependent on the expression of PG, DP and DSG2 as well as ERK1/2 activity. Positive adhesiotropy upon EGFR or SRC inhibition was dependent on the expression of DP, but still effective upon *Dsg2* knockdown. In contrast, positive adhesiotropy upon ADAM17 inhibition was dependent on the expression of *Dsg2*. EGFR inhibition activated the Rho associated kinase (ROCK), and positive adhesiotropy upon EGFR inhibition was achieved through ROCK-mediated enhanced desmosomal assembly.

Together, these findings might not only pave the way to find new treatment options for AC by stabilizing cardiomyocyte cohesion, but could also be suited for other diseases with dysfunctional desmosomes.

1. Introduction

1.1. Cardiac excitation

The heart, being "the engine of life" provides the organism with blood and thereby with oxygen. Therefore, it is of utmost importance that the human heart can beat correctly and at a desired pace to pump a sufficient amount of blood through the cardiovascular system. In the embryo, the heart is the first organ that is fully developed and functional (1). Through heart muscle contraction during the systole and relaxation during the diastole, blood is pumped into blood vessels and finally to the organs. In order to contract, cardiomyocytes, the cardiac muscle cells, have to be electrically excited. Cardiac excitation starts at the sinus node, a group of spontaneously electrically excited cardiomyocytes in the right atrium (Figure 1). The sinus node is the cardiac pacemaker; under resting conditions, it sets a frequency of 60-80 heartbeats per minute by means of electrical impulses (2). These then spread across the atria to the atrioventricular (AV) node, which delays the impulse, ensuring that all blood is pumped from the atria into the cardiac ventricles. Then, the electrical impulse is transmitted via the His bundle to the Purkinje fibers, which excite the ventricular myocardium, leading to a ventricular contraction and blood being pumped into the blood vessels. The electrical impulses are caused by an electric potential existing at cell membranes, which in turn is caused by ion gradients between the cytoplasm and the extracellular space, called resting potential (2). A highly orchestrated sequence of opening and closing of specific ion channels leads to the generation of an electrical signal, the action potential, through depolarization and repolarization of the cardiomyocytes. The electrical signal is transmitted from one cell to the next via gap junctions (GJ). The translation of the electrical signal into a mechanical contraction of the heart muscle is called excitation-contraction coupling (E-C coupling). Increased intracellular Ca^{2+}-levels during the systole lead to Ca^{2+} binding to cardiac troponin, leading to conformational changes and sliding of the actin-myosin filaments and thereby contraction of the cardiomyocyte. During the diastole, the sarcoplasmic Ca^{2+} ATPase (SERCA) pumps Ca^{2+} back into the sarcoplasmic

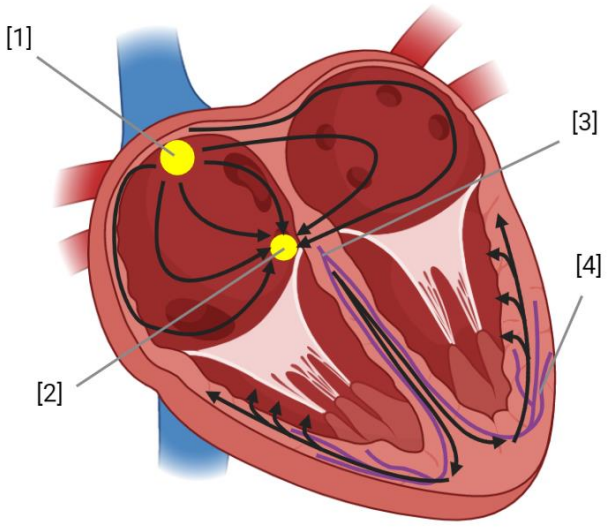

Figure 1 - electrical conduction in the heart.
Electrical impulses are created in the sinus node [1], transmitted to the AV node [2] via the atrial myocardium, propagated by the His-bundle [3] and finally transmitted to the ventricular myocardium via the Purkinje fibers [4].
This cartoon was created using Biorender.com

reticulum (3). The biochemical processes that ultimately result in cardiac contraction are tightly controlled since any deviation might potentially cause severe arrhythmias (4).

Proper cardiac excitation and E-C coupling are only possible when functional cell-cell contacts are present. The risk for arrhythmia increases when cardiac excitation is disturbed. Such arrhythmias are seen e.g., after myocardial infarction (MI), where arteries supplying the myocardium are clogged or blocked by a thrombus, causing hypoxia of the cardiac tissue leading to cardiomyocyte injury and death. Unlike most other cell types, cardiomyocytes barely divide; the cells only gain in size after birth (5, 6). Consequently, upon injury, such as MI, the heart tissue has a limited ability to regenerate. Instead, the injured or necrotic tissue is removed and replaced by fibrotic scar tissue. Heart muscle replacement by fibrotic tissue after cardiac injury also leads to tissue stiffening, reduced ability to contract, decreased blood ejection and increased levels of pro-inflammatory cytokines, which cause more tissue damage, increasing the risk of arrhythmia even further (7).

1.2. Cell-cell contacts in the heart

Two adjacent cardiomyocytes are linked to each other by the intercalated disc (ICD) which consists of adherens junctions (AJ), desmosomes and GJs (8). However, proteins that are not part of desmosomes, AJs or GJs are also expressed at the ICD. In total, over 200 proteins have been associated with the ICD, such as integrins, adhesion proteins, ligands and ligand receptors, such as the β-adrenergic receptor or mechanoreceptors (9, 10). The constituent parts of an ICD are briefly explained below and are depicted in Figure 2.

a. Adherens junctions

AJ-like structures are expressed in primitive organisms. However, the presence of classic AJs is associated with the evolution of metazoa (11). AJs connect the actin cytoskeleton of neighboring cells. The actual cell-cell contact is provided by classic calcium dependent adhesion proteins, in short cadherins: P-Cadherin, N-Cadherin (N-CAD) or E-Cadherin (E-CAD). However, in the adult heart, E-CAD and P-Cadherin are usually not expressed (12). N-CAD forms homophilic cell-cell contacts, and its cytoplasmic tail is linked to a catenin. The group of catenins comprises α-, β-, and γ-catenin, the latter one is also known as plakoglobin (PG). The link to the cytoskeleton is provided by further proteins, such as α-actinin or vinculin (13). Together with desmosomes, AJs provide cellular cohesion. Unlike in desmosomes, cadherins are not densely packed in AJs, leaving space for other transmembrane proteins and thereby leading to a more heterogeneous organization (11).

AJs are crucially involved in embryogenesis; however, loss of AJ components in adulthood can also have severe consequences. In mice, loss of N-CAD resulted in embryonic death, whereas

N-CAD mutations led to severe embryonic malformations, including cardiac defects (14). *Cdh2*$^{-/-}$ embryonic stem cells (*Cdh2* being the gene coding for N-CAD) were not incorporated into the heart wall during cardiac formation (15). Not only is N-CAD involved in embryogenesis, but it also plays a major role in ICD maintenance: a conditional knockout of *Cdh2* led to loss of ICDs, spontaneous arrhythmias, impaired cardiac function and resulted in sudden cardiac death (SCD) in mice (16). Though expression of E-CAD could rescue the effects of an N-CAD loss during embryogenesis or in adult mice, mice expressing E-CAD instead of N-CAD in the heart suffered from dilated cardiomyopathy (DCM) with downregulated protein levels of connexin 43 (Cx43) and increased hypertrophy (17). Furthermore, upon loss of AJs, desmosomal assembly was prevented in keratinocytes (18), underlining the importance of proper AJ function and assembly.

b. Desmosomes

The word desmosome is derived from the Greek words "desmo" – bond and "soma" – body. Evolutionary, desmosomes first appeared in vertebrates and are mostly found in tissues that are exposed to mechanical stress (19, 20). Through desmosomal proteins, intermediate filaments (IFs) of one cell are connected to the IFs of the neighboring cell. The adhesion of two cells is provided through the interaction of two transmembrane desmosomal cadherins: desmogleins (DSGs) or desmocollins (DSCs), which interact with each homophilically or heterophilically. Cadherin interactions are strictly dependent on Ca^{2+}. Growing keratinocytes in cell culture medium without Ca^{2+} resulted in loss of desmosomal contacts and decreased cellular cohesion (21). While in skin four different DSGs (DSG1-4) and three different DSCs (DSC1-3) are expressed in a skin layer-specific pattern, in the heart, only DSG2 and DSC2 are expressed. In the skin, the expression of DSC1, DSG1 and DSG4 is increased in the upper layers of the epidermis (stratum corneum and stratum granulosum) and decreasing towards the lower layers (stratum spinosum and stratum basale). In contrast, the expression of DSC2, DSC3 and DSG3 is increasing towards the lower layers of the epidermis, and DSG2 is only expressed in the stratum basale (22, 23). Classical cadherin binding is mediated through the extracellular domains of the proteins by a tryptophan-swap, where the tryptophan residue of a desmosomal cadherin, i.e. DSG2 is bound by a hydrophobic pocket in the extracellular domain of a desmosomal cadherin from a neighboring cell and *vice versa* (24, 25).

Through their cytoplasmic tail, desmosomal cadherins bind to armadillo proteins, particularly PG and plakophilins (PKPs). However, out of the four PKPs (PKP1-4), only PKP2 is expressed in the heart. In the skin, the PKPs also have a layer-specific expression pattern: PKP1 is more expressed in the differentiated (upper) layers of the epidermis, whereas PKP2 is only expressed

in the lower layers of the epidermis (22). The armadillo proteins are then in turn linked to desmoplakin (DP), which anchors the desmosome to the IF, which in cardiomyocytes is usually the type III IF protein, also known as desmin (DES). Impaired desmosomes, e.g., caused by mutations in genes coding for desmosomal proteins can lead to pathologies of the skin, gut and heart (19, 26, 27).

c. Gap junctions

The main function of GJs, unlike AJs or desmosomes, is not to provide cellular cohesion but to provide communication between cells by allowing the passage of small molecules, which are smaller than 1 kDa (13). Each GJ is comprised of two connexons or hemi-channels (one per cell), which consist of six connexins, out of which Cx43 is the most expressed in the adult ventricular myocardium (28, 29). Through GJs, cardiomyocytes form a functional syncytium. By propagating the electrical impulse from one cardiomyocyte to the surrounding cardiomyocytes, GJs play a major role in the E-C coupling in the heart (29).

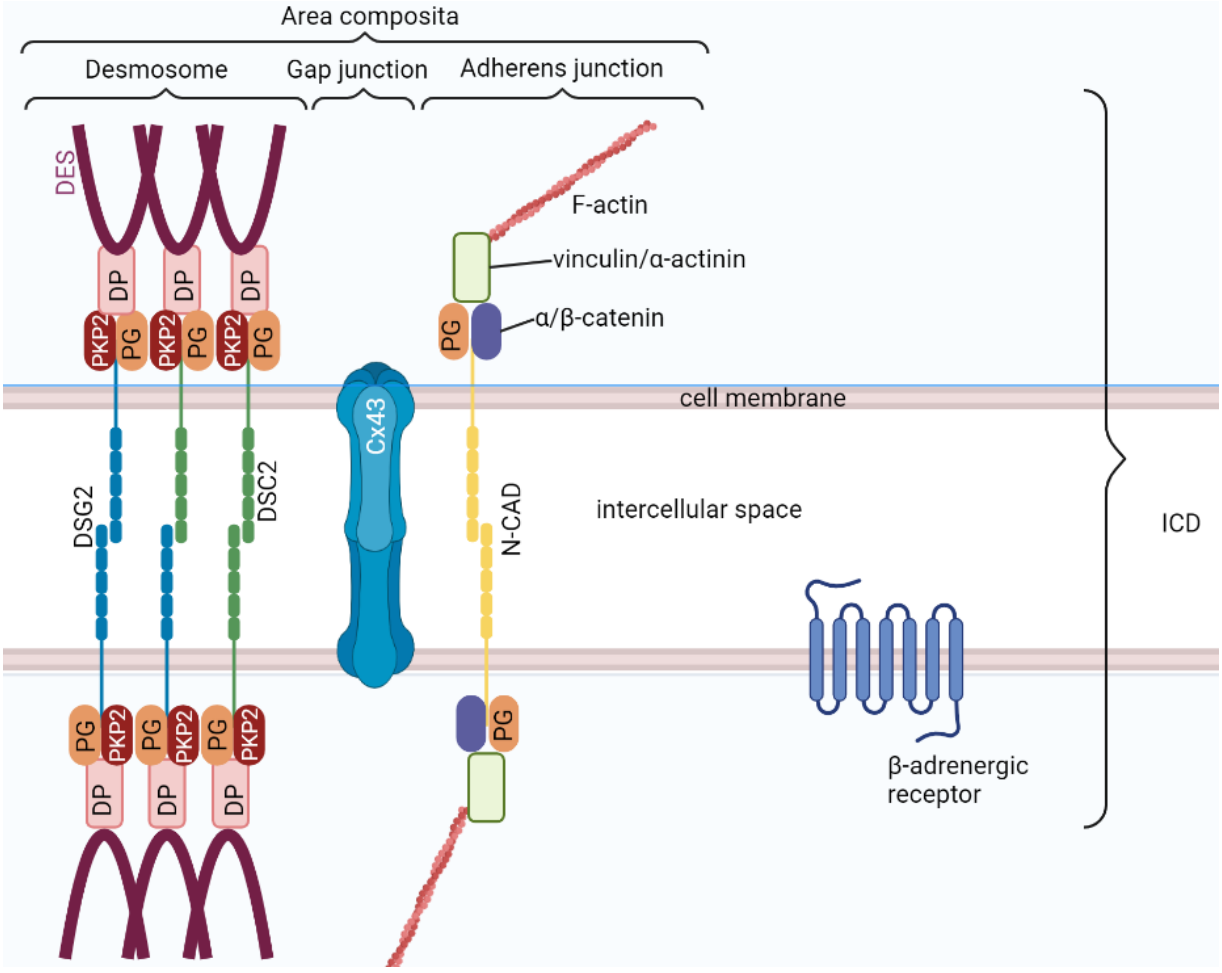

Figure 2 - Structure of the intercalated disc consisting of AJs, desmosomes and GJs.
This cartoon was created using Biorender.com

While in other tissues AJs, desmosomes and GJs can be distinctly separated from each other, in the heart they are often intermingled and desmosomal, AJ and GJ proteins interact with each

other. Especially desmosomal proteins can also be found at AJs or GJs, leading to the term "area composita" (30-32). The fact that a loss of N-CAD in murine hearts led to a loss of AJ and desmosomal proteins from the ICD as well as to a reduction in Cx43 protein levels, further strengthens the concept of the area composita (16).

In endothelia and epithelia, in addition to AJs, desmosomes and GJs, there is one more kind of cellular junction, namely tight junctions, which regulate barrier permeability and are also involved in cellular cohesion.

1.3. Desmosomal signaling

Desmosomes were believed to only provide cellular cohesion, but it is now becoming apparent that desmosomes can also act as signaling hubs by the so-called outside-in signaling (33, 34). This idea is supported by the embryonic lethality of *Dsg2*$^{-/-}$ and *Dsc3*$^{-/-}$ mice, which might not only be caused by loss of adhesion but also by signaling defects during embryogenesis: these embryos die before desmosomes are formed (35-37). Most studies on desmosomal signaling were performed in the skin, where expression of the different desmosomal proteins is correlated to the proliferative and adhesion requirements as well as the differentiation states of the tissue layer. Alterations in cadherin expression, especially cadherin substitutions, can disturb the balance between proliferation and differentiation: Overexpression of DSC3, DSG2, DSG3 or loss of DSC1 increased proliferation and reduced differentiation, whereas loss of DSG3 reduced proliferation (23, 38-40).

In keratinocytes, DSG1 suppressed the epidermal growth factor receptor (EGFR, also known as ErbB1) and downstream extracellular signal regulated kinase 1/2 (ERK1/2) signaling, leading to epidermal differentiation (41). In contrast, loss of DSG1 prevented differentiation in an ERK1/2-dependent manner and enhanced ErbB and mitogen associated protein kinase (MAPK) signaling in keratinocytes (42, 43).

In human colon or colorectal adenocarcinoma cells, loss of DSG2 reduced proliferation and decreased EGFR signaling (44). DSG2 and EGFR interacted in enterocytes and inhibition of EGFR signaling in wildtype as well as DSG2-deficient enterocytes was correlated with reduced cellular cohesion (45). Similar mechanisms were also observed in squamous cell carcinomas, where DSG2 levels were correlated to EGFR protein levels and signaling activity (46). Apart from that, DSG2 enhanced p38MAPK signaling in enterocytes (47) and enhanced AKT, STAT3, mitogen activated protein kinase kinase 1/2 (MEK1/2) and nuclear factor κB (NFκB) signaling in keratinocytes (40). Furthermore, DSG2 interacted with inositol 1,4,5 trisphosphate kinase (IP_3) in enterocytes, whereas loss of DSG2 reduced phospholipase C (PLC)-IP_3 signaling (48). Soluble fragments of DSG2 resulting from DSG2 cleavage by matrix metalloproteinases

(MMPs) or members of the A Disintegrin And Metalloprotease (ADAM) family members have also been shown to be involved in cellular signaling. Soluble DSG2 activated ErbB2/ErbB3, which are members of the EGFR family, thereby leading to an activation of AKT, mammalian target of rapamycin and MAPK signaling (49).

RhoA, Rac1 and Cdc42 activity can be enhanced by *DSG3* overexpression in human keratinocytes, whereas Rac1 inhibition reduced DSG3-induced membrane protrusions, DSG3-actin interaction and DSG3 localization at cell junctions (50). Furthermore, DSG3 can regulate the hippo signaling pathway, whereas a knockdown of *DSG3* decreased expression of target genes of the hippo pathway protein YAP (51). Apart from that, DSG3 formed a complex with SRC and led to SRC activation, whereas loss of DSG3 or its function enhanced p38MAPK signaling and activated p53 (52-54).

Less is known about DSCs in signaling pathways. DSC3 repressed the MAPK/ERK pathway in lung cancer cells (55), whereas loss of DSC2 enhanced EGFR and AKT signaling and thereby increased cell proliferation (56).

