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Bedeutung des Epidermalen Wachstumsfaktorrezeptors für die Physiologie und Pathophysiologie des Herzkreislaufsystems.

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Der epidermale Wachstumsfaktorrezeptor (EGFR) gehört zur Familie der ErbB-Rezeptortyrosinkinasen, die in den letzten 30 Jahren hauptsächlich im Rahmen von neoplastischen Erkrankungen untersucht wurden. Bisherige Untersuchungen *in vitro* sowie durch pharmakologische Inhibition *in vivo* weisen auf eine Beteiligung des EGFR an der Entstehung von Bluthochdruck, alters-assoziierten Gefäßveränderungen und entwicklungsbedingter Herzhypertrophie hin. Der EGFR wird in allen Zellen des kardiovaskulären Systems exprimiert. Die Aktivierung des EGFR erfolgt durch Bindung seiner Liganden als auch durch Transaktivierung. Hierbei binden Substanzen, wie Angiotensin II oder Aldosteron an ihre kanonischen Rezeptoren und führen über intrazelluläre Signalkaskaden zur Aktivierung des EGFR und zu den daraus resultierenden zellulären Veränderungen. Weder die Konsequenz der EGFR-Aktivierung *per se* noch seiner Transaktivierung im kardiovaskulären System sind auf zellulärer Ebene bzw. *in vivo* verstanden.
Zur Erlangung eines besseren Verständnisses wurden im Rahmen dieser Arbeit zwei Knockout-Mausmodelle mit konditionaler bzw. konditional-induzierbarer Deletion des EGFR in glatten Gefäßmuskelzellen (VSMC) bzw. auch in Kardiomyozyten generiert und sowohl *in vivo, ex vivo* als auch *in vitro* analysiert. Es konnte gezeigt werden, dass der EGFR in VSMC und Kardiomyozyten an der Aufrechterhaltung der Gewebshomoöstase beteiligt ist. Er verhindert kardiale Hypertrophie und hält den basalen, vaskulären Tonus aufrecht. Angiotensin II dient er *in vivo* und *ex vivo* als Mediator der vasokonstriktorischen Effekte und in Situationen mit inadäquat erhöhten Aldosteronkonzentrationen vermittelt der EGFR einen Teil der kardiovaskulären Effekte des Hormons bei männlichen Versuchstieren. Damit steigert der EGFR die Herz- und Gefäß-schädigende Wirkung des Renin-Angiotensin-Aldosteron-Systems. *In vitro* konnte gezeigt werden, dass die Wirkung des EGFR zumindest partiell von der transaktivierenden Substanz abhängig ist. Unter anderem führt Aktivierung des EGFR zu einer Aktivierung des ERK 1/2-Signalweges, der intrazellulären Ca^{2+}-Freisetzung und der Förderung der Migration im Zellverbund. Weniger deutlich ist die Beeinflussung der Expression von Markergenen für Fibrose oder Entzündung.

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1. Abkürzungsverzeichnis

A431	-	humane, epidermoid Zelllinie
ADAM	-	A Disintegrin And Metalloproteinase
ADAMTS	-	ADAM mit Thrombospondin-Motiv
AG1478	-	Tyrphostin, Inhibitor des EGFR
AGE	-	Advanced-Glycation-Endproducts
Akt	-	Proteinkinase B, Serin/Threonin-spezifische Proteinkinase
ANP	-	atriales, natriuretisches Peptid
AP-2	-	clathrin adaptor protein complex 2
AS	-	Aminosäure
ATP	-	Adenosintriphosphat
Bad	-	BCL2 antagonist of cell death
BMP	-	*Bone morphogenetic protein*
BNP	-	B-Typ natriuretisches Peptid
BTC	-	Betacellulin
C3H	-	Mausstamm
C57Bl6	-	Mausstamm
Ca^{2+}	-	Calcium
CaMKII	-	Ca2+/calmodulin-dependent protein kinase II
Cbl	-	Cellular homologue of Cas NS-1 oncogene
Ccl	-	CC-chemokine ligand
CD1	-	Mausstamm
C/EBP	-	CCAAT-enhancer-binding proteins
c-fos	-	Bestandteil des Transkriptionsfaktors AP-1
c-jun	-	Bestandteil des Transkriptionsfaktors AP-1
c-myc	-	avian myelocytomatosis viral oncogene homolog
CM	-	Kardiomyozyten
Cre	-	Cre-Rekombinase
Crk	-	CT10 regulator of a tyrosine kinase, Adaptorprotein
CrkL	-	Crk-ähnliches Protein
c-Src	-	Akronym aus cellular und sarcoma, Tyrosinkinase
CTGF	-	connective tissue growth factor
DAG	-	Diacylglycerol
dFmax	-	maximale Kraftzunahme
DMT1	-	Diabetes mellitus Typ 1
E1	-	Tag 1 der embryonal Entwicklung
EGF	-	epidermaler Wachstumsfaktor
EGFR	-	epidermaler Wachstumsfaktorrezeptor
Egr-1	-	Early growth response protein 1

EKB-569	-	kleinmolekularer EGFR-Inhibitor
EKG	-	Elektrokardiogramm
ELK1	-	ETS domain-containing protein
eNOS	-	endotheliale NO-Synthase
eps	-	Epidermal growth factor receptor kinase substrate 8
ErbB	-	Erythroblastoma Virus Gen Produkt v-ErbB
ERK	-	Extracellular signal regulated kinase
ET-1	-	Endothelin-1
ET_B-Rezeptor	-	Typ B Endothelin-Rezeptor
Gab1	-	GRB2-asszoiiertes Bindeprotein 1
GAP	-	GTPase-aktivierende Proteine
GH	-	Wachstumshormon
GIT-1	-	ARF-GTPase-aktivierendes Protein 1
GPCR	-	G-protein gekoppelte Rezeptoren
GPR30	-	G-protein gekoppelter Rezeptor 30, evtl. Östrogenrezeptor
Grb2	-	Growth factor receptor-bound protein 2
H9C2	-	kardiale Myoblastenzelllinie der Ratte
HB-EGF	-	Heparin-gebundenes EGF
HCC	-	Hepatozelluläres Karzinom
HeLa	-	humane Zervixkarzinomzelllinie gewonnen von Henrietta Lacks
HIF-1α	-	Hypoxia inducible faktor 1α
HRG	-	Heregulin
HUVEC	-	human, umbilical vein endothelial cells
IC_{50}	-	mittlere inhibitorische Konzentration
IC-4	-	EGFR-Inhibitor
ICAM-1	-	Intercellular adhesion molecule
iEGFR	-	induzierbare EGFR-Knockout-Maus
IgE	-	Immunglobulin E
IκB	-	intrazelluläres Protein das NFκB hemmt
IL	-	Interleukin
iNKT	-	invariante, natürliche T-Killerzellen
IP3	-	Inositoltrisphosphat
JAK	-	Januskinasen
JM	-	juxtamembranäre Domäne
JNK	-	c-Jun N-terminale Kinasen
loxP	-	Zielsequenz der Cre-Rekombinase
KO	-	knockout
MAPK	-	Mitogen-activated protein kinase
MCP-1	-	monocyte chemoattractant protein-1 o. CC-chemokine ligand 2
MEK	-	Mitogen-activated protein kinase kinase

MF1	-	Mausstamm
Mg^{2+}	-	Magnesium
MLC	-	leichte Kette des Myosins
MLC-2v	-	ventrikuläre, leichte Kette des Myosins, Typ 2
µM	-	Mikromolar
MMP	-	Matrixmetalloprotease
mTOR	-	Mammalian target of rapamycin
NaCl	-	Kochsalz
NF-κB	-	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nM	-	nanomolar
NMB	-	nicht meßbare Bindung
NO	-	Stickstoffmonoxid
NOX	-	NADPH-Oxidasen
Nrf2	-	Nuclear factor (erythroid-derived 2)-like 2
NRG	-	Neuregulin
P-Intervall	-	Dauer der Ausbreitung der Vorhoferregung im EKG
P0	-	Tag O nach der Geburt
p38	-	p38-mitogenaktivierte Proteinkinasen
$p47^{phox}$	-	Neutrophil cytosol factor 1
p85	-	regulatorische Untereinheit der PI3-Kinase
PAR	-	Protease activated receptor
PCB	-	polychlorierte Biphenyle
PGE_2	-	Prostaglandin E_2
PI3	-	Phosphatidylinositol-3
PKC	-	Proteinkinase C
PKI-166	-	kleinmolekularer EGFR-Inhibitor
PLC	-	Phospholipase C
Prl	-	Prolaktin
PTB	-	Phosphotyrosine-bindende Domäne
Pyk2	-	Proline-rich tyrosine kinase 2
QRS	-	Ausbreitung der Erregung über die Herzkammern im EKG
QTc	-	auf die Herzfrequenz korrigierte Dauer der Kammererregung
Rac	-	Ras-related C3 botulinum toxin substrate
Raf	-	rapidly accelerated fibrosarcoma, Proteinkinase
Ral	-	Ras-related *protein*
Ras	-	Rat sarcoma, kleines G-Protein
RG50864	-	Tyrphostin, EGFR-Inhibitor
ROS	-	reaktive Sauerstoffspezies
SH2	-	Src-homology 2 Domäne
SH3BP-1	-	SH3 Domain Binding Protein 1

Shc	-	SH2-containing collagen-related proteins
Shp2	-	PTPN11, Tyrosinphosphatase
SM22	-	Transgelin, Smooth Muscle Protein 22-alpha
SOS	-	Son of sevenless, GTP-Austauschfaktoren
Sp1	-	specificity protein 1, Transkriptionsfaktor
STAT	-	signal transducer and activator of transcription
SV129	-	Mausstamm
sVEGFR1	-	löslicher VEGF-Rezeptor Typ 1
TACE	-	TNF-α converting enzyme, ADAM-17
TGF	-	Transforming growth Faktor
TNF-α	-	Tumor-necrosis factor α
TRAIL	-	Tnf-related apoptosis inducing ligand
TRPM6	-	Transient receptor potential cation channel, subfamily M, member 6
U937	-	humane Monozyten-Zelllinie aus einem Lymphom
Vav	-	Vav Guanine Nucleotide Exchange Factor
VCAM-1	-	Vascular cell adhesion protein 1
VEGF	-	*Vascular Endothelial Growth Factor*
VSMC	-	glatte Gefäßmuskelzellen
Wa-1	-	Waved-1, Mauslinie, Loss-of-function Mutation von TGF-α
Wa-2	-	Waved-2, Mauslinie, Loss-of-function Mutation des EGFR

2. Einleitung

Die ErbB-Rezeptoren sind eine Familie von vier Rezeptortyrosinkinasen, die bisher hauptsächlich Beachtung als Förderer des Tumorwachstums, z.B. bei Brustkrebs, Ovarialkarzinom, Lungentumoren, Kolonkarzinom, und Pankreaskarzinom, fanden[8]. Zu den ErbB-Rezeptoren gehört neben dem ErbB2 (Her-2), ErbB3 und ErbB4 auch der epidermale Wachstumsfaktorrezeptor (EGFR)[5]. Inzwischen ist aber auch eine pathogenetische Bedeutung des EGFR bei nicht-neoplastischen Erkrankungen, wie der polyzystischen Nierenerkrankung[9], Psoriasis[10,11] und Asthma bronchiale[12] beschrieben. Seit der EGFR vor über 30 Jahren erstmalig als Zielstruktur für die Tumortherapie vorgeschlagen wurde[13-15], sind erfolgreich Medikamente, wie Trastuzumab (Herceptin®), Cetuximab (Erbitux®), Gefitinib (Iressa®) oder Erlotinib (Tarveva®), die gegen ErbB-Rezeptortyrosinkinasen gerichtet sind, in die Behandlung von Tumorpatienten integriert worden[8,13]. Allerdings werden die ErbB-Rezeptoren nahezu ubiquitär exprimiert und ihre physiologische Bedeutung ist noch nicht vollständig geklärt, unter anderem, weil Mäuse mit Deletion der Rezeptoren meist embryonal oder kurz postnatal versterben[16]. Daher kann es nicht überraschen, wenn bei Anwendung eines Teils der oben genannten Tumortherapeutika Nebenwirkungen auftreten. So entwickelten Patientinnen bei Gabe von Trastuzumab, besonders bei gleichzeitiger Einnahme von Zytostatika, schwere Herzhypertrophien bis hin zum Herzversagen[17].