PKP2 and EGFR interacted in different cell lines, and overexpression of PKP2 resulted in increased EGFR activation, dimerization and signaling, leading to increased cell proliferation and migration, whereas a knockdown of *Pkp2* decreased EGFR signaling (57). Furthermore, the interaction of EGFR with ErbB2 was increased upon overexpression of PKP2. These results were confirmed *in vivo*, where treatment of mice with tumors with a short hairpin RNA targeting *Pkp2* (sh*Pkp2*) decreased tumor growth as compared to shCtrl mice (57). Apart from that, PKP2 translocated to the nucleus, where it interacted with RNA Polymerase III (58), whereas PKP1 interacted with the eukaryotic translation initiation factor 4A1 and thereby promoted translation (59). In an adenorectal carcinoma cell line, knockdown of *PKP2* or a double-knockdown of *PKP2* and *PKP3* decreased expression of PKP3, PG, DSG2 and DSC2 and reduced protein kinase C (PKC) activity. In these cells, PKP2 but not PKP3 was found in complex with PKC (60). PKP2, either by regulation of RhoA activity and localization or by regulation of PKC signaling (or both), was required for the incorporation of DP into desmosomes (61, 62). Inhibition of the RhoA effector Rho-associated kinase (ROCK) decreased DP incorporation into desmosomes during desmosomal assembly (61). Loss of DP increased cell motility via enhanced Rac1 and decreased RhoA activity, and was accompanied by enhanced p38MAPK activation, whereas RhoA activation prevented enhanced cell motility (63). Knocking down *Pkp2* by siRNA in HL-1 cardiomyocytes led to the differential expression of several micro RNAs (miRs) via the E2F1 pathway (64), as well as activation of the hippo signaling pathway (65). In $Pkp2^{+/-}$ mice or in cardiomyocytes with a *Pkp2* knockdown the

transforming growth factor β (TGFβ) pathway was activated and led to fibrosis and p38MAPK signaling, whereas p38MAPK inhibition by 5Z-7-Oxozeaenol decreased the pro-fibrotic response, but not the pro-adipogenic response (66). The transcriptome of *Pkp2* conditional knockout (cKO) mice revealed over 1000 genes to be differentially regulated. Amongst them, the insulin signaling pathway, paracrine signaling pathways and the calcium signaling pathway were downregulated (67).

PG suppressed the signaling of the EGFR effector molecule SRC and subsequently RhoA and Rac1 signaling, and thereby increased cellular cohesion and reduced cell motility (68-70). Increased PG protein levels in prostate cancer cell lines decreased proliferation and invasiveness, enhanced cellular cohesion as well as stabilization of desmosomes through enhanced protein expression of DP (68). Furthermore, upon loss of PG, p38MAPK was activated (71). PG acted as a tumor suppressor through interaction with the tumor suppressor protein p53, thereby regulating p53 transcriptional activity and thus the expression of p53 targets, particularly members of the 14-3-3 protein family (72). Reduced protein levels of PG can be found in different tumors, and are associated with reduced apoptosis in response to apoptotic stimuli in keratinocytes through regulation of cytochrome c release from mitochondria and thereby regulation of caspase-3 activity, whereas increased expression of PG reduced proliferation (73). Increased cytoplasmic levels of PG, e.g., caused by mutations leading to a decreased incorporation of PG into desmosomes or AJs, favor a translocation of PG to the nucleus, where, due to its high similarity to β-catenin, PG competes with β-catenin for its binding partner, the T-cell factor/lymphoid enhancer factor (TCF/LEF) and inhibit Wnt signaling. The β-catenin/Wnt signaling pathway will be explained in more detail later (see 1.5.2.3). Apart from PG, β-catenin/Wnt signaling can also be suppressed by E-CAD, DSC1, DSC2, DSG2, PKP2 or DP (40, 44, 56, 65, 74-77). On the other hand, in mice overexpressing DSC3 in suprabasal layers, β-catenin signaling was increased (39). In contrast, N-CAD can either suppress or enhance β-catenin/Wnt signaling, depending on the cellular context (78, 79). In pemphigus vulgaris (PV), an autoimmune disease caused by autoantibodies (IgGs) directed against DSG1 and/or DSG3 leading to acantholysis and blistering of skin and mucosa, autoantibody binding led to changes in signaling. Binding of PV-IgGs to DSG1 and/or DSG3 increased intracellular Ca^{2+}-levels, as well as phosphatidylinositol-4 kinase (PI4K) signaling via PLC/IP_3 and activated the Fas/Fas ligand cell death pathway (80, 81). Furthermore, PV-IgG binding activated p53, p38MAPK, ERK1/2 and SRC (52, 82). In turn, p38MAPK activated EGFR and inactivated RhoA (81, 83, 84). In murine keratinocytes, decreased nuclear localization of PG was observed after PV-IgG treatment, which did not lead to changes in β-

catenin-mediated signaling, but increased the expression of c-Myc and consequently cell proliferation, indicating that PG represses c-Myc transcription (85). Upon epithelial-to-mesenchymal transition, a so-called cadherin switch often occurs with increased N-CAD protein levels leading to an activation of Rho and Wnt signaling (86, 87).

Together, these data provide evidence that desmosomes do indeed not only provide cellular cohesion but that desmosomal components are also involved in signaling processes.

1.4. Regulation of cellular cohesion

Wound healing and cell renewal are essential aspects of epidermal tissues. Cellular cohesion plays a major role in these processes. In the skin, desmosomes are usually hyperadhesive, which describes a Ca^{2+}-independent adhesive state. Upon wound healing, desmosomes leave the hyperadhesive state, leading to a decrease in cellular cohesion and tissue remodeling (88). Loss of cell cohesion is often found in metastasizing tumors. A reduction in number of desmosomes is associated with poor prognosis in cancers through loss of desmosomal adhesion, thereby easing the metastatic process. In addition, alterations in signaling mediated by desmosomal components were also observed in cancers (89). Since apart from providing cellular cohesion, desmosomal cadherins can also be involved in signaling, loss of desmosomal components can favor malignancies by increasing proliferation (90). The differential expression of desmosomal proteins can lead to changes in signaling of Wnt/β-catenin, EGFR, c-Myc, AKT or changes in MMP activity (44, 53, 56, 57, 68, 74-76, 89). Both up- as well as downregulation of desmosomal proteins have been found in tumors.

The layer-specific expression of desmosomal cadherins and armadillo proteins in the skin is regulated on a transcriptional level via signaling pathways, such as Notch signaling, Ephrin, Wnt signaling, protein kinase A (PKA) or PKC signaling (22, 33). On a post-transcriptional level, miRs, long noncoding RNAs and RNA-binding proteins can regulate desmosomal protein expression. Furthermore, desmosomal proteins can be subject to posttranslational modifications, including phosphorylation, *N*-glycosylation, *O*-glycosylation or palmitoylation (33, 34, 91). For example, in human keratinocytes SRC phosphorylated PKP3, leading to a detachment of PKP3 from DSG3 and subsequently to a weakened cellular cohesion (92). In addition, desmosomes and AJs can be internalized upon EGFR signaling or after protein shedding by sheddases, such as caspases, MMPs or ADAMs (93, 94).

EGF treatment affected cell-cell adhesion in carcinoma cells and led to phosphorylation of tyrosine residues of PG and β-catenin, which was later shown to be the consequence of EGFR activation (95, 96). Furthermore, tyrosine phosphorylation of β-catenin, PG, E-CAD or p120 catenin was linked to decreased cellular cohesion by decreasing AJ stability and adhesive strength (97). Unspecified cadherins and β-catenin were found in complex with protein tyrosine phosphatase μ (PTPμ) in rat hearts. In contrast, PTP inhibition led to an accumulation of tyrosine-phosphorylated cadherins and it was suggested that these phosphorylations destabilize cellular cohesion (98). One of the mechanisms leading to decreased cellular cohesion upon tyrosine phosphorylation of β-catenin is the dissociation of phosphorylated β-catenin from the AJs to the cytoplasm (99). Phosphorylation of PG at tyrosine residues 693, 724 or 729 by EGFR decreased cellular cohesion by disrupting the desmosomal complex, resulting in a translocation of PG to the cytoplasm in keratinocytes as well as in squamous cell carcinoma cell lines, whereas EGFR inhibition promoted desmosomal assembly (100-102). Furthermore, PG translocation to the nucleus upon its phosphorylation at tyrosine residues led to a repression of TCF/LEF transcription, since PG-TCF/LEF complexes do not bind DNA efficiently (103). However, through interaction with LEF1, PG induced *Dsc2* gene expression and inhibited *Dsc3* expression (104). On the other hand, tyrosine phosphorylation of PG was critical for the development of cell junctions in differentiating keratinocytes (105).

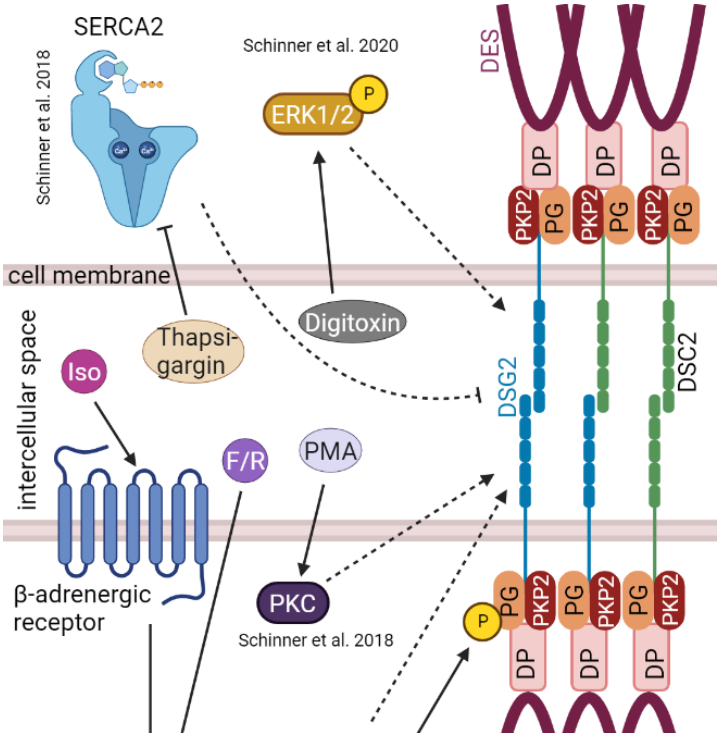

Figure 3 – Mechanisms known to affect cardiomyocyte cohesion. Positive adhesiotropy was induced upon adrenergic signaling, PKC activation and digitoxin treatment. SERCA2 inhibition decreased cardiomyocyte cohesion. Adrenergic signaling phosphorylated PG at S665 and enhanced DSG2 localization at the membrane. DSG2 localization was also enhanced at the membrane upon PKC activation or digitoxin treatment. In the case of digitoxin treatment, this effect was dependent on ERK1/2 phosphorylation.
This cartoon was created using Biorender.com

By enhancing cellular cAMP levels, adrenergic signaling activated PKA, which led to phosphorylation of PG at S665 and strengthened cardiomyocyte cohesion (106). In contrast, digitoxin increased cardiomyocyte cohesion in an ERK1/2-dependent manner, dependent on PG but independent of PG-S665 (107). Furthermore, PKC activation enhanced cardiomyocyte cohesion, whereas

SERCA2 inhibition inhibited it (25). Apart from that, the knowledge on how cardiomyocyte cohesion is regulated is limited (Figure 3) (32).

Since in arrhythmogenic cardiomyopathy (AC) desmosomal turnover is disturbed, it is crucial to get a better understanding of the regulation of cardiomyocyte cohesion, as this might provide the base for a novel therapeutic approach.

1.5. Arrhythmogenic cardiomyopathy

1.5.1. Disease phenotype

AC is a familial heart disease, the hallmarks of which are inflammation of cardiac tissue, arrhythmias and replacement of the myocardium by fibro-fatty tissue with ventricular wall thinning to as thin as 1 mm (being described as "paper-thin"), whereas in healthy people, 5-8 mm is normal (108, 109). First descriptions of a disease resembling AC date to the middle of the 1700s by Giovanni Maria Lancisi, whereas more detailed and precise descriptions were published in the 1970s (110). In 2010, a task force set new criteria for AC diagnosis: These criteria allow a definite (2 major or 1 major and 2 minor or 4 minor criteria), borderline (1 major and 1 minor or 3 minor criteria) or possible (1 major or 2 minor criteria) diagnosis of AC. For an AC diagnosis, the criteria met by a patient have to belong to different groups out of the six criteria categories: I) global and/or regional dysfunction and structural alterations, II) tissue characterization of the wall, III) repolarization abnormalities, IV) depolarization or conduction abnormalities, V) arrhythmias and VI) family history (111).

The prevalence of AC is 1:1000-1:5000, men are more often affected than women, and depending on the genetic background, the inheritance is autosomal-dominant or recessive. In some patients, mutations are found in more than one gene and these patients often have a more severe disease manifestation (112).

In the majority of AC patients, mutations in genes coding for proteins of the desmosome are found, which is why AC is considered a disease of the desmosome or the ICD (113). Even before causative mutations were identified for AC, alterations in the desmosomal structure were observed by electron microscopy (114), and it was hypothesized that the alterations at the ICD may be the cause of decreased cell-cell adhesion. In AC patients as well as in different AC models with various underlying mutations, shortened and/or fewer ICDs as well as fewer GJs were observed (112). To date, several mutations have been classified as causative mutations for AC. The corresponding proteins include proteins of the area composita (115-122), ion channels, proteins of the sarcoplasmic reticulum and proteins of the nuclear envelope (123-128) (Table 1). Specific mutations in the genes coding for DP (gene truncation) or PG (deletion of 2 bp; $JUP^{2157del2}$, *JUP* being the gene coding for PG) lead to cardio-cutaneous phenotypes, in

particular the Carvajal syndrome or Naxos disease (129, 130). Apart from that, some mutations have been associated with AC, but have not been yet confirmed to be causative for the disease, such as mutations in the gene coding for SH3 domain-containing protein 2 (SORBS2), filamin C (FLN-C) or mutations in the tight junction protein (*TJP1*) gene, coding for zonula occludens 1 (ZO-1). These mutations might enhance the susceptibility or worsen the outcome of the disease (131-133). However, for 30-40% of AC patients, no underlying mutation has been identified. The variety of causative mutations of AC shows the complexity of the disease and indicates that different pathomechanisms might be involved. Furthermore, the "one gene-one disease" paradigm is being put into question since mutations in genes which are mutated in AC can also lead to other cardiac diseases. Some mutations associated with AC have also been found in healthy individuals in population-based genome sequencing projects, indicating that these mutations are not necessarily causing AC by themselves. Additional factors, such as viral infections, alterations in miR expression, extensive physical exercise (in particular endurance activity) or psychosocial stress are involved in AC pathogenesis and can aggravate the disease (134-138). Interestingly, in murine studies, it was found that moderate physical exercise or voluntary exercise was beneficial in the case of other cardiomyopathies and also in some cases of AC (139, 140).

Table 1 – causative mutations in AC

Area composita proteins	Other proteins
• PKP2 • PG • DP • DSG2 • DSC2 • DES • Z-disc protein alpha actinin (ACTN2) • lim-domain binding 3 protein (LDB3) • N-CAD • titin (TT)	• sodium voltage-gated channel alpha ($Na_v1.5$) • ankyrin-B (ANKB) • human ryanodine receptor (RyR2) • phospholamban (PLN) • transmembrane protein 43 (TMEM43) • lamin A/C (LMNA) • transforming growth factor-3 (TGFB3) • α-T-catenin (CTNNA3)

In the early stages, AC is almost asymptomatic, however, upon disease progression, electrical abnormalities visible in the ECG, syncopes and palpitations are observed in addition to arrhythmias. Further, morphological changes in the heart in the form of fibro-fatty replacement of the muscle tissue take place. Usually fibrosis progresses from the outside (epicardium) to the inside (endocardium) of the heart, leading to ventricular wall thinning which favors aneurysmal dilatation (141). During the later stages of the disease, cardiomyocytes become hypertrophic (142). Cardiac hypertrophy can be a compensatory response at first; however, if sustained for a long time, it can lead to heart failure through decompensation, ventricular dilation, fibrosis and

arrhythmias (143). If untreated, AC ultimately leads to increased risk of SCD, especially during physical activity (144, 145). However, patients are at risk for SCD even before they feel the disease symptoms. So far, only symptomatic treatment options are available, ranging from reduction of physical activity, changes in lifestyle, treatment with antiarrhythmic drugs, including β-blockers or class III antiarrhythmics, catheter ablation, implantation of an implantable cardioverter-defibrillator or heart transplantation (146, 147). A better understanding of the underlying causes and the pathomechanisms of AC might be a first step towards novel treatment options.

1.5.2. Pathomechanisms of AC

1.5.2.1. Arrhythmia

As obvious from the name, arrhythmias are one of the hallmarks of AC and are one of the first symptoms that present themselves in patients with AC (148). Three major aspects contribute to arrhythmias in AC: Cx43, $Na_v1.5$ and Ca^{2+}-homeostasis.

a. Cx43

Decreased Cx43 expression in cardiomyocytes can (but most not) lead to arrhythmias, whereas inducible $Cx43^{-/-}$ mice suffer from fibrosis in addition to arrhythmias, which are potentially lethal (149-151). Abnormalities in Cx43 localization have been associated with several conditions, such as MI or ischemia-reperfusion injury, but also AC and other cardiomyopathies (152). In HL-1 cardiomyocytes, Cx43 expression at the membrane was reduced when cellular cohesion was impaired (25). Mislocalization of Cx43 as well as decreased Cx43 expression was observed in Boxer dogs, a common AC model, as well as in some AC patients. This phenomenon often correlated to a mutation or mislocalization of PG or to *PKP2* mutations and to altered Cx43 phosphorylation patterns. Furthermore, decreased Cx43 expression or mislocalization was observed in cardiomyocytes derived from induced pluripotent stem cells (iPSCs) of AC patients with mutations in *LMNA* or the $PKP2^{c.2013delC}$, $PKP2^{c.972InsT}$ or $PKP2^{c.148del4}$ mutations, as well as in neonatal rat ventricular myocytes (NRVMs) with a *Pkp2* knockdown, a *Dsp* knockdown or knockout (*Dsp* being the gene coding for DP), or the $Pkp2^{235C>T}$ or $Jup^{2157del2}$ mutations (153-168). The decreased Cx43 protein levels in *Dsp* knockdown NRVMs were caused by an ubiquitinylation of Cx43 (168). Reduced Cx43 expression or mislocalization was also found in several murine AC models: the cardiac-specific congenital and inducible $Jup^{-/-}$ mice, $Dsg2^{cKO}$ mice, mice expressing a mutant form of *Dsg2* lacking exons 4 and 5 ($Dsg2^{E4-5}$), $TMEM43^{S358L}$ mice, as well as cardiac-specific $Dsp^{-/-}$ mice (168-180). In heterozygous DP^{R451G} mice, Cx43 localization was decreased slightly but not significantly under normal conditions, whereas after transverse aortic constriction, Cx43 was

reduced at the ICD (181). Mislocalization of Cx43 was associated with conduction abnormalities in *Dsg2*^{cKO} mice, in *Dsg2*$^{E4-5}$ mice and in conditional *Dsp*$^{+/-}$ mice (159, 173). Decreased but normally localized Cx43 protein levels were observed in mice overexpressing DSG2 or DSG2^{N271S} (human DSG2^{N266S} correlate). DSG2^{N271S} mice had arrhythmias and prolonged QRS complexes, which are a sign of decreased conduction velocity, probably caused by reduced sodium currents (182, 183). Prolonged QRS complexes were also observed in *Dsg2*$^{E4-5}$ mice (173). In cardiac-specific *Jup*$^{-/-}$ mice, QRS complexes with a lower amplitude and prolonged PR intervals were observed (180), however, in inducible *Jup*$^{-/-}$ mice, no changes in the ECG were found (179). In cardiac-specific *Jup*$^{-/-}$ mice, which have decreased levels of DSG2 at the ICD, treatment with a DSG2 linking peptide (DSG2-LP), which stabilizes DSG2, led to increased DSG2 localization at the ICD, as well as colocalization of DSG2 with Cx43 and reduced arrhythmias (175).