Der Mineralocorticoidrezeptor und der Angiotensinrezeptor 1 sind in die Entstehung von kardiovaskulären Erkrankungen involviert. Bis zum heutigen Tag konnte der Pathomechanismus durch den der Angiotensinrezeptor 1 und der Mineralocorticoidrezeptor kardiovaskuläre Schäden induzieren noch nicht abschließend geklärt werden. Zum Teil liegt dies sicher auch darin begründet, dass die Bedeutung des EGFR, der essentiell für die pathophysiologische Wirkung u. a. dieser Rezeptoren ist[18], bisher noch nicht ausreichend untersucht werden konnte. So ist abzuklären welche Bedeutung der EGFR in glatten Gefäßmuskelzellen für die physiologische Blutdruckregulation und die Blutgefäßhomöostase besitzt. Auch ist die Funktion des EGFR in Kardiomyozyten noch nicht bekannt. Reguliert er in diesen Zellen, die im Adulten nicht zur Proliferation befähigt sind, das Zellwachstum? Fördert der EGFR die Expression von Genen die zur Entstehung von Fibrosen, Entzündungen oder Veränderungen der Synthese von Reaktiven Sauerstoffspezies beitragen? Übt der EGFR die gleichen Effekte sowohl in glatten Muskelzellen als auch in Kardiomyozyten aus? Welche Effekte vermittelt die Aktivierung des EGFR unter pathologischen Bedingungen und folgen die Effekte, unabhängig von der pathologischen Situation, einem bestimmten Prinzip? Bisherige Untersuchungen die sich diesen Fragestellungen gewidmet haben, haben entweder mit Hilfe von pharmakologischen Inhibitoren oder Nagermodellen mit globaler Veränderung des EGFR gearbeitet. Diese Herangehensweise birgt jedoch den Nachteil, dass aufgrund der ubiquitären Expression des EGFR die Ergebnisse nur schwer zu interpretieren sind. So könnten zum Beispiel positive Effekte auf die Funktion der Blutgefäße bei Inhibition des

EGFR durch schädliche Effekte auf das Herz maskiert werden. Zusammengenommen waren diese Überlegungen der Aufsetzpunkt für die vorliegende Arbeit. Das Ziel war es die physiologische und pathophysiologische Bedeutung des EGFR für das Herz-Kreislaufsystem näher zu untersuchen, mit besonderer Berücksichtigung der arteriellen Blutgefäße sowie der *in vivo* Situation. Hierbei sollte ein besonderes Augenmerk darauf gelegt werden, die zellspezifischen Effekte zu demaskieren.
In dieser Arbeit werden Daten vorgestellt die mit konditionalen und induzierbaren Knockout (KO)-Mausmodellen gewonnen wurden, die eine Deletion des EGFR in glatten Gefäßmuskelzellen (VSMC) und/oder eine starke Reduktion des EGFR in Kardiomyozyten aufweisen. Hierbei haben wir sowohl *in vivo*, *ex vivo* als auch *in vitro* die Bedeutung des EGFR für das Herz-Kreislaufsystem untersucht. Am Anfang dieser Arbeit erfolgt eine Einführung in die bisherigen Erkenntnisse zur Bedeutung des EGFR und seiner Liganden.

2.1. ErbB-Rezeptortyrosinkinasefamilie

Die ErbB-Rezeptortyrosinkinasefamilie hat sich aus einem Rezeptor mit einem Liganden bei Würmern, über einen Rezeptor mit vielen Liganden in Fliegen zu einer Familie mit vier Rezeptoren und vielfältigen Liganden in Säugetieren entwickelt. ErbB-Rezeptoren vermitteln ihre Funktion als Dimere, wobei jeder ErbB-Rezeptor mit den anderen Mitgliedern der Familie interagieren kann[5]. Sie bildet damit ein Netzwerk, das sehr unterschiedliche Signalwege aktiviert[5,19] (s. **Abbildung 1**). Die Erstbeschreibung des EGFR erfolgte 1984 durch Axel Ullrich[20]. Die ErbB-Rezeptoren gehören zu den Prototypen der Rezeptortyrosinkinasen. Ihre Funktion und Bedeutung wurde bereits ausführlich in Übersichtsartikeln behandelt, unter anderem[5,6,19,21]. Hier soll nur ein kurzer Überblick über die Funktion der ErbB-Rezeptoren gegeben werden.
Alle vier Rezeptoren besitzen eine extrazelluläre Ligandenbindungsregion, eine einfache Transmem-brandomäne, eine kurze, intrazelluläre, juxtamembranäre Domäne, gefolgt von der Tyrosinkinasedomäne. C-Terminal befinden sich die Phosphotyrosinbindungsstellen für die Effektormoleküle[6,19]. Allerdings ist für den ErbB2 kein Ligand bekannt[19,22] und ErbB3 weist keine Tyrosinkinaseaktivität auf[19,23]. Die Ligandenbindung führt zu einer Konformationsänderung die die Tyrosinphosphorylierung in *trans* erlaubt[6,24,25] (s. **Abbildung 2**). Die extrazelluläre Domäne von ErbB2 scheint permanent eine ähnliche Konformation wie die extrazelluläre Domäne des EGFR nach Bindung des Liganden aufzuweisen. Damit lieg der ErbB2 permanent „semi“ aktiviert in der Zellmembran vor[19,26].

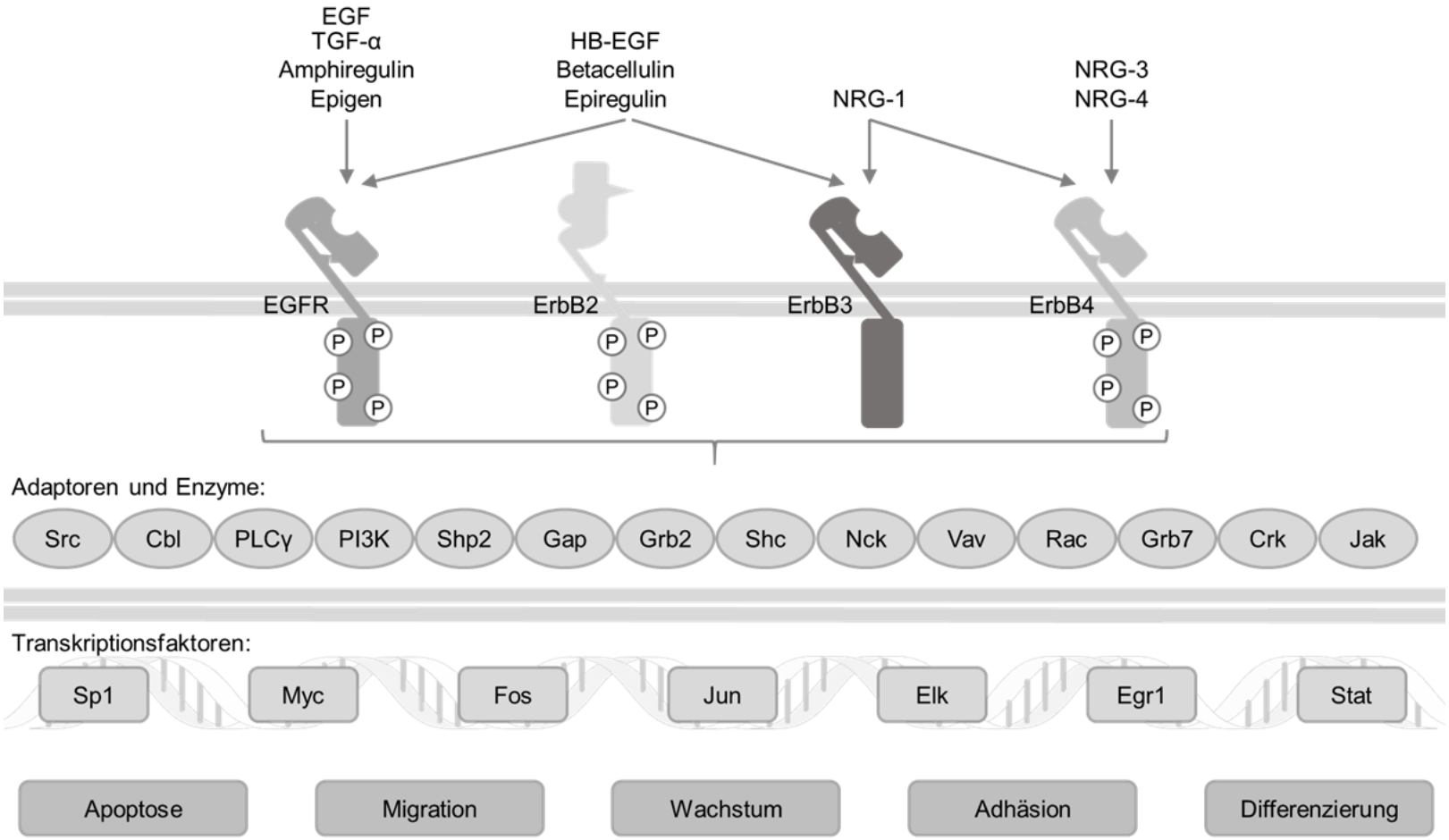

Abbildung 1: Grafische Übersicht über das Signalnetzwerk der ErbB-Rezeptortyrosinkinasefamilie. EGFR = Epidermaler Wachstumsfaktorrezeptor, ErbB2 = Epidermaler Wachstumsfaktorrezeptor Typ 2, ErbB3 = Epidermaler Wachstumsfaktorrezeptor Typ 3, ErbB4 = Epidermaler Wachstumsfaktorrezeptor Typ 4, EGF = epidermaler Wachstumsfaktor, TGF-α = Tumorwachstumsfaktor-α, HB-EGF = Heparin-bindender EGF, NRG = Neuregulin, weitere Abkürzungen sind im Abkürzungsverzeichnis zu finden. Abbildung modifiziert nach Yarden & Sliwkowski[5].

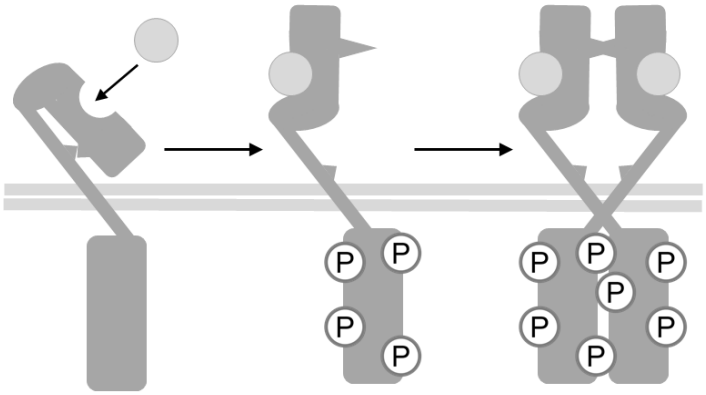

Abbildung 2: Dimerisierung des EGFR. Durch die Bindung des Liganden wechselt der Rezeptor von seiner gestauchten, inaktiven Form in seine ausgeklappte, aktive Form. In diesem Funktionszustand kann er dann mit einem weiteren Rezeptor der ErbB-Familie dimerisieren. Im Gegensatz zu Insulinrezeptoren findet die Interaktion der zwei Dimerisierungspartner nur zwischen den Rezeptoren, nicht zwischen Rezeptoren und Liganden statt. Abbildung modifiziert nach Bessmann *et al*[7].

Die Interaktion der ErbB-Rezeptoren mit Adaptormolekülen bzw. Enzymen erfolgt durch deren SH2(Src-homology)- bzw. PTB(Phosphotyrosinbinde)-Domänen[6,27].

Neben der Zusammensetzung des Rezeptordimers scheint auch die Aktivierungsstärke für die zellulären Effekte der ErbB-Rezeptoren mitverantwortlich zu sein[28]. Die Aktivierungsstärke wird auch durch den Liganden moduliert, da diese bezüglich des einzelnen ErbB-Rezeptors aber auch in Abhängigkeit von dessen Dimerisierungspartner zum Teil deutliche Unterschiede in der Affinität aufweisen[29] (s. **Tabelle 1**). Von den aktivierten Signalwegen können einige durch alle ErbB-Rezeptoren aktiviert werden, andere sind spezifisch für die einzelnen Rezeptoren. So ist die Aktivierung des Mitogen-activated protein kinase (MAPK)-Signalweges über die Aktivierung von Shc (SH2-containing collagen-related proteins) und/oder Grb2 (Growth factor receptor-bound protein 2) ein gemeinsames Merkmal der Aktivierung aller ErbB-Rezeptoren[30]. Auch die Phosphatidylinositol-3-Kinase (PI3-Kinase) kann direkt (ErbB3)[28] oder indirekt durch die meisten ErbBs aktiviert werden[31].

Die Information über die Aktivierung des Rezeptors wird sowohl im Zytosol als auch im Nukleus weiterverarbeitet. Änderungen in der Genexpression ermöglichen es den Zellen sich an die neuen Gegebenheiten anzupassen[6]. Eine vereinfachte Version des ErbB-Signalnetzwerkes ist in **Abbildung 1** dargestellt.

Zusammenfassend lässt sich feststellen, das durch die unterschiedliche Kombination von Liganden und Rezeptoren sowohl die Signalstärke als auch der Signalweg variiert werden kann und damit durch die ErbB-Rezeptoren ein feinabgestimmtes Signalnetzwerk gebildet wird, das wichtige zelluläre Prozesse moduliert. Da in dieser Arbeit hauptsächlich die Bedeutung des EGFR behandelt werden soll, wird auf die anderen ErbB-Rezeptoren und ihre Liganden nur in Ausnahmefällen eingegangen.

Tabelle 1: IC_{50}-Werte der unterschiedlichen ErbB-Liganden für die verschiedenen Rezeptordimere (modifiziert nach Jones *et al*[29])

	IC_{50} [nM]					
	[EGF]			[HRG]		
Ligand	**ErbB1/1**	**ErbB1/2**	**ErbB3/3**	**ErbB2/3**	**ErbB4/4**	**ErbB2/4**
HRGα	NMB	NMB	550	48	510	7.4
HRGβ	NMB	NMB	5.4	0.2	5.1	0.1
trx-EPR-α	2800	24	NMB	230	NMB	110
BTCα	1.4	1.7	NMB	NMB	3.6	0.2
HB-EGFα	7.1	3.4	NMB	NMB	NMB	310
EGFα	1.9	1.2	NMB	NMB	NMB	49
TGFα	9.2	6.4	NMB	NMB	NMB	340
trx-BiR	2.7	0.7	1100	32	23	0.9

HRG = Heregulin, BTC = Betacellulin, NMB = nicht meßbare Bindung oder die berechnete IC_{50} ist größer als 5µM.

2.2. Funktion des EGFR

Die Sequenz des EGFR wurde 1984 durch Axel Ullrich[20] publiziert. Posttranslational kann der EGFR unter anderem glykosyliert werden, diese Modifikationen können bis zu 20% seines Molekulargewichtes ausmachen. Funktionell sind diese Veränderungen für die Translokation des EGFR zur Zelloberfläche und seine Funktionalität von Bedeutung[32,33]. In polarisierten Zellen wird der EGFR hauptsächlich in die basolaterale Membran eingebaut[34]. Hier kommt es zur Assoziation mit Lipid-rafts, die durch die Aminosäuresequenz der Extrazellulärdomäne[35,36] vermittelt wird. Der EGFR kann mit allen drei ErbB-Rezeptoren dimerisieren[37] und dient als Relaisstation für die Kreuzaktivierung verschiedener Signalwege[37] (**Abbildung 1)**. Die Phosphorylierung des EGFR und damit die Aktivierung nachgeschalteter Signalwege ist abhängig von i) dem Dimerisierungspartner, ii) der Art der Liganden und iii) der Konzentration der Liganden.
Durch die Carboxy-terminale Domäne des EGFR wird sowohl die intrazelluläre Weiterleitung der Signale vermittelt, als auch die Herrunterregulation und Endozytose des EGFR[32]. Zudem ermöglichen die Aminosäurereste 984-996 die Interaktion des EGFR mit Aktin[32,38].

2.2.1. Kanonische Signalwege

Die Effekte des EGFR in vivo und in vitro werden durch die von ihm aktivierten Signalwege bestimmt. Die Differenzierung welcher ErbB-Rezeptor für die Aktivierung der verschiedenen Signalwege notwendig ist, wird jedoch erschwert, da die ErbB-Rezeptoren a) mit allen anderen Mitgliedern Dimere bilden können, b) einige der Signalwege auch von mehreren ErbB-Rezeptoren aktiviert werden können5,31 und c) es Proteine gibt, die bei niedrigen Ligandenkonzentrationen aktiviert werden, während andere erst bei hohen Ligandenkonzentrationen aktiviert werden[39]. Die Interaktion des EGFR mit weiteren Proteinen erfolgt über deren SH2 bzw. PTB-Domänen. Bis 2008 wurden bereits über

hundert Proteine beschrieben, die mit dem EGFR selber interagieren und über 200 Proteine die EGFR-abhängig modifiziert werden[40]. Des Weiteren ist bei der Untersuchung der durch den EGFR induzierten Signalwege noch zu bedenken, dass der EGFR internalisiert werden kann und einige Proteine hauptsächlich mit den bereits internalisierten Rezeptoren interagieren, und so deren Signalweiterleitung fördern[28]. Zu den vier wichtigsten Signalwegen die durch den EGFR aktiviert werden gehören 1) der Ras-Raf-MEK-ERK-Signalweg, 2) der PI3-Kinase-Akt-Signalweg, 3) der PLCγ und 4) der STAT- Signalweg[4] (s. **Abbildung 3**).

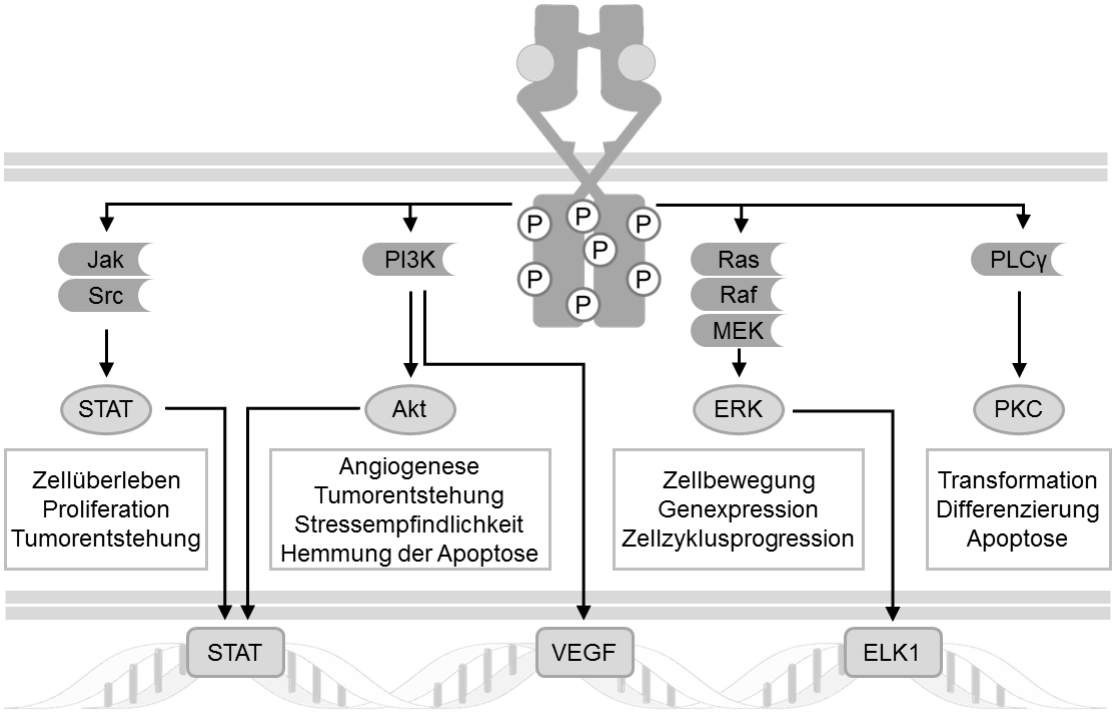

Abbildung 3: Kanonische Signalwege des EGFR. Durch Bindung seiner Liganden und damit Aktivierung des EGFR kommt es zur Förderung der STAT, PI3-Kinase, MAP-Kinase oder Phospholipase Cγ abhängigen Zellveränderungen wie Zellüberleben, Proliferation, Migration oder auch Angiogenese. Abbildung modifiziert nach Nyati *et al*[4].