b. $Na_v1.5$

Mutations in the *SCN5A* gene, which codes for the voltage-gated sodium channel $Na_v1.5$, have been associated with several cardiac diseases, amongst them, DCM, AC and the Brugada syndrome, a cardiomyopathy with disturbed ion channels (184, 185). Loss of $Na_v1.5$ reduced HL-1 cardiomyocyte cohesion (186). Loss of Cx43 impaired $Na_v1.5$ distribution and function (149, 157, 159, 187). Inducible Cx43^{D378stop} mice showed strong arrhythmias which were attributed to a mislocalization of $Na_v1.5$. These mice had a high lethality within 21 days of knockout induction (188). Knockdown of *Dsp* by siRNA in HL-1 cardiomyocytes resulted in decreased Cx43 as well as $Na_v1.5$ protein levels along with altered ion currents (189). Furthermore, PKP2 and DSG2 have each been shown to form a complex with $Na_v1.5$ as well as Cx43 in cardiomyocytes (25, 172, 183, 190-192). In HL-1 cardiomyocytes with a lentiviral *Pkp2*-knockdown as well as in *Pkp2*$^{+/-}$ mice, decreased sodium currents were observed, which in the case of HL-1 cardiomyocytes was attributed to a decreased localization of $Na_v1.5$ at the membrane (193, 194). The abnormal sodium currents observed in PKP2^{K859R} iPSC-derived cardiomyocytes were reversed by inhibiting the glycogen synthase 3 β (GSK3β) using the GSK3β inhibitors SB216763 (SB21) or CHIR99021 (195). In HL-1 cardiomyocytes stably expressing LMNA^{Q517X}, $Na_v1.5$ protein expression at the membrane was decreased, leading to impaired action potentials (196). Changes in ion currents were observed in zebrafish carrying the *Jup*^{2157del2} mutation and were attributed to an impaired trafficking of ion channels to the membrane, as observed in *Jup*^{2157del2} NRVMs (170). Furthermore, decreased levels of $Na_v1.5$, as well as SAP97, which is responsible for the membrane trafficking of $Na_v1.5$, were observed

in AC patients (153, 170). In DSG2^{G638R} iPSC-derived cardiomyocytes from AC patients, sodium currents were decreased along with *SCN5A* mRNA expression levels (197).

c. Ca^{2+}-homeostasis

Proper Ca^{2+}-homeostasis is essential for cardiomyocyte contraction. In AC, Ca^{2+}-homeostasis can be affected by altered levels of Ca^{2+}-handling proteins, as seen in *Dsg2*$^{E4-5}$ mice, where decreased mRNA levels of *Pln* and *Atp2a2* (coding for SERCA2) were observed (142). Furthermore, in a PLN^{R14del} murine AC model, changed mRNA splicing of over 200 genes was observed, amongst them, genes coding for proteins of the 'cardiac cell action potential' cluster were enriched (198). Under resting cellular conditions, GJ hemichannels are closed, whereas increased intracellular or low extracellular Ca^{2+}-concentrations enhance GJ hemichannel opening probability, which can affect electrical conduction and signal propagation (199). RyR2 and Cx43 can directly interact at the ICD. Increased intracellular Ca^{2+}-levels, together with RyR2 activation, led to GJ hemichannel opening (199). In *Pkp2*^{cKO} right ventricular cardiomyocytes, spontaneous Ca^{2+}-sparks, decreased expression of RyR2, increased membrane permeability and increased Ca^{2+}-levels in the cytoplasm and mitochondria were observed (67, 200). Inhibition of Cx43 GJ hemichannel opening by GAP19 in *Pkp2*^{cKO} right ventricular myocytes blunted the effect of the *Pkp2* knockout (200). Furthermore, *Pkp2*^{cKO} hearts had an increased susceptibility for arrhythmias paralleled by impaired Ca^{2+}-currents, whereas in *Pkp2*^{cKO} mice with a heterozygous *Gja1* knockout (*Gja1* being the gene coding for Cx43), Ca^{2+} currents were normalized (200). Apart from that, *Pkp2*^{cKO} mouse hearts had decreased levels of the calcium channel $Ca_v1.2$, calsequestrin 2 and ANKB, increased Ca^{2+}-leakage and prolonged action potentials (67). In human cardiac mesenchymal stromal cells, decreased levels of PKP2 led to a dysregulation of the Ca^{2+}-handling machinery with increased levels of SERCA2 and calcium/calmodulin-dependent protein kinase II (CAMKII) phosphorylation, whereas $Ca_v1.2$ was decreased (201).

In AC patients, decreased protein levels of DP led to a downregulation of integrin 1βD, which in turn increased phosphorylation of RyR2 at S2030 and thereby led to delayed after-depolarizations and a spontaneous Ca^{2+} release from the sarcoplasmic reticulum, especially upon adrenergic stimulation (202). SERCA inhibition decreased cellular cohesion by decreasing DP and DSG2 localization at the membrane in HL-1 cardiomyocytes (25).

Dysregulation of Ca^{2+}-signaling and Ca^{2+}-overload in mitochondria led to cardiomyocyte apoptosis and necrosis, another hallmark of AC (203, 204). Involvement of mitochondrial damage resulting in inflammation and cardiomyocyte death has been hypothesized, but not studied extensively in AC pathogenesis (205). Indeed, in *Dsg2*$^{E4-5}$ mice, forced exercise in the

form of swimming increased inflammation and cardiomyocyte necrosis through increased cytosolic Ca^{2+}-levels via mitochondrial dysfunction (144). Furthermore, in cardiac-specific *Dsp*$^{-/-}$ mice, proteins associated with pyroptosis, mitochondria-related apoptosis and necroptosis together referred to as PANoptosis, were increased (206).

1.5.2.2.Apoptosis, necrosis and inflammation

Post-mortem, cardiac inflammation was observed in up to 70% of AC patients (207). Increased apoptosis or necrosis has also been observed in AC patients' hearts (142, 208-213), AC patient-derived iPSC-derived cardiomyocytes (177), *Jup*^{2157del2} NRVMs, murine AC models, such as the cardiac-specific DP^{R2384H} mice, epicardial cell-specific *Dsp*$^{-/-}$ mice, *Dsg2*$^{mut/mut}$ mice with a premature stop codon, *Dsg2*$^{E4-5}$ mice, cardiac-specific *Jup*^{2157del2} or *Jup*$^{-/-}$ mice (12, 115, 144, 170, 214-218). Similar to changes in the ECG, increased apoptosis and necrosis accompanied by inflammation in the heart is an early symptom of AC (182). Apoptosis in AC might be caused by inflammatory and pro-apoptotic cytokines (219). On the other hand, necrosis causes increased inflammation of cardiac tissue (217) and it is not clear, which occurs first, apoptosis or inflammation.

Immune cell infiltration close to fibrotic areas has been observed in AC boxer dogs, *Dsg2*$^{E4-5}$ mice, *Dsg2*^{cKO} mice and in *Des*$^{-/-}$ mice (166, 173, 207, 217, 218, 220-222). Levels of apoptosis were positively correlated to fibrosis and immune cell infiltration in AC (223). However, even when no immune cell infiltration was present, cardiomyocytes of AC patients secreted inflammatory cytokines (224). The levels of interleukin 1β (IL1β), IL6, IL6 receptor (IL6R), IL8, monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1β (MIP1β), tumor necrosis factor α (TNFα) as well as TNFα receptor 1 and 2 were increased in the blood serum or plasma of some AC patients (225, 226). IL6, TNFα and IL17 decreased PG levels at the ICD (225). Plasma levels of C-reactive protein (CRP) were more increased after ventricular tachycardia in AC patients as compared to patients with ventricular tachycardia without AC (227). *Jup*^{2157del2} NRVMs also secreted inflammatory cytokines into the cell culture medium (170). *Dsg2*$^{E4-5}$ mice had increased levels of IL1α, IL1β, IL2, IL4, IL6, IL6R, IL13, TNFα, interferon γ (IFNγ), chemokine (C-X-C motif) ligand protein 5 (CXCL5), CXCL1, CXCL13, MCP1, granulocyte-macrophage colony-stimulating factor as well as NFκB in the heart (218, 219, 228). NFκB inhibition by Bay 11-7082 reversed the AC phenotype by restoring normal protein localization of Cx43 and PG, preventing apoptosis and the secretion of inflammatory cytokines in *Jup*^{2157del2} NRVMs. Furthermore, NFκB inhibition restored heart function, prevented fibrosis and inflammation, and normalized PG, Cx43 and GSK3β localization in *Dsg2*$^{E4-5}$ mouse hearts (228). Treatment of AC patient-derived *Pkp2*$^{c.2013delC}$

iPSC-derived cardiomyocytes with Bay 11-7082 alleviated inflammation by reducing cytokine production (228). In *Jup*^{2157del2} mice and *Dsg2*$^{mut/mut}$ mice with a premature stop codon, inflammation could be prevented by inhibiting GSK3β using SB21 (214). In *Dsg2*$^{mut/mut}$ mice, GSK3β inhibition improved SERCA function (229).

In *Des*$^{-/-}$ mice and in some AC patients, the complement system was activated (222, 230). Furthermore, circulating levels of acylation-stimulating protein, a product of the complement system, were correlated to AC disease severity and increased risk for heart failure (231).

An autoimmune involvement in AC was already hypothesized in 1989 (114). However, only in 2018, a first report showed that in AC boxer dogs and some AC patients, anti-DSG2 antibodies are present, followed by another study in AC patients showing autoimmune antibodies directed against heart and ICD proteins (232-234).

Altogether, the inflammatory processes and possible autoimmune component link AC to granulomatous myocarditis and PV (225).

1.5.2.3.Fibrosis, adipogenesis, lipogenesis and the Wnt pathway

Upon disease progression, apoptotic and necrotic areas in the heart are replaced by fibrotic and fatty tissue. However, recently, it has been suggested that the shift towards adipogenesis might be one of the early drivers of AC (235). Mice with a *Dsg2* mutation where no tryptophan swap is possible (DSG2-W2A mice) showed increased integrin αV/β6 levels at the ICD, which in turn induced TGFβ production and thereby led to fibrosis and arrhythmia (236). One of the main mechanisms of adipogenesis and lipogenesis in AC is the Wnt pathway. The Wnt pathway is one of the best-studied biological pathways. However, its effect in AC and other cardiac pathologies, such as MI, is controversial (1, 8, 237), indicating that a tight regulation of the Wnt pathway is necessary. The majority of the data indicate that Wnt pathway inhibition favors the progression of AC. Usually, cytosolic β-catenin which is not associated with AJs, is quickly degraded. Upon activation of the Wnt signaling pathway, GSK3β is phosphorylated, leading to its inactivation. Consequently, β-catenin remains hypophosphorylated, thereby preventing its ubiquitinylation and degradation (1, 237, 238). β-catenin translocates to the nucleus and binds to TCF/LEF by displacing Groucho. Through interaction with further proteins, such as the cAMP responsive element binding protein and BCL9, transcription that was repressed before, is activated (1), leading to a transcriptional program that promotes myogenesis and inhibits adipogenesis. Therefore, inhibition of the Wnt pathway, thus β-catenin degradation, leads to an adipogenic and fibrotic switch in transcription (239-241). Due to its structural similarity to β-catenin, PG can compete with β-catenin for TCF/LEF binding in the nucleus (238). This way,

PG inhibits β-catenin signaling, as PG-TCF/LEF complexes are not as efficient as β-catenin-TCF/LEF complexes in binding DNA and initiating transcription (238, 242).

In several AC models, alterations in the Wnt pathway have been found. Mutations, either in *Jup*, or in genes coding for other desmosomal components, such as DP or PKP2, reduced incorporation of PG into desmosomes and increased nuclear PG localization. Therefore, PG might play a unique role in the pathogenesis of AC, as its expression and localization were found to be altered even in AC models or AC patients, where the causative mutation was not in the *JUP* gene (124, 127, 153, 154, 160, 243, 244). In mice, PG overexpression or expression of a truncated PG led to nuclear localization of PG, increased fibrosis, adipogenesis, lipogenesis and increased mortality, which was attributed to suppressed canonical Wnt signaling (245). In Boxer dogs, β-catenin translocated from the ICDs towards the endoplasmic reticulum, and it was hypothesized that this decreased Wnt signaling and favored the development of an AC phenotype (246).

In cardiac fibro-adipocyte progenitors, deletion of *Dsp* increased Wnt signaling, which favored the differentiation towards adipocytes (247). In HL-1 cardiomyocytes with a siRNA-mediated *Dsp* knockdown as well as in *Dsp*$^{-/-}$ or DP^{R2384H} mice, increased adipogenesis or lipogenesis and fibrogenesis resulting in an AC phenotype with altered protein localization of PG, DES, β-catenin and Cx43 was observed (241, 248). However, in a different *Dsp*$^{-/-}$ mouse line, no changes in PG localization were found, whereas protein levels of DSG2, DSC2, PKP2 and Cx43 were decreased (171). Decreased β-catenin activation was found in DSG2^{Q558X} mice along with an altered miR profile and decreased levels of β-catenin downstream targets c-Fos, c-Myc and cyclin D1 (249). Increased GSK3β activation and decreased β-catenin activation was found in TMEM^{S358L} mice along with increased apoptosis and could be prevented by GSK3β inhibition (174). Two AC patient-derived *PKP2*-mutant iPSC-derived cardiomyocyte lines, namely *PKP2*$^{c.2484C>T}$ and *PKP2*$^{c.1841T>C}$, showed increased lipogenesis. In addition, in *PKP2*$^{c.2484C>T}$ iPSC-derived cardiomyocytes, altered ion currents as well as increased apoptosis, but no fibrogenesis was observed (243, 250). Furthermore, treatment of *PKP2*$^{c.972InsT/N}$ iPSC-derived cardiomyocytes with the GSK3β-inhibitor SB21 activated the Wnt pathway, rescued the adipogenic phenotype, but did not alter cellular junctions (177). SB21 also improved electrophysiological parameters in *Jup*^{2157del2} zebrafish. In *Jup*^{2157del2} NRVMs and mice, in *Dsg2*$^{mut/mut}$ mice (premature stop codon), as well as in *Pkp2*^{1851del123} NRVMs, SB21 treatment restored the mislocalization of PG and Cx43 (170, 215). Furthermore, SB21 prevented apoptosis in *Jup*^{2157del2} mice and increased N-CAD localization at cell junctions in *Jup*^{2157del2} NRVMs (214). In *PKP2*$^{c.354delT}$ as well as in PKP2^{K859R} iPSC-derived cardiomyocytes, PG

translocated from the cellular junctions towards the cytoplasm; however, no fat accumulation was observed (195).

In different cardiac-specific *Jup*$^{-/-}$ mouse lines, β-catenin expression was increased (106, 179, 180). While in congenital *Jup*$^{-/-}$ mice, no changes in the nuclear localization of the protein and no subsequent changes in the Wnt signaling were observed (180), tamoxifen-inducible cardiac-specific *Jup*$^{-/-}$ mice showed increased β-catenin protein levels along with increased Wnt signaling (179). Interestingly, *Jup*$^{-/-}$ mice do not seem to have fatty, but rather only fibrotic infiltrates of the cardiac tissue (179).

In human AC hearts activation of the hippo signaling pathway, a pathway that can inhibit Wnt signaling, was found (65). Furthermore, in sh*Pkp2*-treated HL-1 cardiomyocytes, changes in the hippo signaling pathway were observed, along with increased phosphorylation of β-catenin and decreased Wnt signaling activity (65). On the other hand, in another study, utilizing sh*Pkp2*-treated HL-1 cardiomyocytes, no changes in Wnt or hippo signaling were found. Instead, changes in the miR expression pattern were observed and loss of miR-184 was found to be a driver of pro-lipogenic transcription (64). Increased lipogenesis was also observed in DSG2^{N271S} mice, though PG localization appeared normal (182). Taken together, these data indicate that adipogenesis and lipogenesis must not necessarily be related to changes in Wnt or hippo signaling.

Altogether, abnormalities in ion channel distribution, decreased cellular adhesion and increased fibro-fatty infiltrations (which do not conduct the cardiac excitation) lead to reduced and heterogeneous conduction. Finally, the “precision machine” heart (the engine of life) no longer functions properly: the stage is set for severe and life-threatening arrhythmias in AC patients (Figure 4).

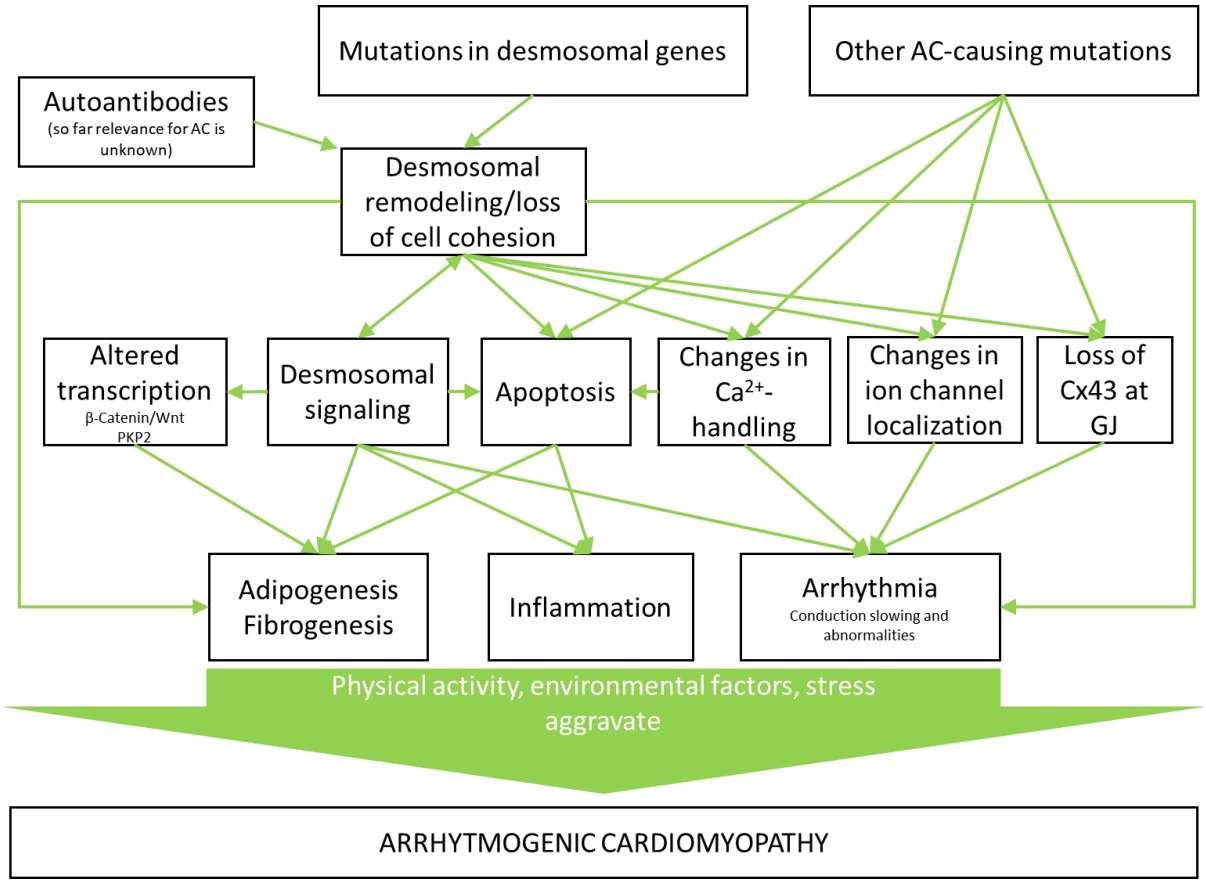

Figure 4 - Mechanisms of AC, adapted from (141).