2.2.2. Transaktivierung

Einleitung

Die Transaktivierung des EGFR wurde erstmals 1994 von der Arbeitsgruppe um Hans J. Rahmsdorf beschrieben[41]. Durch die Transaktivierung kann der EGFR auch Signale anderer Hormone, vasoaktiver Substanzen wie Endothelin-1[42-44], Noradrenalin[5], Prostaglandin E_2 (PGE_2)[45] oder Angiotensin II[46] weiterleiten. Aber auch Glukose[47], reaktive Sauerstoffspezies[48], oxidierte Lipide[49], ultraviolettes Licht[41], Änderungen des Zellvolumens[50] und Dehnung[51] können durch Transaktivierung des EGFR zelluläre Signalwege aktivieren und damit die Anpassung des Organismus an veränderte Umweltbedingungen ermöglichen. Über diesen Mechanismus ist der EGFR in

pathologische Prozesse wie die Schädigung des Herzens durch Ischämie und Reperfusion, Atherosklerose, Nierenerkrankungen, Bluthochdruck und Asthma bronchiale involviert. Es werden zwei Mechnismen der EGFR-Transaktivierung unterschieden. Bei dem ersten Mechanismus wird der EGFR-Liganden-unabhängig aktiviert (s. **Abbildung 4**). Hier kommt es, ohne das ein EGFR-Ligand an seinen Rezeptor bindet durch die Aktivierung von intrazellulären Signalkaskaden zur Phosphorylierung des EGFR. Dies wurde erstmals für das Wachstumshormon[52] und Prolaktin[53] beschrieben. Beim zweiten Mechanismus kommt es zur Liganden-abhängigen Aktivierung des EGFR, dies ist der Mechanismus über den G-Protein gekoppelte Rezeptoren (GPCR) vermutlich einen großen Teil ihrer Einflüsse auf Zellproliferation, Zellüberleben und Migration ausüben (s. **Abbildung 4**). Bei diesem Mechanismus induziert z.B. Angiotensin II die Freisetzung von EGFR-Liganden von der Zelloberfläche. Durch Bindung dieser Liganden wird in der Folge der EGFR aktiviert. Der so genannte „Triple-membrane-spanning"-Mechanismus wurde erstmals von der Arbeitsgruppe um Axel Ullrich für Carbachol beschrieben[54].

EGFR-Liganden-unabhängige Transaktivierung

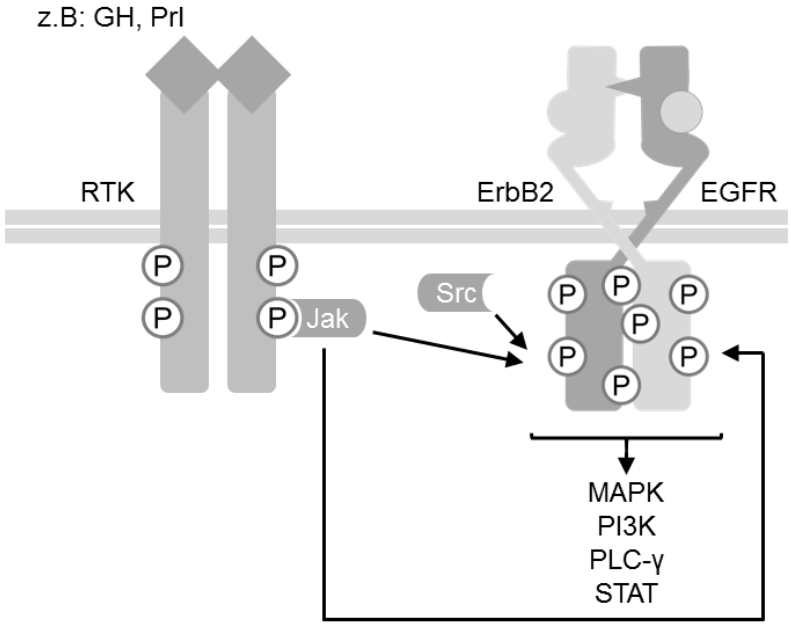

Abbildung 4: Liganden-unabhängige Aktivierung des EGFR und des ErbB2. Die Aktivierung von Rezeptortyrosinkinasen durch ihre kanonischen Liganden, z.B. Wachstumshormon (GH) oder Prolaktin (Prl) führt über intrazelluläre Tyrosinkinasen, z.B. Jak oder Src, zur Aktivierung des EGFR bzw. des ErbB2. In der Folge kommt es zur Aktivierung der EGFR abhängigen Signalwege wie der Aktivierung der MAP-Kinasen. Abbildung modifiziert nach Holbro & Hynes[6].

Bei der Liganden-unabhängigen Transaktivierung des EGFR dient der EGFR als Gerüst und die Aktivierung seiner Kinasedomäne ist nicht notwendig für die Weiterleitung des Signals[6] (**Abbildung 4**). D.h. es erfolgt eine Phosphorylierung des EGFR an Tyrosinresten die nicht zu den klassischen Autophosphorylierungsstellen gezählt werden. Damit werden zusätzliche Signalwege aktiviert, wie z.B. die Aktivierung der PI3-Kinase durch den EGFR[6,32,55,56]. Yamauchi *et al*[52] konnten als erste zeigen, dass das Wachstumshormon sowohl *in vivo* als auch *in vitro* zu einer Tyrosinphosphory-

lierung des EGFR in der Leber von Mäusen und in der Zellkultur führt. Diese EGFR-Phosphorylierung ist abhängig von JAK2 aber nicht von der EGFR-Kinase Aktivität[52,57]. Das Wachstumshormon induziert vermutlich die Phosphorylierung an Y1068 des EGFR, das Teil eines Grb2-Bindungsmotivs ist. Daher führt das Hormon zu einer verstärkten Assoziierung von EGFR und Grb2. Durch die Transaktivierung des EGFR kommt es zu einer erhöhten ERK-Aktivierung[57].

Auch G-Protein gekoppelte Rezeptoren können über die Rekrutierung von β-Arrestinen oder Src zu einer EGFR-Liganden-unabhängigen Transaktivierung führen[58-60]. Durch die Aktivierung von Src durch GPCRs kann die intrazelluläre Tyrosinkinasedomäne des EGFR aktiviert werden[46,60,61]. Hierbei sind nicht die Autophosphorylierungsstellen des EGFR die Zielstrukturen von Src, sondern die Tyrosinreste Y869, Y915, Y944 und Y1125[32,55,56]. Die Transaktivierung des EGFR durch Src vermittelt die Effekte des MAPK-Signalweges[47,48], des PI3-Kinase-Signalweges[43,48] und des STAT-Signalweges.

Auch Steroidhormone vermitteln einen Teil ihrer Wirkung durch die Liganden-unabhängige Transaktivierung des EGFR. So stimuliert Aldosteron die Expression des EGFR und der EGFR wiederum vermittelt einen Teil der schnellen, nicht-genotropen Wirkungen des Mineralocorticoidrezeptors, dessen Ligand Aldosteron ist[62]. Es wird vermutet, dass diese Wirkung durch eine Subpopulation der Steroidrezeptoren erfolgt, die an der Plasmamembran lokalisiert sind[62].

EGFR-Liganden abhängige Transaktivierung

Bei der Liganden-abhängigen Transaktivierung des EGFR (**Abbildung 5**) binden vasoaktive Substanzen, Neurotransmitter, Zytokine etc. an ihren kanonischen Rezeptor, meist ein GPCR[54,63-65]. Durch einen intrazellulären Signalweg werden extrazellulär Proteasen aktiviert, meist MMPs oder ADAMs, die EGFR-Liganden von der Zellmembran abspalten. Hierbei handelt es sich meist um HB-EGF. Allerdings wurde im Rahmen der Transaktivierung auch schon die Freisetzung von TGF-α beschrieben[66]. Der freigesetzte Ligand bindet auto- oder parakrin an den EGFR und fördert dessen Aktivierung[44,60,67] und damit die Aktivität der kanonischen Signalwege des EGFR wie MAPK und PLC-γ1. Da bei der Liganden-abhängigen Transaktivierung das Signal dreimal die Zellmembran passiert, spricht man vom „Triple-membrane-spanning"-Mechanismus. Wenn man die Anzahl der verschiedenen GPCRs betrachtet und ihre variable Expression abhängig vom Gewebe, dann kommt diesem Mechanismus vermutlich eine große physiologische und pathophysiologische Bedeutung zu.

Zu den Substanzen die den EGFR über diesen Mechanismus transaktivieren können gehören unter anderem Angiotensin II [68] oder auch adrenerge-Agonisten[5].

Der genaue molekulare Mechanismus ist noch nicht abschließend geklärt. So sind weder die Liganden-freisetzenden Proteasen noch die intrazellulären Signalwege für alle transaktivierenden Substanzen ermittelt. Serotonin, Endothelin-1 oder Thrombin, sollen die Transaktivierung des EGFR in z.B. Adipozyten durch die $G_{\alpha q}$/11-Untereinheiten

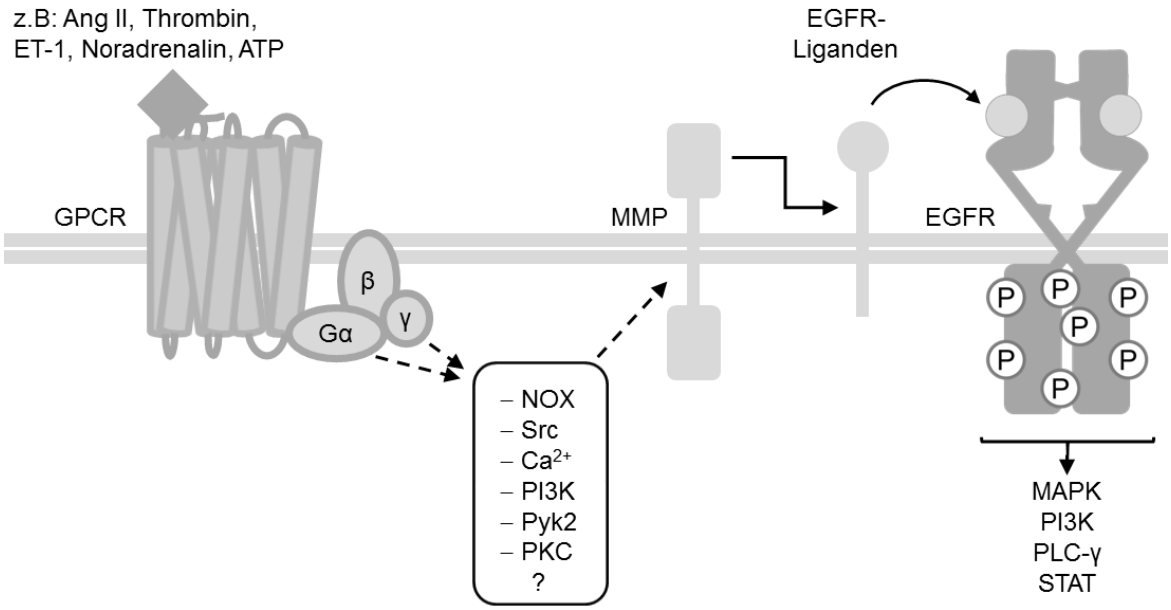

Abbildung 5: Liganden-abhängige Transaktivierung des EGFR. Durch Bindung von verschiedenen Substanzen an ihre kanonischen Rezeptoren, hauptsächlich GPCRs kommt es über intrazellulärer Signalkaskaden zur Aktivierung von MMPs. Diese setzen die EGFR-Liganden, hauptsächlich HB-EGF, frei und führen damit zur Aktivierung des EGFR und seiner nachfolgenden Signalwege, wie MAP-Kinasen, PI3-Kinasen, Phospholipase Cγ oder von STAT. Modifiziert nach Schreier *et al*[3]; Ang II = Angiotensin II

vermitteln[69]. Bei anderen Liganden wird die Transaktivierung des EGFR allerdings durch die $G_{\beta\gamma}$-Untereinheiten vermittelt[60,70]. Dieser Mechanismus scheint über die Aktivierung von MMP-14 und die anschließende Freisetzung von HB-EGF vermittelt zu werden[60].
In der Summe lässt sich sagen, dass die Liganden-abhängige Transaktivierung des EGFR vermutlich abhängig ist von dem induzierenden Liganden, der zugrundeliegenden Zelle und auch dem pathophysiologischen Kontext. Auch die physiologische und pathophysiologische Bedeutung dieses Mechanismus ist noch nicht abschließend geklärt, jedoch scheinen besonders die mitogenen Effekte der GPCR-Liganden durch den EGFR vermittelt zu werden[5].