1.6. The role of PKA, p38MAPK, ERK1/2, PKC, EGFR and ADAM17 in the heart

As mentioned above, in keratinocytes and enterocytes, several pathways are known to influence cellular cohesion, including PKA, EGFR, PKC and the MAP kinases p38MAPK and ERK1/2. Furthermore, MMPs or ADAMs are known to be involved in cellular cohesion by shedding of adhesion molecules or release of transmitters which activate or repress signaling pathways. Since desmosomes can serve as signaling hubs, and in AC desmosomal contacts are disturbed, desmosomal signaling might also be affected in AC. Furthermore, signaling pathways can affect desmosomal composition. Therefore, to get a better understanding of the underlying mechanisms of AC it is crucial to investigate signaling pathways that regulate desmosomal cohesion. Since this work focuses on the role of PKA, p38MAPK, ERK1/2, PKC, EGFR and ADAM17 in cardiomyocyte cohesion, the next sections contain a brief introduction to the role of these proteins especially in the heart as well as on cellular cohesion in other tissues. So far, only an involvement of PKA and PKC in the regulation of cardiomyocyte cohesion was reported (10, 106).

1.6.1. PKA

Upon binding of catecholamines to the β-adrenergic receptor in the heart, which is a G-protein coupled receptor (GPCR), its G-protein subunit is released into the cytoplasm and activates adenylyl cyclases which convert cellular ATP to cAMP, the main activator of PKA signaling (251). The central role of cAMP signaling via PKA in the heart is to control E-C coupling upon adrenergic stimulation (252). In this aspect, cAMP signaling is involved in the regulation of inotropy (contraction force), chronotropy (contraction rate) and lusitropy (ability to relax) by regulating Ca^{2+}-handling proteins and the contractile apparatus (253, 254). Loss of PKA renders cardiomyocytes irresponsive to adrenergic signaling, but also leads to a slight dysregulation of Ca^{2+}-handling without cardiac adverse effects (255). Apart from regulating E-C coupling, PKA signaling in the heart also affects apoptosis, mitochondrial homeostasis and gene transcription (252). A cardiotoxic role of PKA activation especially upon MI, ischemia or during hypertrophy has been well established (251). Apart from that, PKA was found to be involved in diabetic cardiomyopathy, Takutsobo cardiomyopathy as well as anthracycline-induced cardiomyopathy (251). On the other hand, nuclear and cytoplasmic PKA signaling might have opposite effects, since nuclear PKA activity favors, whereas cytoplasmic PKA signaling inhibits hypertrophy (256, 257). Furthermore, PKA can phosphorylate and thereby inhibit GSK3β, which in turn enhanced *Gja1* mRNA levels in murine hearts upon MI (258). Besides, myocardial injury following MI is milder when cAMP levels are elevated prior to MI by β-adrenergic agonists, adenylyl cyclase activators or phosphodiesterase (PDE) inhibitors (251).

The β-adrenergic receptor, which is the main activator of PKA signaling in the heart, has been shown to be localized at the ICD (10), thus misassembled ICDs might affect adrenergic signaling in the heart. In fact, adrenergic signaling induced positive adhesiotropy in cardiomyocytes, however was not effective to induce positive adhesiotropy in the absence of PG (106). Nevertheless, adrenergic signaling reduced arrhythmias in HL-1 cardiomyocytes with a siRNA-mediated *Jup* knockdown, indicating that adrenergic signaling can affect GJs in the absence of PG (25). Conversely, cholinergic signaling induced by acetylcholine (ACh) decreased cardiomyocyte cohesion via a phosphatidylinositol-3 kinase (PI3K)-AKT axis and prevented adrenergic signaling-mediated PG phosphorylation and positive adhesiotropy (259).

1.6.2. p38MAPK and ERK1/2

In si*JUP* keratinocytes, where p38MAPK activation was increased, inhibition of p38MAPK enhanced cellular cohesion, whereas it was ineffective in si*DSP* keratinocytes (71). Furthermore, inhibition of p38MAPK prevented loss of cell cohesion in keratinocytes treated

with mucosal PV-IgG (m-PV-IgG, containing autoantibodies targeting DSG3), mucocutaneous PV-IgG (mc-PV-IgG, containing autoantibodies targeting DSG1 and DSG3), or pemphigus foliaceus IgG (PF-IgG, containing autoantibodies targeting DSG1) (260). Increased p38MAPK or ERK1/2 activity in the heart seems to be a two-sided sword. ERK1/2 inhibition protected mice against ischemia/reperfusion injury by reducing cardiac inflammation, as well as cardiomyocyte apoptosis and autophagy (261). Increased ERK1/2 activity is involved in hypertrophic cardiomyopathy, maladaptive hypertrophy in response to anthracycline treatment and in adaptive cardiac hypertrophy e.g., upon cardiac stress to prevent apoptosis and cardiac injury. ERK1/2 downregulation has been suggested to favor the transition to maladaptive hypertrophy during pressure overload and subsequent heart failure (262). In mice expressing constitutively active MEK1, the upstream kinase of ERK1/2, increased hypertrophy was observed. Interestingly, these mice showed increased resistance to cardiac apoptosis (143), indicating that the role of ERK1/2 in cardiomyocytes is rather complex. It has been suggested that ERK1/2 signaling mediates hypertrophy, whereas p38MAPK is more associated with apoptosis during post-MI remodeling of the heart (263). After ischemia-reperfusion injury as seen after MI, p38MAPK was activated and induced apoptotic pathways, whereas inhibition or knockout of p38MAPK, especially the main cardiac isoform p38MAPKα, reduced apoptosis, cardiac hypertrophy and fibrosis (264-267). In a pacing-induced heart failure model in rabbits, phosphorylation of p38MAPK was significantly increased along with fibrosis and hypertrophy and was correlated to the number of apoptotic cells (268). On the other hand, a cardioprotective role of p38MAPK upon MI was shown in rats (269). Inhibition or a cardiac-specific knockout of p38MAPKα induced hypertrophic cardiomyopathy with reduced cardiac function, and the severity of the cardiomyopathy was correlated to the degree of p38MAPK inhibition (270). Furthermore, under certain conditions, e.g., after anoxic preconditioning, p38MAPK activity was anti-apoptotic in cardiomyocytes (271). On the other hand, in diabetic mice suffering from diabetes-induced cardiac dysfunction or diabetic cardiomyopathy, p38MAPK activation was increased in the heart, accompanied by hypertrophy and increased apoptosis. In contrast, inhibition or knockdown of p38MAPK reduced hypertrophy, apoptosis and reduced inflammatory cytokine levels, leading to cardioprotection (272-275). In hearts explanted from patients with DCM, kinase activities of p38MAPK, SRC, ERK, c-Jun, JNK, BMK1 and p90RSK were upregulated (276). In PKP2 or DP-deficient NRMVs, TGFβ was upregulated and led to the transcription of pro-inflammatory and pro-fibrotic genes via p38MAPK signaling, whereas p38MAPK inhibition prevented the changes in gene expression (66). All in all, data indicate that different pools or isoforms of p38MAPK might have different functions in the

heart, and that the activity of these kinases needs to be tightly regulated to hold the balance between their cardioprotective and cardiotoxic effects. Given the role of ERK1/2 and p38MAPK in regulation of cell cohesion in other cell types, these proteins might be of interest in regulation of cardiomyocyte adhesion.

1.6.3. PKC

The PKC family consists of three major groups of PKCs which were classified by the mechanism that activates their signaling: a) classical PKCs, which are activated by Ca^{2+} and diacylglycerol (DAG), b) novel PKCs, which are activated only by DAG, independent of Ca^{2+} and c) atypical PKCs, which are independent of both, Ca^{2+} and DAG (277). Knockout of a specific PKC isozyme can lead to the compensatory upregulation of other PKC isozymes (277). Depending on the isozyme in question and the physiological context, PKC signaling can be cardioprotective or cardiotoxic. Upon ligand binding, PKCs translocate to the cytoplasm where they are directed to specific target sites through receptors of C kinase proteins (278).

PKC activation is associated with the cardiac inflammatory response, whereas PKC inhibition reduced the secretion of inflammatory cytokines in the heart (277, 279, 280). Furthermore, PKC isozymes differentially regulate MMPs or ADAMs, and thereby affect extracellular matrix composition, adhesion molecules, as well as the secretion of membrane-bound mediators into the extracellular space (281-283). Cardiac fibrosis is differentially regulated by PKC isozymes in the heart (284-287). Inhibition of PKCα and PKCβ one week post-MI improved cardiac function (288). Aberrant PKCα or PKCβ activity induced cardiomyocyte hypertrophy via ERK1/2 and enhanced protein synthesis (278, 289, 290), whereas PKCε activity was associated with compensated hypertrophy without heart failure (291). Overexpression of PKCβ or its constitutive activity led to cardiomyopathy resembling diabetic cardiomyopathy (292). PKCθ inhibition ameliorated diabetic cardiomyopathy in mice by reducing fibrosis (293). On the other hand, PKCθ-knockout in mice led to a phenotype resembling DCM with cardiomyocyte hypertrophy and apoptosis, cardiac fibrosis and cardiac dysfunction (294). Inhibition of PKCε resulted in a potentially lethal cardiomyopathy that resembled DCM (295). Furthermore, cardiomyocyte-specific expression of an inhibitory protein fragment for PKCδ decreased SERCA protein levels and resulted in lethal myofibrillar cardiomyopathy in mice (296). Apart from that, novel PKC activation is involved in ischemic preconditioning, through which the heart is partially protected from ischemia-reperfusion injury, whereas inhibition of novel PKCs abolished the cardioprotectiveness of ischemic preconditioning (297-300).

In AC patients, PKC protein levels were decreased, which was also observed in murine AC models, where PKC localization at the ICD was reduced compared to wildtype mice (65).

Decreased PKC levels were also observed in sh*Pkp2*-treated HL-1 cardiomyocytes, leading to an activation of the hippo signaling pathway, since PKC inhibits the hippo signaling pathway through phosphorylation of NF2 and Merlin (65). Furthermore, PKC activation induced positive adhesiotropy in HL-1 cardiomyocytes (25) and might therefore be of interest in AC.

1.6.4. EGFR

The EGFR family consists of four receptor tyrosine kinases: EGFR, ErbB2, ErbB3 and ErB4. EGFR can be activated by several ligands, including EGF, amphiregulin, heparin-bound EGF (HB-EGF), TGFα, betacellulin or epiregulin. These ligands are expressed as transmembrane precursors and are cleaved by ADAMs or MMPs (301). Upon ligand binding, EGFR dimerizes, leading to its autophosphorylation and subsequent signaling events through phosphorylation or binding of signaling effectors (302). EGFR activation can also occur independently of ligand binding by transactivation; then other proteins, such as angiotensin II (Ang II), the death receptor 5, PKC or the β-adrenergic receptor activate EGFR (303-307).

Proteins of the EGFR family are crucial during embryogenesis and knockout or mutation of either one member of the EGFR family led to severe, sometimes lethal cardiovascular defects (308). The role of EGFR in adult cardiomyocytes is complex. EGFR signaling decreased cardiomyocyte apoptosis and was needed for proper cardiac contractility, ischemic preconditioning and was protective during ischemia-reperfusion injury or adrenergic signaling-induced cardiac damage, whereas EGFR inhibition or mutation led to myocardial dysfunction (309-312). Mice with a cardiac-specific EGFR knockdown first seemed healthy; however, cardiac function decreased starting from the age of nine weeks with subsequent cardiac remodeling at the age of ten months. However, chronic adrenergic stimulation rescued cardiac function without leading to hypertrophy. Furthermore, these mice had alterations in Ca^{2+}-homeostasis through decreased PLN and cardiac troponin C phosphorylation. Untreated, these mice had a median survival of 13 months (313). EGFR transactivation by the cardiac death receptor 5 led to hypertrophy without fibrosis or apoptosis in mice (303). Vascular and cardiomyocyte-specific *Egfr* knockout mice had increased inflammation, fibrosis, hypertrophy and increased lethality (314). EGFR was involved in morphine-induced protection from ischemia-reperfusion injury in rats and treatment with EGF showed similar effects as morphine (315). Chronic exposure of mice to the EGFR inhibitors EKB-569 and AG1478 increased cardiac fibrosis, especially in female mice (316). Apart from that, EGFR downregulation decreased cardiac function in mice and led to hypertrophy, interstitial fibrosis, altered Ca^{2+}-homeostasis and ultimately to death. EGFR inhibition-sensitive changes in cytokine expression upon adrenergic stimulation were mainly attributed to cardiac fibroblasts (317). After MI,

cardiac EGFR expression was increased, however, enhanced EGFR protein levels were mainly found in fibroblasts rather than in cardiomyocytes (318). In mice, EGFR inhibition by gefitinib prevented cardiomyocyte apoptosis, hypertrophy and expression of inflammatory cytokines induced by chronic isoprenaline treatment, but did not affect fibrosis, whereas gefitinib treatment alone, similar to isoprenaline, induced fibrosis (317). However, opposite results were obtained when subjecting mice chronically treated with isoprenaline to erlotinib (307). Deletion or mutation of ErbB2, another member of the EGFR family, led to DCM in mice and decreased survival after pressure overload and increased sensitivity to anthracycline-induced cardiomyocyte injury (301, 319). In diabetic rats, EGFR protein levels were increased and led to vascular dysfunction. In contrast, acute EGFR activation protected the heart from ischemic injury, whereas EGFR inhibition worsened cardiac function (320). On the other hand, EGFR and ErbB2 inhibition by lapatinib or inhibition of EGFR alone by AG1478 attenuated diabetes-induced vascular dysfunction and reversed the diabetes-induced changes in gene expression as well as the increase of NFκB, ROCK, and ERK1/2 protein levels and activity (321-323). Furthermore, in diabetic mice or mice with diabetic cardiomyopathy, EGFR inhibition ameliorated cardiac fibrosis, endoplasmic reticulum stress and endothelial dysfunction, reduced cardiac remodeling, hypertrophy as well as myocardial injury, cardiomyocyte apoptosis, reactive oxygen species generation, oxidative damage and restored SERCA2a localization (324-326). Furthermore, EGFR inhibition protected the heart from ischemia-reperfusion injury by reducing inflammation, apoptosis, fibrosis, and endoplasmic reticulum stress in a diabetic setting (327). EGFR inhibition also prevented obesity-induced cardiac fibrosis, injury and inflammation in mice, thereby attenuating hyperlipidemia-induced cardiomyopathy (328).

Overexpression of ErbB2 led to hypertrophy, fibrosis and increased lethality in response to adrenergic signaling, whereas lapatinib-treatment reversed the ErbB2 effects (329). Enhanced EGFR signaling through overexpression of HB-EGF accelerated cardiac fibroblast proliferation and enhanced cardiomyocyte hypertrophy, whereas overexpression of HB-EGF two or four weeks after MI increased fibrosis and apoptosis (330). EGFR activation was observed after MI in mice, whereas silencing of amphiregulin reduced EGFR activation and improved cardiac function by reducing fibrosis but not apoptosis (331). Inhibition of ADAM12, an ADAM that can transactivate EGFR through HB-EGF shedding, prevented cardiac hypertrophy by decreasing EGFR signaling (332). Reduced EGFR protein levels were associated with reduced fibrosis in healthy mice and reduced pressure overload-induced cardiac dysfunction (333). Furthermore, EGFR signaling was associated with renal fibrosis, whereas EGFR inhibition decreased renal inflammation (334, 335). EGFR inhibition protected cardiomyocytes against

aldosterone-induced damage (336). Ang II via EGFR signaling induced cardiac fibrosis, inflammatory cytokine production and hypertrophy, whereas SRC inhibition, EGFR inhibition, downregulation or mutation, or mutation of the Ang II receptor lacking the ability to transactivate EGFR, reduced these effects (337-341). Though mice with mutated EGFR showed remodeling upon aldosterone treatment, they were protected from aldosterone-induced endothelial dysfunction (342). Furthermore, erlotinib treatment prevented abdominal aortic aneurysm formation as well as hypertrophy, fibrosis and upregulation of IL6 and TGFβ in mice upon treatment with Ang II and β-aminopropionitrile (343). In rats treated with ACh, cardiac fibroblast proliferation and collagen content was increased through nicotinic ACh receptor (nAChR) signaling via EGFR, whereas inhibition of nAChR signaling decreased fibrosis, reduced EGFR phosphorylation and ameliorated cardiac function in rats with pulmonary hypertrophy (344). Altogether it seems that EGFR signaling needs to be tightly controlled in the heart since its dysregulation leads to cardiovascular pathologies (345). Since EGFR inhibition affected keratinocyte cohesion, and EGFR is regulated in several cardiac pathologies, regulation of EGFR activity might be of interest in the pathogenesis of AC.

1.6.5. ADAM17

Dysregulation of ADAM17, also known as TNFα converting enzyme (TACE), is involved in several diseases, including cardiovascular or renal pathologies, cancers, acute pancreatitis, inflammatory and autoimmune disorders, nervous system disorders or liver diseases (346-350). ADAM17 can be activated by GPCRs, PKC, p38MAPK or other ADAMs or MMPs (346, 351). Ang II increased ADAM17 protein levels and activity in murine hearts via p38MAPK (352). Furthermore, Ang II-mediated EGFR effects can be modulated by ADAM17 (353). ADAMs and EGFR can be activated by cholinergic signaling, which is also known to reduce cellular cohesion in cardiomyocytes (259, 344, 354).