2.3. Bedeutung des EGFR für das kardiovaskuläre System

Die Bedeutung der ErbB-Rezeptoren für die Funktion und Homöostase des Herz-Kreislauf-Systems wurde erstmals erkannt, als Frauen die einen EGFR-Inhibitor im Rahmen der Krebstherapie erhielten, eine Herzhypertrophie entwickelten. Inzwischen gibt es eine Vielzahl von Berichten, die die Bedeutung von allen vier Mitgliedern der ErbB-Familie für die Entwicklung des kardiovaskulären Systems unterstreichen[13,71-80]. In **Tabelle 2** ist eine Übersicht über den kardiovaskulären Phänotyp der verschiedenen Mauslinien mit Deletion eines der vier ErbB-Rezeptoren gegeben.

Ob alle vier Rezeptoren im Herzen exprimiert werden ist noch unklar. In Herzen von neonatalen und adulten Ratten scheint ErbB4 stärker exprimiert zu sein als ErbB2, während ErbB3 so gut wie nicht nachweisbar ist[90-92]. Über das Vorhandensein des EGFR sind widersprüchliche Daten vorhanden.

Tabelle 2: Auflistung der verschiedenen KO-Mausmodelle der Rezeptoren der epidermalen Wachstumsfaktor-Familie

KO	Letalität	Überlebenszeit	Kardiovaskulärer Phänotyp	Quelle
$EGFR^{-/-}$	abhängig vom Hinter-grundstamm vor der Implantation, in der Mitte der Trächtigkeit oder 2-3 Wochen postnatal	max. 2-3 Wochen post natal	Erhöhte Herzfrequenz	16,81,82
$EGFR^{Wa-2}$	keine	wenige Tage postnatal (Laktation)	verdickte und vergrößerte Aortenklappen	73,83-86
$EGFR^{Dsk5}$	keine	keine Angaben	keine Angabe	87
$EGFR^{Wa-5}$	zwischen Tag E10.5–12.5	keine	keiner beschrieben	88,89
ErbB2	embryonal	E10.5	Reduzierte Wanddicke, verringerte Trabekelbildung, reduzierter intrakardialer Blut-fluß	76
ErbB2	embryonal	vor E11	Keine Trabekelbildung, normale bis hypomorphe endokardiale Tasche, normaler Ausflusstrakt,	75
ErbB3	embryonal	E13.5	Reduzierte Klappenbildung (Mesenchym fehlt), fehlerhafter Klappenschluss (Reflux)	76
ErbB4	embryonal	E10-11	Abbruch der Herzentwicklung, Fehlende Trabekelbildung, reduzierter intrakardialer Blut-fluß	74

2.3.1. Herz *in toto*

Unter physiologischen Bedingungen scheint die Expression des EGFR im Herzen hauptsächlich in den Koronargefäßen zu erfolgen, weniger in den Kardiomyozyten[93]. Vergleicht man die mRNA für den EGFR semiquantitativ zwischen jungen und adulten Tieren, so nimmt diese mit dem Alter ab[94]. Die Funktion des EGFR im Herzen ist noch nicht bekannt. Unter pathophysiologischen Bedingungen wurden sowohl protektive[95-97] als auch schädigende Effekte[98,99] des EGFR für die Funktion des Herzens nachgewiesen.

Transaktivierung

In vivo kann die Transaktivierung des EGFR im Herzen durch Katecholamine[58,97], Endothelin-1[80,100], Angiotensin II[94,100] und Glukose[98,99,101] nachgewiesen werden. Aber auch eine $G_{\alpha q}$-Protein unabhängige Aktivierung durch weitere Liganden von GPCRs ist möglich[96]. Der EGFR in Kardiomyozyten scheint sowohl den Akt- als auch den ERK1/2-Signalweg zu aktivieren[96,97]. Damit wird das Zellüberleben gefördert und das Herz vor Ischämie/Reperfusionsschäden geschützt.

Hypertrophie

Im allgemeinen wird die Herzhypertrophie als ein adaptiver, physiologischer Prozess betrachtet. Die Zunahme des Herzgewichtes soll als Antwort auf einen gestiegenen Blutdruck, die Wandspannung, Kammergröße und kontraktile Funktion des Herzens aufrecht erhalten[102]. Neben diesen, initial positiven, Effekten ist die Herzhypertrophie ein unabhängiger Risikofaktor für Herzkreislauferkrankungen, die bei fehlender Behandlung zum Herzversagen führen kann[102].

Die Bedeutung des EGFR für die Entstehung, Progression bzw. Aufrechterhaltung der Herzhypertrophie ist noch nicht abschliessend geklärt. Bisher wurden einzelne Untersuchungen zur Beeinflussung der Herzfunktion an *Wa-2* Mäusen durchgeführt, deren Ergebnisse aber teilweise widersprüchlich sind. So berichten Barrick *et al*[103] von einer Verdopplung des Herzgewichtes dieser Mäuse, einer reduzierten Verkürzung (fractional shortening) und einem erhöhten Druckgradienten über die Aortenklappe. Bedingt werden diese Veränderungen durch eine Verdickung der Herzklappen[103]. Griol-Charhbili *et al*[104] hingegen zeigten lediglich eine Zunahme des Herzgewichtes von ~20%. Die chronische Inhibition des EGFR durch kleinmolekulare EGFR-Inhibitoren (AG1478 bzw. EKB-569) hingegen verändert das Herzgewicht nicht, führt aber zu einer reduzierten systolischen Herzfunktion bei weiblichen Mäusen[13]. Welche zusätzlichen Faktoren den Grad der Hypertrophie bestimmen ist noch nicht untersucht worden. In der Summe deuten die Befunde daraufhin, dass der EGFR im Herzen zur Aufrechterhaltung der physiologischen Herzfunktion benötigt wird. Unter pathophysiologischen Bedingungen scheint der EGFR die Herzhypertrophie zu fördern. So kann durch Antisense-RNA gegen den EGFR bei hypertensiven Ratten die Zunahme des Herzgewichtes reduziert werden[94]. Auch in einem Modell der Isoprenalin-induzierten Herzhypertrophie konnte die Gabe von Gefitinib, einem kleinmolekularen Inhibitor der Tyrosinkinase des EGFR, die Zunahme des Herzgewichtes und der Apoptose im Herzen reduzieren, nicht jedoch die Herzfibrose[105]. Im Gegensatz dazu, reduzierte die Gabe von PKI-166, ebenfalls ein EGFR-Inhibitor, lediglich den linksventrikulären, enddiastolischen Druck in hypertonen Ratten (5/6-Nephrektomie) aber nicht das Herzgewicht[93]. Auch die durch Angiotensin II-induzierte Herzhypertrophie soll durch den EGFR vermittelt werden[106].

Der zugrunde liegende Mechanismus, durch den der EGFR die Herzhypertrophie verhindert, ist vollständig ungeklärt. Weder ist bekannt, in welchen Zellen der EGFR seine

anti-hypertrophe Wirkung entfaltet (Kardiomyozyten, Fibroblasten oder Endothelzellen?), noch wurde die Signalkaskade untersucht. Die Aktivierung von Src und nachfolgend die Transaktivierung des EGFR soll bei kurzfristiger Überexpression von $G_{\alpha q}$ zur Herzhypertrophie beitragen[96].

Fibrose

Neben einer Vergrößerung des Herzens weisen die Mäuse mit hypomorphem EGFR (*Wa-2*) eine erhöhte interstitielle und perivaskuläre Fibrose auf, die jedoch in der Stärke ihrer Ausprägung abhängig vom Hintergrundstamm der Mäuse ist[103]. In einer interessanten Studie haben Barrick *et al*[13] Mäuse mit zwei EGFR-Inhibitoren über 90 Tage gefüttert. Dabei zeigt sich bereits unter basalen Bedingungen eine Zunahme der interstitiellen Fibrose durch Gabe von EGFR-Inhibitoren. Dass Inhibitoren des EGFR einen eigenen profibrotischen Effekt haben, konnte in einer weiteren Studie bestätigt werden, hier durch Gabe von Gefitinib[105]. Barrick *et al*[13] konnten einen geschlechtsabhängigen Effekt des EGFR nachweisen. So waren männliche Tiere vor der verstärkten Fibrose des Herzens geschützt. Dies könnte durch ein erhöhtes Expressionsniveau von EGF bei Männchen erklärt werden[107,108]. Dies, zusammen mit der Erhöhung der Transkription von EGF, ErbB2 und B-typ natriuretischem Peptid (BNP) in den Herzen von AG1478 behandelten C57Bl6 Männchen, könnte die Kardiotoxizität reduzieren[13].

Zelltod

Die Befunde zur Bedeutung des EGFR für die Apoptose im Herzen sind bisher noch widersprüchlich. Einerseits steigert die Hemmung des EGFR die Apoptoserate in kardialen Zellen und reduziert die Expression von Bcl-2l1, einem anti-apoptotischen Protein[13]. Auch scheint der EGFR einen Teil der antiapoptotischen Wirkung von Neuregulin-1 im Herzen zu vermitteln, da dieser Effekt durch AG1478 hemmbar ist[109]. Zusätzlich reduziert die Transaktivierung des EGFR durch β-adrenerge Rezeptoren die Apoptose im Herzen[58,97,110]. Andererseits reduziert die Hemmung des EGFR die Diabetes-assoziierte Apoptose von Kardiomyozyten[98] und auch in einem Herzhypertrophiemodell (β-adrenerge Stimulation) wurde eine reduzierte Apoptose im Herzen durch die Hemmung des EGFR beobachtet[105]. Leider wurde meines Wissens in keiner dieser Publikationen untersucht, welche Zellen im Herzen von der Apoptose betroffen sind. So würde eine vermehrte Apoptose von Kardiomyozyten eine verschlechterte Herzfunktion erwarten lassen, während eine vermehrte Apoptose von Fibroblasten für die Funktion des Herzens evtl. sogar förderlich wäre.