Over 80 substrates of ADAM17 have been identified so far, including proteins that mediate cell adhesion, but also EGF, TNFα, IL6 and other cytokines, IL6R and the β-adrenergic receptor (302, 335, 347, 351, 355).

Mice with reduced ADAM17 protein levels were more susceptible to inflammatory bowel disease (IBD) and developed atherosclerosis through defective EGFR or TNFα receptor 2 signaling (356, 357). Apart from that, these mice had cardiac hypertrophy, opaque eyes and waved hair follicles (357). In line with that, *ADAM17* loss of function mutations were associated with a phenotype resembling inflammatory skin and bowel disease, as well as ventricular dilation, with increased DSG2 expression in keratinocytes and decreased TNFα production

(358, 359). Furthermore, cardiomyocyte-specific *Adam17* knockdown mice developed cardiomyopathy and had suppressed angiogenesis after MI and decreased survival rates (360). On the other hand, after acute MI as well as in patients with myocarditis, ADAM17 and TNFα levels were increased and were correlated to in-hospital complications or severity of myocarditis (361-363). Furthermore, ADAM17 inhibition reduced fibroblast migration *in vitro* and reduced fibrosis and improved cardiac function after acute MI *in vivo* (364). ADAM17 is involved in cardiac hypertrophy, whereas miR or shRNA-mediated knockdown of *Adam17* decreased hypertrophy (365). Inhibition or deletion of ADAM17 protected mice from EGFR-mediated kidney fibrosis upon acute kidney injury and reduced inflammation upon renal injury in mice, whereas in patients with chronic kidney disease, ADAM17 was upregulated (366). Increased ADAM17 protein levels were found in murine diabetic hearts, whereas a cardiomyocyte-specific knockout of ADAM17 improved cardiac function and remodeling in diabetic mice and reduced cardiomyocyte apoptosis (367). In patients with DCM or hypertrophic obstructive cardiomyopathy, increased ADAM17 and TNFα levels were observed, whereas protein levels of tissue inhibitor of metalloproteinases 3 (TIMP3), an inhibitor of ADAM17, were decreased (368, 369). Furthermore, TIMP3 deficiency led to DCM with impaired cardiac function, hypertrophy and increased soluble TNFα levels, as well as to heart failure or interstitial renal fibrosis (370-372). Simultaneous inhibition of MMPs and TNFα prevented heart failure in TIMP3-deficient mice (372). TIMP3 overexpression reduced tissue damage and mortality after ischemia-reperfusion injury or doxorubicin-induced cardiac injury by decreasing infarct size and cardiomyocyte apoptosis via inhibition of MAPK signaling (373). Increased ADAM17 mRNA and protein levels were observed in two week old *Dsg2*$^{E4\text{-}5}$ mice, whereas in adult mice ADAM17 levels were comparable to wildtype mice (218).

All in all, these data suggest that a basal activity of ADAM17 is needed for physiological tissue homeostasis, whereas overexpression or increased ADAM17 activity can favor pathologies. Given that ADAM17 is upregulated in several cardiovascular pathologies as well as in hearts of two week old AC mice and can shed DSG2 and TNFα, inhibition of ADAM17 might be beneficial in AC.

Together, these data show that PKA, PKC, p38MAPK, EGFR or SRC signaling as well as ADAM17 activity are involved in several cardiac pathologies and in some cases have already been associated with AC or with regulation of cellular cohesion. Regulating the activity of these proteins might affect cardiomyocyte cohesion and might thereby be beneficial in AC.

2. Aims of the thesis

Arrhythmogenic cardiomyopathy (AC) is a genetic disease, that untreated can lead to sudden cardiac death, which in some cases occurs even before patients felt symptoms of the disease. The hallmarks of AC are arrhythmia, fibro-fatty replacement of the myocardium and cardiac inflammation. Mutations in genes coding for proteins of the desmosome can cause AC through destabilization of cardiomyocyte cohesion and changes in ion channel localization. To date, treatment possibilities for AC are only symptomatic treatment options, such as changes in lifestyle, antiarrhythmic drugs, implantable cardioverter-defibrillators, or heart transplantation. To improve quality of life, it is crucial to find treatment options that go beyond symptomatic treatment and prevent disease progression. Since strengthening cardiomyocyte cohesion might improve the AC phenotype, in this study, mechanisms to strengthen cardiomyocyte cohesion were assessed in HL-1 cardiomyocytes and in *Jup*$^{-/-}$ mice, representing an AC model. Modulation of signaling pathways in AC is of interest, especially since desmosomes do not only provide cellular cohesion but can also regulate cellular signaling by serving as signaling hubs. After identifying signaling pathways which increase cellular cohesion in cardiomyocytes, the underlying mechanisms of enhanced cardiomyocyte cohesion will be investigated. These possibilities to strengthen cardiomyocyte cohesion can pave the way for finding new therapeutical approaches of AC beyond symptomatic treatment. Furthermore, the findings can also be of interest for other cardiac pathologies and other diseases caused by dysfunction of desmosomes with which AC shares common aspects.

3. Discussion

Impaired desmosome turnover which weakens cellular cohesion is one of the drivers of AC leading to arrhythmia, cardiac injury, fibrosis and adipogenesis. Therefore, in order to find therapeutic options beyond symptomatic treatment for AC, it is necessary to get a better understanding of desmosomal adhesion which is crucial for cardiomyocyte cohesion. In this work, several, in part interdependent mechanisms regulating cardiomyocyte cohesion were discovered and are summarized in Figure 5. These findings might pave the way in finding new therapeutic approaches for AC beyond symptomatic treatment.

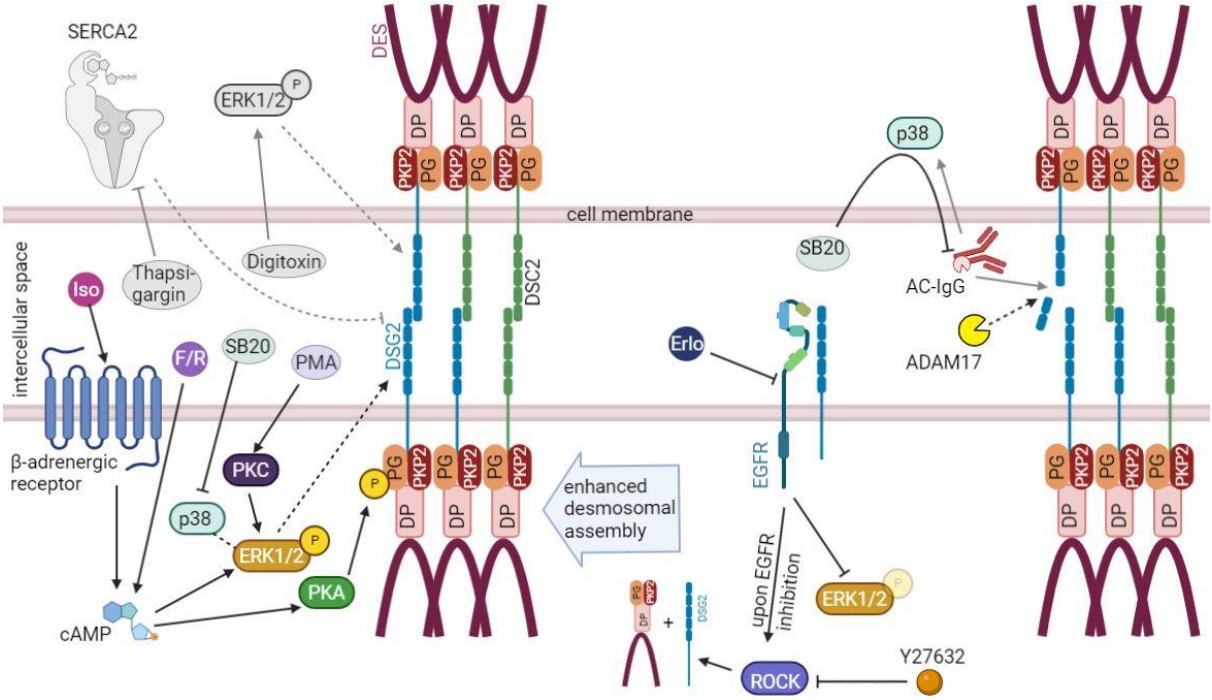

Figure 5 – mechanisms regulating cardiomyocyte cohesion.
Activation of adrenergic signaling and PKC induced positive adhesiotropy. Furthermore, inhibition of p38MAPK, EGFR or ADAM17 also enhanced cardiomyocyte cohesion. Positive adhesiotropy upon adrenergic signaling, PKC activation and p38MAPK inhibition was dependent on ERK1/2. EGFR inhibition-mediated positive adhesiotropy was mediated through enhanced desmosomal assembly dependent on ROCK activity. In contrast, ADAM17 inhibition did not lead to enhanced desmosomal assembly. Apart from that, p38MAPK inhibition prevented the AC autoantibody-mediated loss of cardiomyocyte cohesion.
Greyed out parts of the image show what was known before (left) or what was shown by other lab members (DSG2 cleavage through autoantibodies, right).
This cartoon was created using Biorender.com

In this work, *Jup*$^{-/-}$ mice were utilized as a murine AC model. *Jup*$^{-/-}$ mice show extensive fibrosis already at the age of six weeks (13, 374). Furthermore, these mice have arrhythmia, decreased levels of DSG2 at the ICD and increased β-catenin protein levels (106, 175, 179, 180, 374). While the knockout of either, *Jup* or *Ctnnb1* (coding for β-catenin) is not lethal, a conditional knockout of both genes led to conduction abnormalities, severe arrhythmia, ICD disassembly and SCD in mice (375).

In the current thesis, it was shown that *Jup*$^{-/-}$ mice have increased EGFR protein expression and increased p38MAPK activation compared to wildtype littermates, indicating a possible involvement of these signaling pathways in the pathogenesis of AC. Furthermore, in heart lysates obtained from *Pkp2*$^{-/-}$ mice, increased EGFR levels were found. Increased EGFR protein levels were also observed in samples obtained from the left atrium of AC patients as compared to samples from patients with DCM. The effectiveness of several pathways enhancing cardiomyocyte cohesion, termed positive adhesiotropy, was assessed. As shown before (25, 106), adrenergic signaling via PKA, induced by Forskolin and Rolipram (F/R, Forskolin being an adenylyl cyclase activator and Rolipram a PDE4 inhibitor) or isoprenaline (a β-adrenergic agonist), as well as PKC activation by phorbol 12-myristate 13-acetate (PMA) led to positive adhesiotropy in HL-1 cardiomyocytes and in wildtype mice, and PKC activation was also effective to enhance cardiomyocyte cohesion in *Jup*$^{-/-}$ mice. Inhibition of p38MAPK by SB202190 (SB20), inhibition of EGFR by erlotinib, inhibition of SRC by PP2 or inhibition of ADAM17 by TAPI-1 induced positive adhesiotropy in HL-1 cardiomyocytes, in wildtype mice and in *Jup*$^{-/-}$ mice. Positive adhesiotropy was paralleled by enhanced DSG2 localization at the membrane of HL-1 cardiomyocytes in all cases apart from PKC activation. Furthermore, DP localization was enhanced at the membranes upon inhibition of EGFR, SRC or ADAM17. The enhanced localization of DSG2 as well as DP at the ICD was confirmed in wildtype cardiac slice cultures upon inhibition of EGFR or SRC, whereas in *Jup*$^{-/-}$ cardiac slice cultures, only DSG2 was increased at the ICD after EGFR or SRC inhibition. Apart from that, in HL-1 cardiomyocytes, adrenergic signaling, PKC activation and p38MAPK inhibition induced desmosome hyperadhesion independent of ERK1/2 activity. In HL-1 cardiomyocytes, positive adhesiotropy mediated by adrenergic signaling, PKC activation or p38MAPK inhibition was dependent on the expression of PG, DP and DSG2 as well as ERK1/2 activity. ADAM17 inhibition-induced positive adhesiotropy was dependent on the expression of DSG2 (the other desmosomal components were not assessed). Positive adhesiotropy upon inhibition of EGFR or SRC was only dependent on the expression of DP, but was still effective upon knockdown of *Dsg2*. Erlotinib-mediated positive adhesiotropy was achieved through enhanced desmosomal assembly, which was dependent on EGFR inhibition-mediated ROCK activation. In contrast, positive adhesiotropy upon ADAM17 inhibition was not mediated by enhanced desmosomal assembly.

3.1. The role of desmosomal proteins in cardiomyocyte cohesion

Cellular cohesion is provided by desmosomes and AJs, the components of which intermingle in the heart, reflected by the term area composita (30, 31). Therefore, a deficiency or mutation

of components of cellular junctions can destabilize tissue integrity through weakened cellular cohesion. However, siRNA-mediated knockdowns of *Dsg2*, *Jup* or *Dsp* in HL-1 cardiomyocytes did not alter cellular cohesion, as observed before (259). This indicates that other junctional proteins might compensate for the siRNA-mediated reduction of desmosomal proteins or that the amount of protein remaining after siRNA-mediated knockdown was sufficient to provide unchanged cellular cohesion. On the other hand, decreased cellular cohesion in murine cardiac slices obtained from *Jup*$^{-/-}$ mice as compared to wildtype controls was observed, showing that a knockout of *Jup* in a murine model is not directly comparable to a siRNA-mediated, time-limited knockdown in HL-1 cardiomyocytes.

Furthermore, while in HL-1 cardiomyocytes with a siRNA-mediated *Jup* knockdown, PKC activation and p38MAPK inhibition were not effective in inducing positive adhesiotropy, in *Jup*$^{-/-}$ murine cardiac slice cultures, these signaling pathways enhanced cellular cohesion. At first glance, these differences between the cell culture model and the pathogenic mice seem to put into question the suitability of HL-1 cardiomyocytes for AC research. The differences in the results obtained from experiments performed in HL-1 cardiomyocytes and murine cardiac slice cultures might be caused by the different compensatory effects of a siRNA-mediated *Jup* knockdown in HL-1 cardiomyocytes and a cardiomyocyte-specific, congenital knockout of *Jup* in mice. Another aspect that might explain the differences between HL-1 cardiomyocytes and murine cardiac slice cultures is the different origin of the cells: HL-1 cardiomyocytes are murine atrial immortalized cardiomyocytes, whereas the murine cardiac slices were obtained from cardiac ventricles. Furthermore, HL-1 cardiomyocytes are immortalized, not fully differentiated cells, where only clusters of cells beat, whereas cells are differentiated and aligned in a tissue. Importantly, in murine cardiac slices obtained from wildtype mice, all positive adhesiotropic effects observed in HL-1 cardiomyocytes were reproducible, indicating that HL-1 cardiomyocytes are at least partially comparable to the *ex vivo* mouse model. Though in *Jup*$^{-/-}$ mice not all positive adhesiotropic pathways were functional, they might still induce positive adhesiotropy in other AC models, where PG, DSG2 or β-catenin protein levels and localization are not affected. Since HL-1 cardiomyocytes are not ideal, research could be repeated in other cardiomyocyte cell lines, some of which are commonly used in AC research: H9C2 cells, NRVMs and iPSC-derived cardiomyocytes. However, these models also have their downsides: NRVMs and H9C2 cells are immature cells, whereas culturing iPSC-derived cardiomyocytes is time-consuming and expensive (376). Taken together, HL-1 cardiomyocytes are a cost- and time-efficient possibility to perform AC research, which can and should be validated in *in vivo* or *ex vivo* animal AC models, such as transgenic mice, rats or Boxer dogs.

Positive adhesiotropy induced by adrenergic signaling, PKC activation or p38MAPK inhibition was dependent on the expression of the desmosomal proteins DP, DSG2 and PG in HL-1 cardiomyocytes, as observed after siRNA-mediated knockdown of the corresponding genes. ADAM17 inhibition was not effective to induce positive adhesiotropy in HL-1 cardiomyocytes with a siRNA-mediated *Dsg2* knockdown. In contrast, EGFR inhibition induced positive adhesiotropy after knockdown of *Dsg2*, but not after a *Dsp* knockdown. Nevertheless, EGFR inhibition-mediated positive adhesiotropy was still mediated by DSG2, as shown by enhanced localization of DSG2 at the membrane, and increased DSG2 interaction probability observed by AFM measurements upon EGFR inhibition by erlotinib. These differences between positive adhesiotropy upon adrenergic signaling, PKC activation, p38MAPK inhibition or EGFR inhibition indicate two things: a) positive adhesiotropy can be induced via different mechanisms, which was also suggested by the differential regulation of ERK1/2 activity by the treatments (see 3.3) and b) since after siRNA-mediated *Dsg2* knockdown there was still some DSG2 protein left, this protein might be sufficient for the positive adhesiotropic effect of EGFR inhibition, whereas for other positive adhesiotropic treatments normal DSG2 protein expression might be needed in HL-1 cardiomyocytes.

Even though adrenergic signaling was not effective to induce positive adhesiotropy after siRNA-mediated knockdown of *Jup* or *Dsg2* in HL-1 cardiomyocytes, adrenergic signaling reduced si*Jup* or si*Dsg2*-induced arrhythmia, indicating that adrenergic signaling can affect GJs in the absence of PG or DSG2 (25). Future experiments involving murine AC models and *in vivo* experiments could shed more light on the beneficial role of adrenergic signaling in GJ function.

All treatments that induced positive adhesiotropy (adrenergic signaling, PKC activation, p38MAPK inhibition, EGFR inhibition, SRC inhibition, ADAM17 inhibition) enhanced localization of DSG2 at the membrane of HL-1 cardiomyocytes, indicating that DSG2 might be a key driver of positive adhesiotropy, which was also observed in other studies (10, 107, 175, 374). However, the mechanism by which DSG2 protein localization was increased at the cell membrane seems to differ between the treatments inducing positive adhesiotropy.

In HL-1 cardiomyocytes with siRNA-mediated *Dsg2* knockdown, PKC activation, inhibition of p38MAPK or ADAM17 did not result in positive adhesiotropy. However, despite very low protein levels of DSG2 in $Jup^{-/-}$ mice (106), PKC activation, p38MAPK, EGFR, SRC or ADAM17 inhibition induced positive adhesiotropy in murine cardiac slice cultures obtained from $Jup^{-/-}$ mice. Possible explanations for the differences between HL-1 cardiomyocytes and murine cardiac slice cultures were discussed above.