Radikal Stress

Reaktive Sauerstoffspezies (ROS) behindern die kontraktile Funktion des Herzens, in dem sie Proteine, die für die Kopplung von Erregung und Kontraktion verantwortlich sind modifizieren. Zudem können sie Kinasen und Transkriptionsfaktoren aktivieren, die in die

Entstehung der Herzhypertrophie involviert sind. Zusätzlich beeinflussen ROS auch die Apoptose[111]. Wobei ROS nicht nur schädlich sein müssen, sondern auch für protektive Effekte verantwortlich gemacht werden. Krieg *et al*[112] konnten zeigen, dass die durch Acetylcholin vermittelte, mitochondriale ROS-Synthese durch HB-EGF vermittelt wird. Interessanterweise hat AG1478, ein relativ spezifischer Inhibitor des EGFR, bereits unter basalen Bedingungen eine fördernde Wirkung auf die ROS-Produktion und über diesen Signalweg eine reduzierende Wirkung auf die Größe eines Herzinfarktes. In einem Typ1-Diabetes Modell für Mäuse ist die Phosphorylierung des EGFR im Herzen erhöht, wird dieser inhibiert so ist die Bildung von Peroxynitrit und 3-Nitrotyrosin und die Diabetes-assoziierte Kardiomyopythie reduziert[98,99].

Herzklappen

Fehlbildungen der Herzklappen sind unter den kardiovaskulären Fehlbildungen die am häufigsten vorkommenden (25-30% der kardiovaskulären Fehlbildungen)[113]. Bei der Bildung der kardialen Polster kommt es zu einer endothelialen-mesenchymalen Transdifferenzierung einer Endothelzell-Subpopulation. Diese wandern in das kardiale Gel ein, vermehren sich dort und wandeln sich zu mesenchymalen Zellen[113]. Nach Aktivierung, vermutlich durch HB-EGF, reduziert der EGFR die mesenchymale Zellproliferation[113,114]. Bei ersten Untersuchungen zur Bedeutung des EGFR im Herzen, wurden besonders die Herzklappen betrachtet, da hier bereits der Einfluss von Neuregulin-1 und anderen ErbB-Rezeptoren beschrieben worden war. *Wa2*-Mäuse weisen eine Verdickung der Semilunarklappen und eine Aortenstenose auf[73,78], genauso wie Mäuse mit genetischem Knockout des EGFR[73]. Bei der *Wa2*-Maus konnte ein erhöhter Druckgradient über die Aortenklappe nachgewiesen werden[103]. Die Verdickung der Klappen wird verursacht durch eine erhöhte Zellproliferation, Ablagerung von Glycosaminoglykanen und Kollagenen, sowie eine erhöhte Kalzifizierung und inflammatorische Infiltrate in den Aortenklappen[103]. Interessanterweise ist bei *Wa-2* Mäusen der Transkriptlevel (mRNA) des EGFR höher als bei Wildtyp Mäusen[103]. Es konnte zwar eine Korrelation zwischen dem Herzgewicht und der Dicke der Aortenklappen festgestellt werden, aber nicht alle Tiere mit vergrößerten Aortenklappen wiesen auch ein erhöhtes Herzgewicht auf[103]. Dies deutet daraufhin, das evtl. noch ein weiterer Mechanismus zu der Herzhypertrophie durch eine reduzierte EGFR-Aktivität beiträgt. Auch bei adulten Mäusen führt die Hemmung des EGFR zu Veränderungen in der Morphologie von Herzklappen, insbesondere der Aortenklappe[13].

Ischämie-Reperfusion

Durch die Transaktivierung des EGFR fördern β-adrenerge Rezeptoren die Phosphorylierung von Akt und ERK 1/2. Beide Signalwege verhindern das Absterben von Kardiomyozyten und schützen damit vor Ischämie/Reperfusionsschäden[112,115-118]. Neben dem β-adrenergen Signalweg sollen auch weitere GPCRs über diesen Mechanismus den

Schutz des Herzens aktivieren[97]. Welche G-Protein gekoppelte Rezeptoren diesen Effekt ebenfalls ausüben, ist jedoch noch nicht vollständig verstanden.
In der Summe scheint der EGFR im Herzen sowohl eine protektive als auch eine schädigende Funktion zu besitzen. Was zu der Überlegung geführt hat, dass der EGFR für die chronischen Veränderungen bei Erkrankungen eher schädlich ist, während er in akuten Prozessen eher eine positive Wirkung ausübt[98].

2.3.2. Isolierte Kardiomyozyten

Obwohl die Herzmuskelzellen (Kardiomyozyten) verantwortlich für die Kontraktion des Herzens sind, stellen sie doch nur ca. 56% der Zellen im Herzen der Maus und sogar nur 28% beim Menschen[119]. Die restlichen Zellen sind hauptsächlich Fibroblasten[119]. Die kardiale Fibrose, also die Zunahme a) entweder der Anzahl der Fibroblasten oder b) der extrazellulären Matrix, kann durch Herzhypertrophie induziert werden. Es mehren sich jedoch die Hinweise, dass ein umgekehrter Zusammenhang ebenfalls möglich ist, nämlich dass die Herzhypertrophie eine Folge der kardialen Fibrose sein kann. So konnte bei Patienten mit einer Mutation in Proteinen des kardialen Sarkomers eine erhöhte Serumkonzentration des C-terminalen Propeptids des Prokollagens Typ I nachgewiesen werden, ohne dass bereits eine Herzhypertrophie vorhanden war[119].

Transaktivierung

In Kardiomyozyten wurde die Transaktivierung des EGFR unter anderem für Angiotensin II[60,120,121], Phenylephrin[60,122,123], Isoprenalin[97], Endothelin-1[42,124], Acetylcholin[125], Glukose[98] und Dehnung[124] gezeigt. Auch PAR4 scheint einen Teil seiner Wirkung durch die Aktivierung des EGFR zu vermitteln[80].
Die Transaktivierung in Kardiomyozyten kann durch den „Triple-membrane spanning"-Mechanismus erfolgen[121], ob die Transaktivierung durch G_{α}- oder durch $G_{\beta\gamma}$-Untereinheiten[60] vermittelt wird, ist noch nicht geklärt. Die Aktivierung von NADPH-Oxidasen (NOX) bei der Transaktivierung des EGFR ist inzwischen als gesichert anzunehmen. Es ist bisher noch unklar, ob diese den EGFR aktivieren[121] oder ob sie durch den EGFR aktiviert werden[98,126]. So führte die Transaktivierung des EGFR durch Inkubation mit einem Hochglukose-Medium zu einem Anstieg der Expression und Aktivität der NOX-2 und -4 in H9C2-Zellen und neonatalen Rattenkardiomyozyten[98]. Aber auch andere Signalwege sind beschrieben, so scheint Endothelin-1 in Kardiomyozyten den EGFR über die Proteinkinase C zu aktivieren, in Konsequenz der EGFR-Aktivierung wird die Aktivität der MAP-Kinasen und von c-fos gesteigert[42]. Im Gegensatz dazu scheint für die Isoprenalin-induzierte Transaktivierung die Internalisierung des EGFR für die Signalweiterleitung von entscheidender Bedeutung zu sein[97]. Hier konnte eine EGFR-abhängige Aktivierung sowohl von Akt als auch von ERK nachgewiesen werden.
In einer interessanten Studie von Kleine-Brueggeny *et al*[122] konnte gezeigt werden, dass abhängig vom zellulären Phänotyp (Kardiomyozyten- vs. Fibroblasten-ähnliche H9C2

Zellen) die Transaktivierung des EGFR über unterschiedliche Signalwege erfolgt. Während diese Transaktivierung in Kardiomyozyten-ähnlichen Zellen über MMPs vermittelt wird, ist die Transaktivierung des EGFR in Fibroblasten-ähnlichen Zellen nicht von MMPs abhängig. Diese Arbeitsgruppe zeigt das auch die Liganden-unabhängige Transaktivierung über c-Src erfolgen kann. Diese Studie könnte einige der Diskrepanzen in den bisherigen Berichten über die Mechanismen der EGFR-Transaktivierung erklären.
Die Konsequenz der Transaktivierung des EGFR in Kardiomyozyten ist noch nicht ausreichend untersucht. In neonatalen Kardiomyozyten der Ratte führt Dehnung über einen „Triple-membrane-spanning"-Mechanismus zur Transaktivierung des EGFR und damit zu einer gesteigerten Promotoraktivität von BNP. Vermittelt wird dieser Effekt durch eine dehnungsabhängig gesteigerte, NADPH-Oxidasen-abhängige ROS-Synthese, die zu einer erhöhten Aktivität des Endothelin-Promotors führt[124]. Eine andere Studie konnte zeigen, das die Transaktivierung des EGFR in neonatalen Rattenkardiomyozyten die Endothelin-1-abhängige Transkription von c-fos steigert, nicht jedoch die, ebenfalls Endothelin-1-abhängige, Transkription von c-Jun[127].
Zusammenfassend lässt sich feststellen, das es weiterer Untersuchungen bedarf die sowohl die Signalwege als auch die Folgen der Transaktivierung des EGFR in Kardiomyozyten abklären.

Hypertrophie

Die Bedeutung des EGFR für die Hypertrophie von Kardiomyozyten wurde *ex vivo* bzw. *in vitro* bisher weitest gehend an neonatalen Rattenkardiomyozyten untersucht. Allerdings ist auch für diesen Parameter noch keine abschliessende Beurteilung der physiologischen bzw. pathophysiologischen Bedeutung möglich. So konnte durch Überexpression des EGFR mit Hilfe eines viralen Vektors eine Zunahme der Zellgröße induziert werden[128]. Vermutlich wird der Effekt durch die auto- bzw. parakrine Freisetzung von EGFR-Liganden vermittelt.
Die Transaktivierung des EGFR durch Angiotensin II führt, neben der Zunahme der Zellgröße, zu einer Zunahme der Expression von BNP und der fetalen Form der schweren Kette des Myosins[121]. Die Aktivierung des α1-adrenergen Rezeptors verursacht eine Zunahme der Proteinsynthese sowie der Zelloberfläche durch die Aktivierung von STAT3. Diese STAT3 Aktivierung wird durch den Gαq/PKC/MMP/HB-EGF/EGFR-Signalweg vermittelt[129].

Zelltod

Isoprenalin reduziert durch Transaktivierung des EGFR in neonatalen Rattenkardiomyozyten die Apoptose, vermittelt wird dieser Effekt durch die Aktivierung von MEK1/2 und die dadurch verstärkte Transkription von TRAIL (Tnf-related apoptosis inducing ligand)[97]. Exogenes TRAIL selber erhöht ebenfalls die Apoptose von neonatalen Rattenkardiomyozyten.

Differenzierung von Kardiomyozyten

Der EGFR scheint einen Teil der Wirkung von Neuregulin-1 auf die Differenzierung von kardialen, embryonalen Stammzellen zu vermitteln. So kommt es durch Gabe von Neuregulin 1 zu einer erhöhten Expression von Herz-spezifischen Transkriptionsfaktoren, sowie von MLC-2v (ventrikuläre, leichte Kette des Myosins, Typ 2) und dem atrialen, natriuretischen Peptid (ANP), zwei Markern für das Arbeitsmyokard des Herzens. Zudem wird vermehrt α-Aktin exprimiert, ein genereller Marker für das Herz. Die Wirkung von Neuregulin-1 auf die Expression von MLC-2v und ANP war mit AG1478 zu verhindern, im Gegenzug erhöhte sich die Expression von Markern des Erregungsleitungssystems[130].

2.3.3. Kardiale Fibroblasten

Über die kardialen Fibroblasten ist, bezüglich des EGFR bzw. seiner Liganden, noch wenig bekannt. Das bisherige Augenmerk wurde weitgehend auf die Kardiomyozyten gerichtet, da diese für die Funktion des Herzens wichtiger zu sein scheinen.

Die Entwicklung einer kardialen Dysfunktion in hypertensiven Herzen wird immer von einer exzessiven kardialen Fibrose begleitet[131]. In kardialen Fibroblasten induziert Endothelin-1 die Transaktivierung des EGFR durch eine ROS-vermittelte HB-EGF Freisetzung. Diese EGFR Transaktivierung wird durch die Endothelin-1 vermittelte Aktivierung von Shp-2, einer Phosphatase, attenuiert[132].

Auch bei der Isoprenalin-induzierten Herzhypertrophie sind kardiale Fibroblasten involviert, so sind sie für die Sekretion von verschiedenen Zytokinen z.B. die CC-Chemokin Liganden (Ccl)-2, -6, -20, IL2, IL17a oder TNF-α (Tumor-necrosis factor) verantwortlich. Dies kann durch Gefitinib gehemmt werden[105].

2.4. Bedeutung des EGFR für physiologische und pathophysiologische Prozesse in Blutgefäßen *in vivo*

2.4.1. Blutgefäße *in toto*

Transaktivierung

Auch an Blutgefäßen *in vivo* sowie *ex vivo* konnte die Transaktivierung der EGFR nachgewiesen werden. Er soll am vaskulären Umbau beteiligt sein und damit zur Entstehung von Bluthochdruck beitragen. Die Beeinflussung von Arteriogenese, Angiogenese, Restenose[133,134], Atherosklerose, vaskulärer Dysfunktion[135] und der vaskulären Fibrose wurden bereits untersucht. Zudem wird der EGFR für einen Teil der Gefäßveränderungen durch Diabetes mellitus[136] bzw. im Alter[137] mit verantwortlich gemacht. Unter anderem ist für folgende Faktoren eine Transaktivierung des EGFR beschrieben: Angiotensin II[93,94,138-140], Endothelin-1, adrenerge-Agonisten, Mineralocorticoide[141], Leptin[142], Dehnung[143-145], Schubspannung[133] und Flavone[146].

Allerdings wurde auch hier, wie bereits für das Herz erwähnt, hauptsächlich durch pharmakologische Inhibition der Beitrag des EGFR für die Veränderungen der Blutgefäße

untersucht. Damit ist es nicht möglich zu unterscheiden, ob z.B. eine verringerte blutdrucksteigernde Wirkung des Angiotensin II bei Hemmung des EGFR[147] aus einer reduzierten Kontraktion der VSMC resultiert oder durch eine erhöhte NO-Synthese des Endothels verursacht wird.

Gefäßumbau (Remodeling)

Unter Ruhebedingungen scheint der EGFR lediglich einen geringen Effekt auf die Gefäßstruktur zu haben, da bei Mäusen mit einem hypomorphen EGFR keine makro- oder mikroskopischen Unterschiede beobachtet werden konnten[104]. Der vaskuläre Gefäßumbau kann in zwei Hauptformen auftreten. Zum einen als einwärtsgerichtetes, eutrophes Remodeling, bei dem sich der äußere Durchmesser sowie der Lumen-Durchmesser verkleinern, während die Querschnittsfläche der Gefäße annähernd gleich bleibt und das Media/Lumen-Verhältnis sich vergrößert. Zum anderen kann auch hypertrophes Remodeling auftreten, hierbei kommt es zu einem Wachstum der Media durch die das Lumen der Blutgefäße ab und das Media/Lumen-Verhältnis zu nimmt[148]. Welchen Einfluss der EGFR auf die zwei unterschiedlichen Arten des Gefäßumbaus hat ist noch nicht abschließend geklärt. An der A. carotis communis konnte gezeigt werden, dass der Gefäßlumen-verkleinernde Gefäßumbau (inward remodeling) durch Hemmung des EGFR, entweder über AG1478[133] oder einen inhibitorischen Antikörper[134], verringert werden kann. Hierbei reduziert die Hemmung des EGFR die Intimahyperplasie, die Lumenverringerung und erhöht die Reendothelialisierung nach Ballonkatheterisierung[134]. Wird die A. carotis abgebunden, so induziert die Reduktion der Schubspannung die Aktivierung des EGFR[133]. Vermittelt wird dies durch eine Liganden-abhängige Transaktivierung des EGFR mit Aktivierung von ADAM-17[133,134]. Dies wird durch eine Arbeit bestätigt, in der ADAM-17 durch ein dominant-negatives Adenovirus gehemmt wurde. Diese Hemmung führt zu einer verringerten Intimahyperplasie[149].

In den meisten Untersuchungen wird jedoch nicht zwischen den zwei Arten des Gefäßumbaus unterschieden. So beschreiben Ivanov *et al*[150] lediglich, das die Aktivierung des EGFR durch Infusion von EGF über 1 bzw. 3 Tage eine Proliferation von VSMC in der Basilararterie von Ratten induziert. Dieser EGFR-Effekt scheint durch den Calcium-abhängigen Big K^+-Kanal vermittelt zu werden[150].

Inhibition der NO-Synthasen induziert in Nieren von Ratten eine Zunahme der Wanddicke und des Wand/Lumen-Verhältnisses in afferenten Arteriolen. Dieser Umbauprozess kann durch Inhibition des EGFR mit Gefitinib verhindert werden[135]. Allerdings reduziert die Behandlung auch den mittleren arteriellen Blutdruck, daher ist nicht auszuschliessen, dass hier ein Teil der protektiven Wirkung der EGFR-Inhibition alleine durch die Senkung des Blutdruckes bedingt ist.

Fibrose

Die Bedeutung des EGFR in der Entwicklung einer perivaskulären Fibrose ist noch weitgehend unklar. Während Barrick *et al*[103] eine verstärkte perivaskuläre Fibrose bei gestörter Rezeptortyrosinkinaseaktivität beobachten, finden Griol-Charhbili *et al*[104] keinen Unterschied in dem Elastin/Kollagen-Verhältnis in den Blutgefäßen und auch die Expression der Kollagene 1a, 3a, 4a, Fibronectin, Fibrillin-1, Fibulin-1 und Fibulin 5 ist nicht unterschiedlich zwischen Kontrolltieren und *Wa-2*-Tieren.

Werden Wa-2 Mäuse mit Aldosteron/Salz bei gleichzeitiger 5/6-Nephrektomie behandelt, so steigt die Expression von Kollagen1a und 3a. Dieser Effekt fehlt in Kontrollmäusen[104]. Dies deutet daraufhin, dass der EGFR an dieser Stelle einen protektiven Effekt vor Fibrose bietet.

Blutdruck

Die physiologische Bedeutung des EGFR für die Aufrechterhaltung des Blutdruckes ist bisher noch unklar. Bei *Wa-2*-Mäusen zeigt sich kein Unterschied im systolischen Blutdruck im Vergleich zu Wildtyptieren[103,104]. Auch die Gabe von AG1478 über fünf Stunden verändert weder den systolischen, diastolischen noch mittleren Blutdruck bei Ratten[147]. Aus diesen Befunden ergeben sich jedoch keine ausreichenden Daten für die Beurteilung der Gefäßfunktion und damit für die Bedeutung des EGFR im Rahmen der Entstehung des Bluthochdruckes. So wird zunächst der systolische Blutdruck hauptsächlich durch die Funktion des Herzens bestimmt. Daher sind Messungen des diastolischen und mittleren arteriellen Blutdrucks notwendig, um die Bedeutung des EGFR für die Gefäßfunktion in Ruhe abschätzen zu können. Hierbei ist es auch wünschenswert, einen längeren Zeitraum der EGFR-Inhibiton zu beobachten, um langfristige Effekte des EGFR auf die Proteine der kontraktilen Maschinerie untersuchen zu können. Weiterhin ist zu beachten, dass die durch die Blutgefäße aufgebaute Spannung aus einem Gleichgewicht zwischen vasokonstriktorischen und vasodilatatorischen Substanzen resultiert. So kann ein Bluthochdruck z.B. durch eine reduzierte endotheliale NO-Synthese oder durch eine erhöhte Angiotensin II-Aktivität induziert werden, aber auch eine Kombination von beiden Effekten ist möglich. Daher kann aus einer Messung des Ruheblutdruckes keine Aussage getroffen werden, ob die Blutgefäße im physiologischen Regelbereich noch voll funktionstüchtig sind.

Modelle die die pathophysiologische Bedeutung des EGFR untersuchen, weisen eine erhöhte Expression des EGFR in den Aorten von hypertensiven Ratten nach[94,139,140,151]. Hierbei ist zu beachten, dass Kim *et al*[152] keine verstärkte Aktivierung des EGFR bei hypertensiven Ratten beobachten konnten. Im Gegensatz dazu konnte bei Nagern bereits mehrfach gezeigt werden, das die Inhibition des EGFR den Blutdruck in verschiedenen Bluthochdruck-Modellen senkt, z.B. bei spontan hypertensiven Ratten[94], nach 5/6-Nephrektomie[93] oder in Modellen mit erhöhtem Blutdruck durch Mineralocorticoide[104,141], Phenylephrin[153,154] oder Leptin[142]. Interessanterweise kontrahieren mesenteriale

Blutgefäße von Ratten, denen 5/6 ihrer Nieren entfernt wurden, bei Stimulation sowohl mit Angiotensin II als auch mit Phenylephrin schwächer. Dies kann unter anderem durch die Gabe von PKI-166, einem EGFR-Inhibitor, verhindert werden. Die Endothel-abhängige Vasodilatation ist in diesem Modell nicht verändert, während der Endorganschaden der Niere sogar gesteigert ist[93].

Einer der *in vivo* am besten untersuchten Effekte des EGFR ist seine Wirkung auf den Blutdruck-steigernden-Effekt von Angiotensin II, aber selbst hier sind die Ergebnisse uneinheitlich. Die Infusion von Angiotensin II erhöht die Phosphorylierung des EGFR in Aorten von Ratten[152] und die Hemmung des EGFR reduziert die durch Angiotensin II-induzierte Kraftentwicklung in Aortenstreifen um ~40%[155]. Auch kann eine Reduktion des Blutdruckanstiegs durch Angiotensin II bei Gabe von AG1478 in Ratten erzielt werden[147]. Andererseits konnten Chan *et al*[147] weder bei genetischer Mutation (*Wa-2*) noch bei Inhibition des EGFR (AG1478) einen Effekt auf die durch Angiotensin II induzierte Hypertension beobachten[147]. Auch die Kontraktion von Aortenringen[104] von Mäusen bzw. Koronargefäßen von Menschen[156] ist bei Stimulation mit Angiotensin II nicht durch den EGFR beeinflusst.

Ein weiterer relevanter Aspekt der Bedeutung des EGFR im Rahmen der Enstehung eines Bluthochdruckes ist, das der aktivierte Mineralocorticoidrezeptor die Expression des EGFR steigern kann[157]. Damit werden die Blutgefäße für die Transaktivierung des EGFR sensibilisiert und es kommt im Organbad zu einer verstärkten Kontraktion von Blutgefäßen bei Applikation von EGF[158], Angiotensin II[104], Endothelin-1 und adrenergen Substanzen[104,141]. Die Endothel-abhängige Vasorelaxation hingegen wird reduziert, d.h. durch die Behandlung wird eine endotheliale Dysfunktion induziert[104,141]. An humanen, koronaren Mikroarterien konnte *ex vivo* demonstriert werden, dass in Anwesenheit von Aldosteron die Angiotensin II-induzierte Vasokonstriktion vollständig von dem EGFR abhängig ist[156].