While positive adhesiotropy upon adrenergic signaling, PKC activation, p38MAPK or EGFR inhibition is mediated by signaling pathways, in the case of ADAM17 inhibition, positive adhesiotropy might be a purely mechanical issue: Reduced ADAM17 activity enhanced DSG2 protein levels at the membrane, which might be caused by reduced ADAM17-mediated DSG2 cleavage. However, this hypothesis was not proven conclusively in the scope of this thesis. Enhanced localization of DSG2 at the membrane was paralleled by enhanced localization of DP at the membrane upon treatments with TAPI-1, erlotinib and PP2 (the effect was not analyzed upon adrenergic signaling, PKC activation or p38MAPK inhibition), which in the case of ADAM17 inhibition might be a stabilization of the desmosome as a result of reduced desmosomal disassembly, since no changes in assembly were observed. In contrast, in the case of erlotinib and PP2-mediated positive adhesiotropy enhanced desmosomal assembly was confirmed through a Ca^{2+}-switch assay in HL-1 cardiomyocytes. In this assay, depletion of Ca^{2+} leads to the disruption of desmosomal contacts since desmosomal cadherin binding is Ca^{2+}-dependent. Upon Ca^{2+}-repletion, desmosomes are reassembled and the effect of mediators on desmosomal assembly can be assessed. Inhibition of EGFR or SRC after a Ca^{2+}-switch increased cellular cohesion as compared to control-treated samples, indicating enhanced desmosomal assembly. Enhanced desmosomal assembly was also reflected in enhanced localization of DSG2 and DP at the cell membrane of HL-1 cardiomyocytes upon inhibition of EGFR or SRC after a Ca^{2+}-switch.

In HL-1 cardiomyocytes, EGFR was found in complex with DSG2 along with other desmosomal proteins, indicating that EGFR signaling might directly affect the desmosome, and *vice versa*. Interaction of EGFR and DSG2 was previously observed in intestinal epithelial cells. However, in these cells, EGFR inhibition reduced cellular cohesion (45). Furthermore, in squamous cell carcinoma or colon adenocarcinoma cells, knockdown of *DSG2* reduced EGFR protein and activation levels (44, 46). Unlike highly proliferative enterocytes or cancer cells, adult cardiomyocytes barely proliferate: The cells of the intestinal epithelium are replaced within one week, whereas only about 1% of the heart is replaced within one year (377, 378), which might explain the different effects of EGFR inhibition on cardiomyocyte and enterocyte cohesion since EGFR is classically associated with proliferative pathways.

Together, these data strengthen the idea of desmosomes serving as signaling hubs, since EGFR protein levels and p38MAPK activation were increased in *Jup*$^{-/-}$ mice and indicate that some mechanisms which strengthen cardiomyocyte cohesion in wildtype cardiomyocytes, might be suited to enhance desmosomal cohesion even when desmosomal components are mutated or their expression is decreased. Further studies are warranted to investigate the efficacy of these

mechanisms to enhance cardiomyocyte cohesion in AC models with other underlying mutations.

3.2. Adrenergic signaling in positive adhesiotropy and the role of PKA

Since the β-adrenergic receptor was shown to be localized at the ICD (10), misassembled ICDs might affect adrenergic signaling in the heart. Furthermore, decreased PKA signaling caused by hyperlipidemia was observed in diabetic cardiomyopathy (379), whereas in AC patients, decreased β-adrenergic receptor densities were observed (380), indicating that modulating adrenergic signaling might be of interest in AC.

F/R or isoprenaline treatment led to positive adhesiotropy in HL-1 cardiomyocytes paralleled by a PKA and ERK1/2-dependent enhancement of DSG2 localization at the membrane. Treatment with Apremilast, a PDE4 inhibitor, also induced positive adhesiotropy in HL-1 cardiomyocytes (unpublished data). Conversely, carbachol-induced cholinergic signaling reduced cardiomyocyte cohesion, prevented F/R-mediated positive adhesiotropy and transactivated EGFR and MAPK (259, 381).

Positive adhesiotropy induced by adrenergic signaling seems to be dependent on both, ERK1/2 activity and PKA activity since inhibition of either pathway disrupted the positive adhesiotropic effect of F/R-treatment. However, inhibition of ERK1/2 or PKA alone did not alter cardiomyocyte cohesion.

Phosphorylation of PG at S665 might be an effect exclusive to adrenergic signaling-mediated PKA activation since upon erlotinib-mediated EGFR inhibition, PKA was activated according to the PamGene Kinase assay (not validated), but no phosphorylation of PG at S665 was observed (unpublished data). Interestingly, β-catenin can be phosphorylated by PKA leading to enhanced nuclear translocation of β-catenin and to an activation of the Wnt signaling pathway. One of the phosphorylation sites of β-catenin targeted by PKA leading to β-catenin activation is S675 (382, 383) which corresponds to PG-S665. However, while PKA-mediated phosphorylation of β-catenin at S675 results in its activation and translocation from AJs to the nucleus, phosphorylation of PG at S665 enhances its localization at cell membranes (107, 384). Both, PG-S665 and β-catenin-S675 might therefore be beneficial in AC. In HEK293 cells and in fibroblast cell lines PKA phosphorylated and thereby inhibited GSK3β (385), a mechanism that has also been observed in cardiomyocytes and could be protective in AC patients (258) through activation of the Wnt pathway, which should be addressed in future studies.

Adrenergic signaling in cardiac pathologies must be viewed with care since it can have cardiotoxic effects: It is known to induce arrhythmia, and extensive exercise, in the course of which adrenergic signaling is activated, worsens AC progression (25). Apart from that,

β-adrenergic signaling can transactivate EGFR (309), which might lead to loss of cardiomyocyte cohesion. Furthermore, one of the treatment options of AC is administration of antiarrhythmic drugs, i.e., β-adrenergic receptor blockers (136, 137). The finding that adrenergic signaling enhances cellular cohesion, which is disturbed in AC, might at first glance, put into question the β-adrenergic receptor blocker treatment. However, bisoprolol, a β-adrenergic receptor blocker, did not reduce cellular cohesion in HL-1 cardiomyocytes. On the other hand, since the β-adrenergic receptor is localized at the ICD (10), disturbed ICDs in AC might affect β-adrenergic receptor localization. Indeed, reduced β-adrenergic receptor densities were found in AC patients (380), and murine *Jup*$^{-/-}$ hearts did not show increased inotropy or chronotropy in response to F/R treatment (106). Short-time treatment with PDE3 inhibitors was beneficial for cardiac function, whereas long-term treatment increased mortality of DCM patients (386). Preliminary clinical trials indicated that treatment with PDE inhibitors in combination with β-adrenergic receptor blocker therapy might reduce adverse side effects of both treatments in patients with heart failure (387-389). The question remains whether similar mechanisms are present in AC, thus, whether simultaneous adrenergic stimulation or PDE inhibition and β-receptor blocker treatment would still enhance cardiomyocyte cohesion.

In cells, cAMP signaling leading to PKA activation is highly compartmentalized in a spatio-temporal manner, meaning that different pools of cAMP can have very distinct effects. Compartmentalization can be achieved through regulation of cAMP production and degradation through different GPCRs, adenylyl cyclases and PDEs, as well as through different localization of A kinase-anchoring proteins which direct PKA localization (252, 253, 390-392). Therefore, different pools of cAMP might mediate the positive adhesiotropic effect and the hypertrophic and necrotic response upon PKA activation.

Taken together, the data indicate that though adrenergic signaling enhances cellular cohesion in wildtype mice and can affect GJs in the absence of PG and inhibit GSK3β, it might not be well-suited as a treatment option at least in AC patients with *JUP* mutations: *Jup*$^{-/-}$ mice had ruptured ICDs in response to isoprenaline treatment (106), and cardiomyocyte cohesion was not enhanced upon adrenergic signaling in *Jup*$^{-/-}$ mice. Nevertheless, understanding the underlying molecular mechanisms of adrenergic signaling-mediated positive adhesiotropy can help to find a more precise approach, by targeting downstream effectors of adrenergic signaling, which finally might be suited as a treatment option in AC without exerting cardiotoxic effects.

3.3. The role of p38MAPK and ERK1/2 in positive adhesiotropy in cardiomyocytes

p38MAPK and ERK1/2 have a plethora of downstream targets and functions, some of which could be of interest in the pathophysiology of AC. The effects of p38MAPK and ERK1/2 seem to differ between the different pathways which induce positive adhesiotropy. Adrenergic signaling or PKC activation-mediated positive adhesiotropy was paralleled by ERK1/2 activation. Moreover, positive adhesiotropy upon adrenergic signaling, PKC activation, or p38MAPK inhibition was dependent on ERK1/2 activity since ERK1/2 inhibition by U0126 abolished the positive adhesiotropic effect of F/R, isoprenaline, PMA and SB20. On the other hand, EGFR inhibition-mediated positive adhesiotropy was independent of ERK1/2 activation, as ERK1/2 phosphorylation was reduced upon EGFR inhibition.

Inhibition of ERK1/2 alone did not affect HL-1 cardiomyocyte cohesion or DSG2 localization. However, inhibition of ERK1/2 prior to PKC activation reduced membrane localization of DSG2 as compared to controls. In addition, adrenergic signaling and p38MAPK inhibition led to an ERK1/2-dependent enhanced DSG2 membrane localization. In contrast, EGFR inhibition also enhanced the localization of DSG2 and DP at the membrane and increased area composita length, though ERK1/2 activity was reduced. These data indicate that different cellular pathways involving ERK1/2 regulate desmosomal proteins and thereby cardiomyocyte cohesion.

In *ex vivo* skin treated with autoantibodies from PV patients (PV-IgGs), inhibition of ERK1/2 reduced blister formation and was therefore associated with increased cellular cohesion (393). Enhanced ERK1/2 activation was observed in *Dsp* knockdown HL-1 cardiomyocytes, in AC patients with various mutations, in DCM patients and in iPSC-derived cardiomyocytes from DCM patients with a $LMNA^{S143P}$ mutation (202, 394). Furthermore, ERK1/2 inhibition was protective in a murine model for diabetic cardiomyopathy, where it reduced hypertrophy and restored cardiac function by restoring X-box binding protein 1 nuclear localization and transcription (395). Apart from that, in $LMNA^{R225X}$ iPSC-derived cardiomyocytes, representing a DCM model, ERK1/2 inhibition reduced apoptosis (396). On the other hand, in sh*Pkp2*-treated HL-1 cardiomyocytes, ERK1/2 along with PKCα activation was decreased through activation of the hippo signaling pathway (65).

It has been suggested that the effects of ERK1/2 activation in the heart depend on the localization, duration and intensity of the signal (262), which might explain the differential regulation of ERK1/2 activity during positive adhesiotropy. Thus, similar to cAMP signaling,

ERK1/2 signaling might also be compartmentalized in cardiomyocytes. However, to test this hypothesis, future experiments, including murine AC models, are necessary.

All in all, the data obtained in this work suggest that a basal activity of ERK1/2 is needed for positive adhesiotropy since complete inhibition of ERK1/2 as seen after treatment with the MEK1/2 inhibitor U0126, disrupted positive adhesiotropic effects, whereas EGFR inhibition only decreased ERK1/2 activity but did not abolish it. However, it is possible that attempts to stabilize cardiomyocyte cohesion via pathways involving ERK1/2 activation would lead to hypertrophy and facilitate arrhythmias (143, 262, 397).

Upon activation by TNFα, p38MAPK or ERK1/2 activated NFκB signaling via nuclear mitogen and stress activated protein kinase 1/2 in HEK293 cells and thereby activated inflammatory pathways (398). In keratinocytes with a siRNA-mediated *JUP* knockdown, where p38MAPK activity was increased, its inhibition was effective to enhance cellular cohesion, but was ineffective in keratinocytes with a siRNA-mediated *DSP* knockdown (71). In contrast, in intestinal epithelial cells, both, activation and inhibition of p38MAPK weakened cellular cohesion, indicating that in the intestine, p38MAPK signaling must be tightly regulated (47).

p38MAPK inhibition induced positive adhesiotropy in HL-1 cardiomyocytes, paralleled by ERK1/2-dependent enhanced localization of DSG2 at the membrane. In $Jup^{-/-}$ mice, p38MAPK activity was upregulated. Inhibition of p38MAPK induced positive adhesiotropy in murine cardiac slice cultures obtained from $Jup^{+/+}$ and $Jup^{-/-}$ mice. Furthermore, p38MAPK was activated upon treatment with IgGs isolated from AC patients (AC-IgG), whereas inhibition of p38MAPK prevented AC-IgG-induced loss of cardiomyocyte cohesion. On the other hand, positive adhesiotropy upon adrenergic signaling or PKC activation was paralleled by p38MAPK activation. However, direct p38MAPK activation reduced cellular cohesion in HL-1 cardiomyocytes, but did not affect cardiomyocyte cohesion in $Jup^{+/+}$ or $Jup^{-/-}$ mice.

A cardioprotective role of p38MAPK activation upon adrenergic signaling through reduction of β-adrenergic receptor-induced apoptosis in cardiomyocytes has been reported (399). Thus, p38MAPK activation observed upon adrenergic signaling might indeed be a cardioprotective mechanism. Furthermore, p38MAPK activation upon adrenergic signaling or PKC activation might be outbalanced by the mechanisms stabilizing cardiomyocyte cohesion. Another possibility could be that slight p38MAPK activation does not destabilize cardiomyocyte cohesion, whereas strong p38MAPK activation leads to the weakening of HL-1 cardiomyocyte contacts. On the other hand, in murine cardiac slice cultures from $Jup^{+/+}$ as well as $Jup^{-/-}$ mice, p38MAPK activation did not affect cardiomyocyte cohesion.

Similar to p38MAPK inhibition prior to treatment of HL-1 cardiomyocytes with AC-IgGs, p38MAPK inhibition reduced PV-IgG-mediated loss of keratinocyte cohesion (400, 401).

p38MAPK-mediated cardioprotection was observed after MI, whereas p38MAPK inhibition was associated with hypertrophic cardiomyopathy (268-270). In contrast, p38MAPK inhibition has been hypothesized to promote cardiac regeneration upon cardiac injury (402). Post MI, p38MAPK activated apoptotic pathways, whereas mice with a p38MAPKα loss of function amino acid substitution were partially protected from ischemia-reperfusion injury (264-267). Based on these observations, one could argue that the differential effects of p38MAPK inhibition on cardiomyocyte cohesion might be caused by different pools or isoforms of p38MAPK (402).

Mice with diabetic cardiomyopathy had increased p38MAPK protein levels and activity along with hypertrophy, apoptosis and inflammation (272-274). Furthermore, in NRVMs representing AC models lacking DP or PKP2, increased p38MAPK activity induced by TGFβ was observed, whereas in DCM hearts, activity of p38MAPK, ERK and other kinases was increased (66, 276). Therefore, p38MAPK inhibition might be beneficial in AC, as seen in other cardiomyopathies. Indeed, until recently, a phase 3 clinical trial was performed using ARRY-371797, a p38MAPKα inhibitor, to treat DCM caused by *LMNA* mutations. However, this trial was stopped after an interim futility analysis (403, 404). Since the phase 3 clinical trial was not stopped due to safety concerns, and the phase 2 clinical trial showed promising results (405), inhibition of p38MAPK(α) might still be a treatment option for AC, even though it was not successful in *LMNA* mutation-induced DCM. In summary, p38MAPK inhibition can enhance cellular cohesion and decrease inflammation (71, 398, 400, 401). Future experiments could show whether in *Jup*$^{-/-}$ mice, where p38MAPK activation was increased, p38MAPK plays a pathogenic role in AC through destabilization of cellular contacts and induction of inflammation and whether p38MAPK inhibition is a promising approach in *in vivo* AC models.

3.4. PKC activation and cardiomyocyte cohesion

The role of PKC in the heart is even more complex than p38MAPK or ERK1/2 due to the different PKC isozymes which have opposing effects. Activation or overexpression of PKC can be cardiotoxic by increasing ADAM and MMP activity or by enhancing the inflammatory response in the heart (277, 279-283). PKC inhibition reduced fibrosis and cardiac dysfunction after MI and improved Ca^{2+}-homeostasis, reduced fibrosis, inflammation and hypertrophy in heart failure (284-288). Furthermore, PKC activation increased protein synthesis in cardiac fibroblasts after atrial fibrillation but not in fibroblasts isolated from healthy hearts (406). Apart from that, PKC can activate p38MAPK (280). Overexpression of PKCβ in the myocardium led

to cardiomyopathy (292). On the other hand, PKCε inhibition prevented cardiac protection of ischemic preconditioning and led to a cardiomyopathy resembling DCM, whereas PKC activation mimicked ischemic preconditioning and decreased infarct size upon MI (295, 297-299).

PKC activation enhanced HL-1 cardiomyocyte cohesion, as previously described (25), by increasing DSG2 localization at the membrane in an ERK1/2-dependent manner. PKC-mediated phosphorylation of DP is needed for incorporation of DP into the desmosome, a process in which PKP2 is involved as a scaffold, whereas upon loss of PKP2, PKC phosphorylated other targets (62). Enhanced incorporation of DP into desmosomes might be one of the mechanisms behind PKC activation-mediated positive adhesiotropy and should be investigated further. Given that the majority of AC patients have a mutation in the *PKP2* gene, and PKC activation restored DP localization at the membrane in a si*PKP2*-treated squamous cell carcinoma cell line, it is possible that a similar mechanism is beneficial for some AC patients (62, 116, 407). On the other hand, PKC inhibition reduced spontaneous Ca^{2+}-sparks in *Pkp2*^{cKO} right ventricular myocytes, supporting the arrhythmogenic role of PKC activation (200).

The major limitation of the here presented data on PKC in the regulation of cardiomyocyte cohesion is that PMA activates classic and novel PKC isozymes, which might have differential effects on cardiomyocyte cohesion and fate. Though stabilizing cardiomyocyte cohesion is desirable in AC, PKC activation needs to be tightly controlled since it is known to induce arrhythmia (408). In murine cardiac slice cultures obtained from *Jup*$^{-/-}$ mice, PKC activation increased cardiomyocyte cohesion, and in sh*Pkp2*-treated HL-1 cardiomyocytes, as well as in human and murine AC hearts, reduced PKC protein levels were observed (65). Therefore, the above data argue that PKC activation might be a treatment option in AC. In that context, activation of PKC might restore physiological PKC signaling without exerting cardiotoxic effects. Taking the data on cardiac PKC signaling into account, a balance between the arrhythmogenic and the adhesion-stabilizing role of PKC activation needs to be maintained when modulating PKC activity in AC hearts. Understanding the exact molecular mechanism behind PKC activation-induced positive adhesiotropy might provide treatment options by targeting downstream targets of PKC, thereby overcoming the arrhythmogenic side effects of PKC activation.

3.5. Hyperadhesion in the heart

Desmosomal cadherin binding is strictly dependent on Ca^{2+}. However, it has been suggested that the physiological state of intact epidermal keratinocytes is hyperadhesive, which is a strong

adhesion state where desmosomes are independent of Ca^{2+}. During hyperadhesion, desmosomal cadherins are highly organized, which is visible by a dense midline in electron microscopy images of hyperadhesive desmosomes (409). Since Ca^{2+} is tightly regulated in the heart, it is possible that under physiologic conditions cardiomyocytes are also hyperadhesive. In HL-1 cardiomyocytes, hyperadhesion was only observed after modulation of signaling pathways: adrenergic signaling, PKC activation and p38MAPK inhibition increased and even restored cardiomyocyte cohesion in a Ca^{2+}-free environment. The ability to lead to enhanced cardiomyocyte cohesion upon Ca^{2+}-depletion was termed hyperadhesion. In contrast to basal conditions, hyperadhesion induced by adrenergic signaling, PKC activation or p38MAPK inhibition was independent of ERK1/2 activity. The ability of EGFR, SRC or ADAM17 inhibition to induce hyperadhesion in cardiomyocytes was not assessed.

In contrast to cardiomyocytes, in keratinocytes, PKC activation prevented a hyperadhesive state, whereas PKC inhibition drove subconfluent cells into hyperadhesion (88, 410). The different effects of PKC activation on hyperadhesion observed between cardiomyocytes and keratinocytes might be caused by tissue-specific Ca^{2+}-homeostasis.

However, even though or especially because Ca^{2+}-signaling in the heart is tightly controlled, Ca^{2+}-chelation by EGTA, as performed in these experiments, is unphysiological. Nevertheless, understanding the mechanisms behind Ca^{2+}-independent cardiomyocyte cohesion in the heart is important to find new treatment options for AC or other cardiac disorders, where Ca^{2+}-signaling is impaired (67, 200, 411, 412).

3.6. ADAM17 inhibition in cardiomyocytes

The role of ADAM17 is controversial in the heart: Reduced ADAM17 levels or ADAM17 loss of function were associated with cardiomyopathy, ventricular dilation and decreased survival after MI (358-360). On the other hand, elevated ADAM17 was correlated to elevated TNFα levels and to the severity of several cardiovascular diseases, such as MI or myocarditis, whereas ADAM17 inhibition was beneficial and reduced fibrosis, hypertrophy and apoptosis (361-365, 367). Decreased TIMP3 levels, TIMP3 being a physiological ADAM17 inhibitor, were found in patients with DCM or hypertrophic obstructive cardiomyopathy and were associated with heart failure or interstitial renal fibrosis (368-372). Apart from that, TIMP3 overexpression was cardioprotective upon ischemia-reperfusion injury (373).

In mice, ADAM17 deficiency was protective against acute pancreatitis and pancreatic fibrosis, whereas ADAM17 activity was a driver of pancreatic inflammation (350). Furthermore, increased ADAM17 mRNA and protein levels were observed in two week-old *Dsg2*$^{E4\text{-}5}$ mice, whereas in adult mice ADAM17 levels were comparable to wildtype littermates (218). Thus,

ADAM17 might be involved in early AC pathogenesis and be a driver of fibrosis and inflammation.

Inhibition of ADAM17 induced positive adhesiotropy in HL-1 cardiomyocytes and in murine cardiac slice cultures from *Jup*$^{+/+}$ and *Jup*$^{-/-}$ mice. In contrast to EGFR inhibition-mediated positive adhesiotropy, positive adhesiotropy upon ADAM17 inhibition was not mediated by enhanced desmosomal assembly, as shown by means of a Ca^{2+}-switch assay. ADAM17 inhibition-mediated positive adhesiotropy was dependent on DSG2 expression and was paralleled by increased localization of DSG2 and DP at the membrane. Several experiments were performed addressing the hypothesis that ADAM17 inhibition-mediated positive adhesiotropy was mediated by reduced DSG2 cleavage. Attempts to detect changes in DSG2 cleavage upon ADAM17 inhibition by detecting intracellular or extracellular DSG2 cleavage products did not yield conclusive results even after overexpression of a DSG2-GFP construct. Indirect attempts to show reduced cleavage revealed that upon EGS-crosslinking, a trend towards increased DSG2 oligomerization was found at the membrane after ADAM17 inhibition; however, this trend was not statistically significant. Since ADAM17 inhibition does not lead to enhanced desmosomal assembly, increased DSG2 localization at the membrane might be caused by decreased desmosomal disassembly, which would also explain enhanced DP localization at the membrane upon ADAM17 inhibition. However, no definite proof for decreased DSG2 cleavage upon ADAM17 inhibition was found, thus reduced DSG2 cleavage upon ADAM17 inhibition remains a hypothesis.

Phosphorylation of p38MAPK, which can activate ADAM17, was increased in *Jup*$^{-/-}$ mice. However, no change in pro-ADAM17 or ADAM17 protein levels nor in ADAM17 phosphorylation were observed between *Jup*$^{+/+}$ and *Jup*$^{-/-}$ mice. However, ADAM17 can also be activated by p38MAPK independent of the phosphorylation at T735 (413). Further studies are warranted investigating the ADAM17 protease activity in *Jup*$^{+/+}$ and *Jup*$^{-/-}$ hearts, which should include SB20 as p38MAPK inhibitor and TAPI-1 as ADAM17 inhibitor. Interestingly, *TIMP3* mRNA levels were increased in the left ventricle of AC patients (414), which might be a compensatory mechanism in case of increased ADAM17 activity. However, since protein levels were not assessed it is not known whether the upregulation of the mRNA had a functional effect.

Since ADAM17 can transactivate EGFR, the increase in cardiomyocyte cohesion upon ADAM17 inhibition by TAPI-1 might have been caused through inhibition of EGFR signaling. However, upon *Egfr* knockdown in HL-1 cardiomyocytes using siRNA, ADAM17 inhibition still induced positive adhesiotropy, indicating that enhanced cardiomyocyte cohesion upon

ADAM17 inhibition might be independent of EGFR protein expression. However, the knockdown, though statistically significant, was not very efficient, so that the possibility of EGFR involvement in ADAM17 inhibition-mediated positive adhesiotropy remains.

On the other hand, EGF increased the half-life of ADAM17 and ADAM17-mediated DSG2 shedding in A431 cells, indicating that EGF-induced signaling can be upstream of ADAM17 (347). In line with this, SRC-mediated phosphorylation of ADAM17 at Y702 activated protease activity of ADAM17 (415), which might be another aspect of how SRC inhibition contributes to positive adhesiotropy, and which could be assessed by knocking down *Adam17* and inhibiting SRC or EGFR. Apart from the SRC-mediated Y702 phosphorylation, phosphorylation of T735 by PI3K, a kinase involved in carbachol-induced loss of cardiomyocyte cohesion, was needed to activate ADAM17 in mesangial cells (259, 415). When activated through phosphorylation at Y702 and T735, ADAM17 led to a pro-fibrotic upregulation of TGFβ in murine embryonic fibroblasts (415). In autoimmune diseases, PI3K interacting protein 1 (PIK3IP1) was downregulated via IL21 signaling and associated with disease progression, whereas ADAM17 levels were elevated. Inhibition of p38MAPK prevented the IL21-induced ADAM17-mediated reduction of PIK3IP1 levels (416). Since PIK3IP1 is a physiological inhibitor of PI3K, similar mechanisms might be involved in ADAM17 and carbachol-induced loss of cardiomyocyte cohesion. It remains to be elucidated whether similar processes are found in AC.

Subjecting colorectal adenocarcinoma cells for 24 h to TNFα activated p38MAPK, whereas p38MAPK inhibition prevented the TNFα-induced reduction of DSG2 localization at cellular junctions (417). Since enhanced ADAM17 activity is associated with increased TNFα levels, it is possible that a similar mechanism exists in the *Jup*$^{-/-}$ AC model. In that case, positive adhesiotropy mediated by p38MAPK inhibition might at least in part be mediated by ADAM17. This question could be answered by knocking down *Adam17* in HL-1 cardiomyocytes and treating with SB20.

Diabetic mice with a cardiomyocyte-specific *Adam17* knockout had less diabetes-induced cardiac damage than wildtype mice along with significantly increased protein levels but decreased activity of the AMP-activated protein kinase (AMPK) (367). Upon EGFR inhibition by erlotinib in HL-1 cardiomyocytes, increased AMPK activity was indicated by the PamGene Kinase Assay and confirmed by Western blot (unpublished data). Furthermore, AMPK activation enhanced HL-1 cardiomyocyte cohesion (unpublished data). AMPK was shown to be located at the ICD (418), thus defective ICDs might affect AMPK signaling. Decreased AMPK signaling due to a mutation in the gene encoding AMPK was found in a patient with

cardiomyopathy (419). In contrast, activation of AMPK prevented cardiac fibrosis upon MI and was cardioprotective in ischemic cardiomyopathy (420-422). Together, these data indicate that AMPK might be an interesting target for future AC research.

It has been suggested that ADAM17 inhibition is only beneficial in pathologic conditions where the renin-angiotensin system is activated, since ADAM17 can also be transactivated by Ang II (367). Treatment options for AC include angiotensin-converting enzyme inhibitors as well as Ang II receptor blockers, especially in the later stages of the disease. However, no study addressed the activity of the renin-angiotensin system in AC (146, 147, 423).

Due to the multitude of ADAM17 substrates, ADAM17 inhibition can have severe side effects. Therefore, instead of inhibiting ADAM17 function, the pharmacological approach might be to modulate specific activation pathways of ADAM17, which could prevent ADAM17 activation under specific circumstances, whereas other ADAM17 activities would be undisturbed (415). ADAM17 inhibition by small molecules has been the subject of clinical trials to treat rheumatoid arthritis. However, these trials were discontinued due to liver toxicity or lack of efficacy. New and more specific strategies to target ADAM17 include inhibitory prodomains or inhibition of substrate recognition (424). Though these approaches have not been tested in clinical trials, they show that ADAM17 inhibition is of interest in several pathologies. Due to the detrimental effects of an ADAM17 loss of function or strong downregulation (357-359) and the fact that long term ADAM17 inhibition did not lead to positive adhesiotropy in HL-1 cardiomyocytes, it is questionable, whether ADAM17 inhibition is a suitable approach to treat AC. Nevertheless, further research *in vivo* and *ex vivo* murine AC models might lead to a better understanding of AC pathogenesis or help to find a more targeted approach to stabilize cardiomyocyte cohesion and reduce inflammation.

3.7. EGFR inhibition in cardiomyocytes and the role of ROCK

Given that increased EGFR or SRC activity is associated with carcinogenesis, it seems only logical that inhibition of these kinases would stabilize cellular cohesion. However, the biological processes are not that simple. In the heart, both protective and cardiotoxic effects of EGFR signaling have been described.

In $Jup^{-/-}$ mice, EGFR protein levels were increased as compared to wildtype littermates, indicating a potential role of EGFR in the pathogenesis of AC. Indeed, EGFR inhibition by erlotinib or inhibition of the EGFR effector molecule SRC by PP2 led to positive adhesiotropy in HL-1 cardiomyocytes, as well as in murine cardiac slice cultures from $Jup^{+/+}$ and $Jup^{-/-}$ mice. Positive adhesiotropy was paralleled by enhanced localization of DSG2 and DP at the membrane and longer areae compositae in HL-1 cardiomyocytes, and increased localization of

both proteins at the ICD in wildtype mice. In contrast, in *Jup*$^{-/-}$ mice, only DSG2 localization at the ICD was increased.

In mice chronically treated with isoprenaline, gefitinib-mediated EGFR inhibition decreased apoptosis, hypertrophy and inflammation. However, gefitinib induced fibrosis in mice that were not subjected to chronic isoprenaline treatment (317). In line with these findings, it has been suggested that EGFR signaling is essential for heart development and heart function under physiological conditions and that acute EGFR signaling can be cardioprotective, whereas chronic EGFR up- or downregulation can be detrimental especially in cardiovascular pathologies (345, 425). Therefore, EGFR inhibition in AC, where EGFR protein levels are increased, might indeed be cardioprotective and beneficial.

Increased EGFR protein levels observed after MI were mainly found in fibroblasts (318). Since in AC fibrosis is increased, EGFR inhibition in fibroblasts could be beneficial by preventing fibroblast proliferation and attenuating fibrosis (328, 426). However, EGFR inhibition enhanced cardiomyocyte cohesion also in murine cardiac slice cultures from wildtype mice, indicating that inhibition of EGFR directly affects cardiomyocytes. Furthermore, in immunostainings performed in human heart tissue from two AC patients, a slight EGFR signal was observed at the ICD, which was absent in control tissue (unpublished data), indicating that enhanced EGFR levels in AC might also be found in cardiomyocytes

Erlotinib ameliorated renal inflammation in mice upon treatment with TNF-like weak inducer of apoptosis (TWEAK), a member of the TNF family. Furthermore, TWEAK-induced renal inflammation involved an ADAM17-EGFR axis and direct activation of NFκB (335); processes that might also be of interest in AC since inhibition of NFκB was beneficial in murine AC models (228). In diabetic mice, increased NFκB activation through enhanced EGFR levels promoted inflammation, which was prevented upon EGFR inhibition (321, 427). The question remains whether EGFR inhibition could also decrease inflammation in the case of AC. Thus, for a better understanding of the role of EGFR in AC pathogenesis, long term *in vivo* experiments using an EGFR inhibitor are warranted, where the effect on cardiac inflammation should be assessed.

Similar to what was observed in HL-1 cardiomyocytes in the work presented here, in enterocytes, DSG2 was also found in complex with EGFR. However, converse to cardiomyocytes, EGFR inhibition decreased cellular cohesion in enterocytes (45). Furthermore, in enterocytes, loss of DSG2 decreased EGFR signaling (44). In contrast, in hearts of *Jup*$^{-/-}$ mice with decreased protein levels of DSG2, EGFR protein levels were increased as compared to wildtype littermates.

Though acute EGFR inhibition by erlotinib induced positive adhesiotropy in HL-1 cardiomyocytes, knockdown of *Egfr* by siRNA did not affect cardiomyocyte cohesion. This might be due to the relatively weak knockdown efficiency of si*Egfr* (40%), whereas erlotinib was used at IC_{50} concentration. Furthermore, since EGFR was found in complex with desmosomal proteins, a knockdown of *Egfr* might lead to changes at the desmosome, which do not occur upon EGFR inhibition.

Similarly to EGFR inhibition-mediated positive adhesiotropy in cardiomyocytes, EGFR inhibition by AG1478 enhanced cellular cohesion of keratinocytes (428). Furthermore, in response to EGF treatment, DSG2 was internalized via a recycling pathway in a squamous cell carcinoma cell line, whereas E-CAD was proteolytically cleaved (429). However, in cardiomyocytes, EGF treatment did not affect cellular cohesion (unpublished data).

In keratinocytes and enterocytes, knockdown or knockout of *DSG2* reduced SRC phosphorylation and EGFR protein levels (45, 46), which is in contrast to hearts of $Jup^{-/-}$ mice, where low DSG2 levels and high EGFR protein levels were observed. Furthermore, in contrast to positive adhesiotropy upon SRC inhibition in cardiomyocytes, low SRC levels decreased cellular cohesion and increased intercellular space in MDCK cells with disruption of AJs but no changes at the desmosome (430). EGFR and SRC are known to increase β-catenin phosphorylation, leading to its translocation from AJs to the nucleus, thereby weakening keratinocyte cohesion (431-433) and promoting tumor cell invasiveness via AKT (434). The effect of SRC inhibition on cellular cohesion seems to be depending on the context since both SRC inhibition and expression of active SRC destabilized keratinocyte cohesion (105, 435). SRC inhibition prevented loss of keratinocyte cohesion upon treatment with m-PV-IgG, mc-PV-IgG or PF-IgG, as did EGFR inhibition (82, 105, 260, 436). In addition, EGFR inhibition prevented PV-IgG-induced apoptosis (437, 438). However, not all EGFR inhibitors prevented PV-IgG or PF-IgG-induced blistering of the skin (439). Therefore, the efficacy of other EGFR inhibitors other than erlotinib to induce positive adhesiotropy should be assessed in HL-1 cardiomyocytes or in *in vivo* or *ex vivo* animal models.

Positive adhesiotropy induced by EGFR inhibition was not the result of altered DSG2 mobility but rather enhanced desmosomal assembly leading to an increase in DES insertions into desmosomes and enhanced protein levels of DSG2 and DP at the membranes along with enhanced area composita length. Enhanced desmosomal assembly upon EGFR inhibition was already observed in an oral squamous carcinoma cell line as well as in an epidermoid squamous cell carcinoma cell line (101, 102). The increase in DSG2 and DP localization at the membrane of HL-1 cardiomyocytes upon EGFR or SRC inhibition was reflected in immunostainings

performed in murine cardiac slices obtained from *Jup*$^{+/+}$ as well as *Jup*$^{-/-}$ mice by increased DSG2 and DP staining width in wildtype cardiac slice cultures and increased DSG2 staining width in *Jup*$^{-/-}$ cardiac slices after inhibition of EGFR or SRC. Indeed, using electron microscopy, a similar reorganization of ICDs upon positive adhesiotropy after adrenergic signaling was observed before (374).

To further understand the molecular mechanisms behind positive adhesiotropy upon EGFR inhibition, a PamGene Kinase assay was performed. Several kinases were differentially regulated after 30 or 60 minutes of EGFR inhibition, out of which ROCK seemed to be the most promising target. The effect of RhoA inhibition, which is upstream of ROCK, on cellular cohesion varies between cell types and the physiological context (440, 441). Enhanced keratinocyte cohesion was observed in primary keratinocytes cultured in high Ca^{2+}-concentration containing medium and was paralleled by increased RhoA activity (428). Expression of a dominant negative Rho or Rac or inhibition of Rho led to a removal of E-CAD from the cellular junctions in keratinocytes, but not in fibroblasts (442). Furthermore, it was shown that the activity of both, RhoA, as well as Rac was needed to form cadherin-cadherin cellular junctions (443). In keratinocytes, AJ assembly was paralleled by EGFR-mediated Rac activation (444). Apart from that, ROCK inhibition reduced cellular cohesion and increased migration in keratinocytes (440, 445). Furthermore, ROCK inhibition facilitated PV-IgG-mediated skin blistering and loss of cellular cohesion (446, 447). In contrast, RhoA activation strengthened keratinocyte cohesion and prevented PV-IgG-mediated loss of cell cohesion (445). Moreover, p38MAPK inhibition prevented the PV-IgG-induced inactivation of RhoA in keratinocytes (446). Similar mechanisms might be involved in inducing positive adhesiotropy in cardiomyocytes upon p38MAPK or EGFR inhibition in AC since both are increased in AC and can modulate ROCK activity.

However, most studies on ROCK in the cardiovascular system report cardiotoxic effects of ROCK activity. In diabetic cardiomyopathy, increased EGFR levels along with increased ROCK levels were observed (321, 448), and ROCK was increased upon pressure overload in rat cardiomyocytes (449). Activated ROCK signaling led to hypertrophy and fibrotic cardiomyopathy, whereas ROCK inhibition restored Ca^{2+}-homeostasis upon pressure overload-induced cardiac hypertrophy (450-453). Nevertheless, an *in silico* model suggested an involvement of ROCK in the pathogenesis of AC (454). Furthermore, ROCK activated Wnt signaling and thereby negatively regulated adipogenesis (455). Indeed, expression of a dominant-negative ROCK led to AC in mice through translocation of PG to the nucleus. This way, ROCK inhibited Wnt signaling and altered gene expression resulting in an adipogenic

switch (456). Interestingly, in an AC patient with a *PKP2* mutation, RhoA activity and Cx43 protein expression were decreased, whereas in patients that had no *PKP2*-mutation, RhoA activity was enhanced, paralleled by increased Cx43 protein expression (457), indicating that modulation of the RhoA-ROCK axis might not be suited for all AC patients. Furthermore, in *PKP2*$^{c.1760delT}$ iPSC-derived cardiomyocytes, decreased RhoA levels and activity reduced ROCK signaling and thereby enhanced lipogenesis. In line with that, in wildtype iPSC-derived cardiomyocytes, ROCK inhibition induced lipogenesis and reduced cellular cohesion by reducing PKP2 localization at the membrane (458). Upon loss of PKP2, RhoA activity was increased in a squamous cell carcinoma cell line and in HL-1 cardiomyocytes. However, RhoA failed to localize at cell-cell contact sites upon knockdown of *PKP2*, resulting in defective desmosomal assembly (61).

Inhibition of ROCK2, the primary cardiac ROCK isoform, enhanced adipogenesis in 3T3-L1 cells, which was prevented upon inhibition of PI3K (459). On the other hand, cholinergic stimulation led to phosphorylation of myosin light chain 2 (MLC2) via ROCK resulting in a positive inotropic response (460-462), showing that ROCK signaling might not only be beneficial in AC, since positive inotropy is not necessarily desirable in AC patients.

In HL-1 cardiomyocytes, EGFR inhibition enhanced RhoA activation and thereby ROCK activity. Indeed, erlotinib-induced enhanced desmosomal assembly and positive adhesiotropy were dependent on ROCK activity in HL-1 cardiomyocytes. ROCK-dependency of desmosomal assembly was previously observed in a squamous cell carcinoma cell line (61). In myoblasts, cell-cell contact formation led to RhoA activation, whereas disruption of cadherin-cadherin contacts through addition of EGTA inhibited RhoA, which was reversible upon addition of Ca^{2+}. Presence of anti-N-CAD antibodies inhibited RhoA and prevented its activation after a Ca^{2+}-switch (463). Since in HL-1 cardiomyocytes erlotinib-induced positive adhesiotropy was dependent on ROCK, it seems unlikely that increased ROCK activity is just a consequence of increased cell-cell contact formation during desmosomal assembly. Furthermore, RhoA activation by CN04 enhanced cardiomyocyte cohesion (unpublished data), indicating that ROCK may indeed be involved in regulating cardiomyocyte cohesion. In line with that, ROCK inhibition decreased HL-1 cardiomyocyte cohesion and decreased DSG2 localization at the membrane of HL-1 cardiomyocytes.

Given the differential regulation of RhoA activity in AC patients with and without *PKP2* mutations, the effect of an EGFR inhibition in the absence of fully functional PKP2 should be evaluated (457). In *Pkp2*$^{-/-}$ mice, increased EGFR protein levels were observed. Whether

inhibiting EGFR in these mice would also lead to positive adhesiotropy, remains to be elucidated.

Taken together, EGFR inhibition might be beneficial in AC by enhancing cardiomyocyte cohesion through enhanced desmosomal assembly and reduced NFκB-mediated inflammation. If EGFR inhibition was to be considered as a treatment option for AC, one should bear in mind that the dose-response curve of EGFR inhibitors which prevented PV-IgG-mediated loss of keratinocyte cohesion was not linear, but V-shaped, meaning that low and high concentrations of the inhibitor were not effective (439). Compared to all other mediators presented in this work that induced positive adhesiotropy, erlotinib has the advantage that it is already being used in clinics to treat some kinds of cancer. Among the clinically approved EGFR inhibitors, erlotinib seems to have fewer cardiac side-effects (464). Taken together, long term *in vivo* experiments using different EGFR inhibitors and AC models are warranted to assess, whether EGFR inhibition is suited as a treatment option in AC.

3.8. Links to other cardiac or desmosomal diseases

The findings presented in this work are important not only for finding new therapeutic approaches for AC and other cardiovascular diseases, but also for pemphigus or IBD since AC shares common pathological aspects with these diseases.

Cardiomyopathies are a heterogeneous group of diseases with abnormalities in heart muscle function and structure that are not caused by coronary artery disease, hypertension or valve defects (465, 466). Therefore, similar consequences of heart muscle damage can be observed in cardiomyopathies even though the underlying causes differ. Some cardiomyopathies share similar causative mechanisms or mutations (467). For example, mutations of genes coding for proteins of the area composita or *SCN5A* are not only causing AC, but can also be found in DCM, HCM, the Brugada syndrome or lead to heart failure (184, 185, 468). Apart from that, changes in localization of Cx43 can also observed after MI, ischemia-reperfusion injury or other cardiomyopathies, such as HCM, DCM or ischemic cardiomyopathy (152). Similar to what was shown in this work for AC, in DCM, p38MAPK is increased. Apart from that, in DCM, ERK1/2 and SRC phosphorylation are increased, as is ADAM17 activity (276, 368, 369). Furthermore, in DCM increased TNFα levels were found, whereas TIMP3 was decreased (368, 370-372). Thus, inhibition of SRC, p38MAPK or ADAM17 might be of interest in the context of DCM. Furthermore, PKCε inhibition led to a cardiomyopathy resembling DCM, thus also the regulation of PKC levels might be of interest in DCM (295).

Decreased localization of desmosomal proteins at the ICD was observed in patients with sarcoidosis or giant cell myocarditis (225), indicating that stabilization of desmosomal

cardiomyocyte cohesion might be beneficial in these conditions. Furthermore, reduced PG localization at the cardiomyocyte membrane was observed after exposure to TNFα, IL6 or IL17 (225), indicating that stabilization of desmosomal junctions might be beneficial in a wide range of cardiac inflammatory diseases with increased cytokine levels.

Recently it was suggested that AC, similar to IBD and PV has so-called 'hot phases', where disease symptoms are more severe than during 'latent' phases (469). Like AC, pemphigus is a disease of the desmosome. In pemphigus, autoantibodies against DSG1 and/or DSG3 lead to blistering of skin and mucosa. In some pemphigus patients, cardiac involvement, as well as anti-ICD autoantibodies were observed (470-473). Autoantibodies directed against the ICD or against DSG2 were recently found in the blood of AC patients (232, 233). Autoantibodies against cardiomyocyte components were also observed in DCM patients (474, 475). In keratinocytes, inhibition of p38MAPK, ERK1/2 or SRC, as well as PKA activation was protective against PV-IgG-mediated loss of keratinocyte cohesion (393, 400, 436, 476). Indeed, p38MAPK inhibition prevented AC-IgG-mediated loss of cardiomyocyte cohesion, indicating that similar mechanisms might be mediating the effect of PV-IgG and AC-IgG on cellular cohesion. Another aspect linking AC and PV are the cardio-cutaneous diseases, such as Carvajal syndrome and Naxos disease, where mutations of DP or PG lead to AC with woolly hair and palmoplantar keratoderma, respectively (129, 130).

Modulation of PKA activity or its downstream signaling events might be of interest in some, but not all cardiomyopathies. Increased PKA activity was associated with Takutsobo cardiomyopathy and anthracycline-induced cardiomyopathy (477, 478). On the other hand, decreased PKA signaling was observed in DCM (479), which was at least in part caused by mutated PKA substrates (480). Furthermore, hyperlipidemia reduced PKA signaling in diabetic cardiomyopathy (379). Similar to what was shown in heart failure (387-389), a combination of PDE inhibitors and β-receptor blockers might be of interest in AC. In ulcerative colitis patients, treatment with Apremilast, a PDE4 inhibitor, reduced inflammation and fibrosis, and thereby attenuated colon damage (481, 482). Whether Apremilast, which induced positive adhesiotropy in HL-1 cardiomyocytes (unpublished data), would also be effective to reduce inflammation in AC, remains to be elucidated.

Increased p38MAPK activity was found in mice with diabetic cardiomyopathy. In these mice, inhibition or loss of function of p38MAPK reduced hypertrophy, apoptosis and inflammation (272-275), all of which might also be beneficial in AC. Furthermore, a phase 3 clinical trial with DCM patients was performed using ARRY-371797, a p38MAPKα inhibitor, however, it was stopped after an interim futility analysis (403, 404). In contrast, p38MAPK inhibition was

associated with hypertrophic cardiomyopathy (270), indicating that p38MAPK inhibition might not be suited for all cardiomyopathies.

Enhanced p38MAPK activation was not only observed upon treatment of HL-1 cardiomyocytes with AC-IgG and in hearts of *Jup*$^{-/-}$ mice, but also in the intestine of patients with IBD, as well as in keratinocytes following PV-IgG binding, in pemphigus patient skin biopsies, and was correlated to the severity of the inflammation in the case of IBD (400, 483-485). p38MAPK inhibition was protective in mice with IBD, even though TNFα production was increased (486, 487) and prevented loss of keratinocyte cohesion induced by PV-IgG or PF-IgG (400, 488). Furthermore, p38MAPK inhibition was protective in patients with Crohn's disease (CD), one specific IBD (483). However, safety concerns upon p38MAPK inhibition were raised, therefore, it has been suggested to target downstream effectors of p38MAPK (489). CD has been associated with decreased DSG2 protein levels in the intestine (417). Treatment with TNFα, a cytokine that is often increased in CD, led to p38MAPK activation and reduced DSG2 staining at cell junctions in a colorectal adenocarcinoma cell line. SB20 prevented the TNFα-induced p38MAPK activation and restored DSG2 localization (417). Thus, regulation of p38MAPK might be of interest also in other cardiac diseases, in pemphigus and in CD.

Inhibition or knockout of PKCδ, PKCε or PKCθ in mice caused dilated, diabetic or myofibrillar cardiomyopathy (293-296). However, overexpression of PKCβ was also associated with cardiomyopathy in mice (292), indicating that modulation of PKC activity might be of interest in several cardiomyopathies. However, when targeting PKC, attention should be paid to the specific isozymes and their downstream target proteins.

Similar to pemphigus, Darier disease (DD) causes acantholysis of the skin. DD can be caused by *ATP2A2* mutations leading to SERCA2 loss of function, resulting in defective desmosomal and AJ assembly (490). It has been suggested that the loss of functional SERCA2 results in decreased activation and localization of PKCα at the membrane, which causes the defects in AJ and desmosomal assembly. Indeed, PKC activation enhanced keratinocyte cohesion upon loss of SERCA2 function (491). Thus, PKC activation might be of interest in DD. In contrast, upon binding of PV-IgG, activation of PKC was observed (492), whereas inhibition of PKC or PKC downstream effectors prevented PV-IgG-induced loss of keratinocyte cohesion (84, 493-495). Furthermore, PKCα activation was paralleled by increased keratinocyte apoptosis and inflammation in murine skin (496).

Apart from that, several PKC isozymes were protective against IBD by regulating intestinal epithelial barrier integrity and reducing inflammation (497-499). Furthermore, upon CD

progression, reduced expression of protective PKC isozymes in human intestinal biopsies was observed (500).

Taken together, these data indicate that activation of specific PKC isozymes or their downstream targets might be of therapeutical use in DD or IBD patients, but not in PV patients.

The PamGene Kinase assay suggested that PKA might be activated upon EGFR inhibition in cardiomyocytes. Thus, EGFR inhibition might be beneficial during hyperlipidemia, where PKA signaling is decreased (379). Indeed, EGFR inhibition prevented cardiac damage in hyperlipidemia-induced cardiomyopathy (328). Inhibition of ERK1/2, which is downstream of EGFR, was protective in a murine model for diabetic cardiomyopathy and in several iPSC-derived cardiomyocytes with *LMNA* mutations, which served as a model for DCM (395, 396). Furthermore, diabetic cardiomyopathy or diabetes-induced cardiac damage were ameliorated upon EGFR inhibition (321-327). Positive adhesiotropy induced by EGFR inhibition was mediated by ROCK; therefore, ROCK might be of interest in diabetic cardiomyopathy. However, increased ROCK2 activity was observed in mice with diabetic cardiomyopathy and was associated with increased cardiomyocyte apoptosis (448).

Binding of PF-IgGs or PV-IgGs activated EGFR (501), which in the case of PV-IgG was prevented by p38MAPK inhibition (83, 502). EGFR inhibition normalized DSG3 localization upon treatment with PV-IgG and prevented the PV-IgG-mediated loss of keratinocyte cohesion, apoptosis and skin blistering (82, 83, 437, 438). However, these effects were not seen with all EGFR inhibitors (439). Similarly, binding of PV-IgGs activated SRC, whereas SRC inhibition prevented blistering of the skin induced by m-PV-IgGs, mc-PV-IgGs and PF-IgGs (105, 260, 436). Thus, targeting p38MAPK, EGFR or SRC might be of interest in patients with PV or PF.

In macrophages from patients with ulcerative colitis, EGFR activation was observed, and a myeloid cell-specific knockout of *Egfr* ameliorated colitis in mice by reducing inflammatory cytokine levels and increasing anti-inflammatory IL10 cytokine levels (503). In contrast, mice with defective EGFR were more susceptible to colitis (504). Furthermore, upon disease remission, EGFR protein levels were increased in CD patients as compared to active disease state levels (505). Thus, EGFR inhibition might be beneficial in diabetic cardiomyopathy, AC and PV, but not in intestinal diseases.

Increased ADAM17 protein levels were observed upon diabetic cardiomyopathy, DCM and hypertrophic obstructive cardiomyopathy (367-369). Furthermore, ADAM17 and TNFα levels were negatively correlated with left ventricular function and were correlated with disease severity and in-hospital complications upon acute MI and myocarditis (361-363, 369). Knockout of *Timp3* in mice led to DCM and heart failure, which could be ameliorated upon

inhibition of ADAM17 and TNFα (372). Conversely, a cardiomyocyte-specific *Adam17* knockout was protective against diabetic cardiomyopathy in mice (367).

Recently, increased ADAM17 activity was associated with autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (416). Increased ADAM17 protein levels were observed in skin of patients with bullous pemphigoid, an autoimmune disease different from PV with autoantibodies targeting hemidesmosomal components rather than desmosomal proteins (506, 507). However, ADAM17 inhibition did not enhance keratinocyte cohesion and did not reduce PV-IgG-induced loss of keratinocyte cohesion (508). Furthermore, activation of ADAM17 was not observed upon PV-IgG binding, indicating that targeting ADAM17 might be beneficial in bullous pemphigoid but not in PV patients (509).

Systemic *Adam17* knockout mice were more susceptible to colitis by reducing intestinal barrier integrity and regeneration (510). In line with that, decreased ADAM17 protein levels led to IBD with cardiac and cutaneous involvement (357-359). On the other hand, increased ADAM17 protein levels and activity elevated TNFα-shedding and were associated with IBD (511-513). However, increased ADAM17 activity was hypothesized to be a protective mechanism in IBD patients through EGFR activation (510). Specific *ADAM17* single nucleotide polymorphism haplotypes were associated with an increased efficiency of TNFα-blocker treatment, which is a common CD medication (514).

Taken together, regulation of p38MAPK, SRC, EGFR, ADAM17, PKC or PKA activity might be of interest in several cardiovascular pathologies besides AC, including HCM, DCM, ischemic cardiomyopathy or diabetic cardiomyopathy, but also in skin diseases or IBD. However, opposite effects on the heart, skin and intestine were observed upon modulation of some of the here presented signaling pathways. Nevertheless, some findings from AC research can give rise to new research ideas and therapeutic strategies not only for other cardiomyopathies but also other cardiac diseases and even for IBD or pemphigus research.

4. Conclusions and Outlook

In this work, p38MAPK, EGFR, SRC or ADAM17 inhibition were identified as new pathways to stabilize cardiomyocyte cohesion and the mechanisms behind adrenergic signaling and PKC activation-mediated positive adhesiotropy were further investigated. These pathways induced positive adhesiotropy in HL-1 cardiomyocytes and wildtype murine cardiac slice cultures, and only adrenergic signaling did not induce positive adhesiotropy in *Jup*$^{-/-}$ murine cardiac slice cultures. Positive adhesiotropy was paralleled by increased DSG2 localization at the membrane of HL-1 cardiomyocytes in all cases but PKC activation. Inhibition of EGFR, SRC or ADAM17 increased DP localization at the membrane. In HL-1 cardiomyocytes, EGFR or SRC inhibition increased area composita length and enhanced DSG2 and DP staining width in wildtype murine cardiac slice cultures. In *Jup*$^{-/-}$ mice, only DSG2 staining width was enhanced upon EGFR or SRC inhibition. Positive adhesiotropy upon adrenergic signaling, PKC activation or p38MAPK inhibition was dependent on ERK1/2 activity and induced hyperadhesion independent of ERK1/2. EGFR inhibition-induced positive adhesiotropy was mediated by ROCK-dependent enhanced desmosomal assembly. In contrast, positive adhesiotropy induced by ADAM17 inhibition was neither signaling-mediated nor via enhanced desmosomal assembly and might be a mechanical issue through reduced desmosomal disassembly upon reduced DSG2 cleavage. However, most of the investigated pathways enhancing cardiomyocyte cohesion might have their downsides *in vivo*. Adrenergic signaling can induce arrhythmia, was not effective in the *Jup*$^{-/-}$ model and might counteract current AC treatment by β-adrenergic receptor blockers (136, 137, 515). Similarly, PKC activation can also induce arrhythmias and hypertrophy (200, 408). EGFR signaling can be cardiotoxic, and whether EGFR signaling is cardioprotective in AC, remains to be investigated. Finally, reduced ADAM17 levels were associated with inflammatory skin and bowel disease (358, 359), and long term ADAM17 inhibition did induce positive adhesiotropy in HL-1 cardiomyocytes. However, p38MAPK and EGFR were upregulated in *Jup*$^{-/-}$ mice, and EGFR seems to be upregulated in AC patients. Thus, an inhibition of these kinases might restore physiological kinase activity. A more detailed understanding of the underlying mechanisms of positive adhesiotropy upon adrenergic signaling, PKC activation, p38MAPK, EGFR, SRC or ADAM17 inhibition should be addressed in future studies. To find the most suitable target which restores cardiomyocyte cohesion with limited adverse effects in AC, *in vivo* studies with animal models, not only limited to *Jup*$^{-/-}$ mice, are required. Apart from that, the findings presented here might be of use in other cardiovascular pathologies, but also in intestinal or skin diseases.

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Publications

- Cardiomyocyte adhesion and hyperadhesion differentially require ERK1/2 and plakoglobin (2020, JCI Insight)
  doi: 10.1172/jci.insight.140066
- Cardiomyocyte cohesion is increased after inhibition of ADAM17 (2023, Frontiers in Cell and Developmental Biology)
  doi: 10.3389/fcell.2023.1021595
- EGFR inhibition leads to enhanced desmosome assembly and cardiomyocyte cohesion via ROCK activation (2023, JCI Insight)
  doi: 10.1172/jci.insight.163763
- Catalytic autoantibodies in arrhythmogenic cardiomyopathy patients cleave desmoglein 2 and N-cadherin and impair cardiomyocyte cohesion (2023, CMLS)
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