Die blutdruckmodulierende Wirkung der EGFR-Liganden ist bisher hauptsächlich für EGF und TGF-α untersucht. So führt EGF selber zu einem Anstieg des mittleren arteriellen Blutdruckes um 6-8 mmHg[159], hat aber *ex vivo* keinen Effekt auf die Kontraktion von Aortenringen der Ratte[158]. Wird es vor Noradrenalin infundiert, so kann es die Effekte des Noradrenalins reduzieren[159]. Wird es nach der Behandlung mit einem Mineralocorticoid auf Aortenringe der Ratte appliziert, kann es selber eine Kontraktion auslösen[158]. Dies könnte damit zusammenhängen, dass der Mineralocorticoidrezeptor die Expression des EGFR steigert[160].

Ob TGF-α selber eine blutdrucksteigernde Wirkung hat ist meines Wissens bisher nicht untersucht worden. Es konnte jedoch gezeigt werden, das die loss-of-function Mutation von TGF-α (*Wa-1*) den Angiotensin II-induzierten Blutdruckanstieg nicht beeinflusst[161]. Allerdings bleiben bei *Wa-1*-Tieren die Aktivierung von NFκB in Blutgefäßen und die Zunahme der Wanddicke bzw. der vaskulären Zellzahl aus. Die Aktivierung von NFκB erfolgt durch eine NOX/ROS/ADAM-17-abhängige TGF-α Freisetzung. Weder die Deletion

von HB-EGF noch von Epiregulin in Blutgefäßen konnte die Aktivierung von NFκB verhindern[161]. Diese Befunde lassen es möglich erscheinen, dass durch verschiedene EGFR-Liganden unterschiedliche Aspekte der Angiotensin II-Wirkung vermittelt werden.
In der Summe scheint der EGFR bzw. seine Liganden einen geringen bis keinen Effekt auf den Ruheblutdruck zu haben. Bei pathologischen Blutdruckerhöhungen hingegen scheint der EGFR jedoch vermehrt exprimiert bzw. aktiviert zu werden und in der Konsquenz für die blutdrucksteigernde Wirkung verschiedener vasoaktiver Substanzen mit verantwortlich zu sein. In welchen vaskulären Zellen der EGFR *in vivo* den Hauptteil seiner Wirkung vermittelt, ist noch unklar. Da sich Blutgefäße funktionell stark unterscheiden ist des weiteren zu klären, ob der EGFR seine Wirkung im Rahmen des Bluthochdruckes hauptsächlich in großen Leitarterien entfaltet, oder auch in kleineren Widerstandsgefäßen.

Myogener Tonus

Die Druck-induzierte Vasokonstriktion ist eine physiologisch höchstrelevante Reaktion in vielen kleinsten Arterien und Arteriolen. Die Funktion dieser Reaktion ist es bei steigendem Blutdruck die Perfusion des nachgeschalteten Gewebes annähernd konstant zu halten. Zusätzlich wird es damit den Widerstandsgefäßen ermöglicht, auf vasoaktive Substanzen zu reagieren. Die myogene Reaktion erfolgt unabhängig vom Endothel oder von perivaskulären Nerven und ist damit eine spezifische Eigenschaft der glatten Muskelzellen[145,162-164]. Neben anderen Faktoren ist der myogene Tonus abhängig vom EGFR[143-145]. Die Druck-induzierte Vasokonstriktion wird hierbei vermittelt durch eine Aktivierung von MMP-2 bzw. -9, die Freisetzung von HB-EGF[144]. Durch die schrittweise Erhöhung des Blutdrucks kommt es in koronaren Arteriolen zu einer Erhöhung des koronaren Tonus. Dies kann durch die Gabe von EGFR-Inhibitoren verhindert werden[145]. Auch die bei höherem Druck induzierte Phosphorylierung von JAK und STAT in Koronararterien ist abhängig von der Aktivierung des EGFR[145].

Arteriogenese

Im adulten Organismus kommt es zur Neubildung von arteriellen Blutgefäßen hauptsächlich im Rahmen der Wundheilung. Hierbei werden zwei unterschiedliche Konzepte diskutiert, wie diese Blutgefäße entstehen könnten, zum einen durch „de-novo" Bildung, d.h. es bilden sich vollständig neue Arterien, zum anderen durch den Umbau von Kapillaren in Arterien, d.h. bereits vorhandene Blutgefäße passen sich an den gesteigerten Bedarf an[165]. Im Modell der Hinterlaufischämie wird die A. femoralis eines Beines abgebunden. Hierbei zeigt sich, dass die Phosphorylierung des EGFR in Blutgefäßen des ligierten Beines im Vergleich zum unbehandelten Bein zunimmt[166]. Die genetische Deletion des HB-EGF reduziert die Phosphorylierung des EGFR in diesem Modell, führt aber nicht zu einer Veränderung in der Kollateralenbildung[166]. Auch in einem zweiten Modell der pathologischen Arteriogenese ist der EGFR in die Gefäßumbildung involviert. So kann durch Applikation von Erlotinib in den Glaskörper von neonatalen Mäusen die

Revaskularisierung nach Exposition mit erhöhten Sauerstoffkonzentrationen zwar nicht beeinflusst werden, aber die Anzahl der vaskulären Gefäßknäule wird reduziert[167].
Unter pathophysiologischen Bedingungen, nicht jedoch unter physiologischen Bedingungen, fördert ADAM-17 die Neovaskularisierung unter anderem über die Steigerung der Proliferation der Endothelzellen[168]. Der Phänotyp kann *in vitro* durch Gabe von HB-EGF gerettet werden. Dies spricht dafür, dass der EGFR diesen Effekt mitvermittelt.

Atherosklerose

In humanen, atherosklerotischen Plaques ist die Expression des EGFR, HB-EGF, Betacellulin und Epiregulin erhöht[169-171], ähnliches kann in Carotiden von Makaken nach einer mehrjährigen Fütterung mit fettreicher Nahrung beobachtet werden[169]. Auch die Aktivierung des EGFR in atherosklerotischen Plaques ist gesteigert, was den Schluss nahelegt, dass es durch die erhöhte Expression der Liganden zu einer verstärkten Aktivierung des EGFR kommt mit nachfolgender Stimulation von Akt und ERK[169]. Neben der Aktivierung dieser zwei Signalwege scheint der EGFR auch die Aktivität von Merpin-α zu erhöhen[172]. Merpine sind Membran-gebundene Metalloproteasen die in die Entstehung von entzündlichen Veränderungen involviert sind. Die Autoren zeigen, dass es durch die Inkubation von Makrophagen mit oxidiertem Low-density Lipoproteinen zu einer erhöhten Merpin-α-Aktivität kommt. Diese führt zu einer Freisetzung von HB-EGF von der Membran der Makrophagen und im Anschluss zu einer erhöhten Phosphorylierung des EGFR. Durch die Aktivierung des PI3K-Akt-Rac1-p38-Signalweges kommt es zu einer Stimulation der ROS-Synthese[172]. Die Bedeutung des ErbB-Signalweges in den einzelnen Zellen wurde noch nicht im Rahmen der Atherosklerose untersucht.

Vaskuläre Dysfunktion

Bei Mäusen mit einem hypomorphen EGFR (*Wa-2*) ist die endothel-abhängige Vasodilatation von abdominellen Aortenringen leicht verringert[104]. Die Endothel-unabhängige Vasorelaxation hingegen ist unverändert, was darauf schliessen lässt, dass die Veränderung der Relaxation durch Veränderungen in den Endothelzellen bedingt ist. Dies könnte evtl. durch die verringerte Expression der eNOS in diesen Tieren erklärt werden[104]. Dieser Befund konnte in dem pathophysiologisch relevanten Modell der Aldosteron/Salz-Behandlung in Kombination mit 5/6-Nephrektomie reproduziert werden[104]. Interessanterweise sensitivierte diese Behandlung die Blutgefäße für Phenylephrin und Angiotensin II abhängig vom EGFR[173]. Diese Ergebnisse sind zumindest zum Teil gegensätzlich zu Befunden von Ulu *et al*[93]. Hier erfolgte eine Desensitivierung der thorakalen Aorta durch 5/6-Nephrektomie für Phenylephrin und Angiotensin II abhängig vom EGFR.
Über die Funktion der einzelnen Liganden der ErbB-Rezeptorfamilie für die vaskuläre Dysfunktion ist noch so gut wie nichts bekannt. Es konnte allerdings gezeigt werden, dass

die Injektion von Betacellulin in den Glaskörper zu einer erhöhten Permeabilität der Gefäße der Retina führt[174].

Diabetes

Diabetes mellitus kann Veränderungen an den Blutgefäßen hervorrufen, wie z.B. veränderte Reaktivität, Hypertension, Atherosklerose, Mikro-Angiopathie und Herzversagen[136]. Das erhöhte Ansprechen auf Vasokonstriktoren und die reduzierte Vasodilatation sind typische, vaskuläre Konsequenzen des Diabetes mellitus[136]. Die zugrundeliegenden Mechanismen sind jedoch noch nicht vollständig aufgeklärt.

Bei Ratten mit Typ1-Diabetes kommt es zu einer erhöhten Phosphorylierung des EGFR in mesenterialen Gefäßen die zur Aktivierung von ERK1/2 und p38 (p38-mitogenaktivierte Proteinkinase) führt[47]. Die Auswirkungen dieser erhöhten EGFR-Phosphorylierung sind bisher noch nicht genau untersucht worden. So hat die Gabe von AG1478 bei Mäusen mit Spontanmutation eines Leptinrezeptors keine Auswirkung auf den Blutdruck, die Blutglukosekonzentration oder die Insulinresistenz, normalisiert aber den erhöhten myogenen Tonus[162]. Auch bei Ratten mit Streptozocin-induziertem Typ1-Diabetes verändert AG1478 nicht die Blutglukosekonzentration[136], verhindert aber die erhöhte die Vasokonstriktion in mesenterialen Blutgefäßen durch Angiotensin II oder Endothelin-1.

2.4.2. Glatte Muskulatur

Wie bereits im vorhergehenden Abschnitt erläutert scheint der EGFR in die Entstehung verschiedener pathophysiologischer Veränderungen der Blutgefäße involviert zu sein. Auch konnte, hauptsächlich bei Nagetieren, eine erhöhte Phosphorylierung des EGFR in VSMC unter pathophysiologischen Bedingungen festgestellt werden, so zum Beispiel bei spontan hypertensiven Ratten[175] oder alten Ratten[137]. Da alle Mitglieder der ErbB-Familie und die meisten ihrer Liganden in VSMC exprimiert werden[37] wurden Untersuchungen in isolierten VSMC durchgeführt.

Signalweg

In VSMC ist durch die Stimulation des EGFR die Aktivierung der folgenden Proteine beschrieben: ERK 1/2[145,175-177], STAT3[145], JAK[145], PI3K[176], PLC-γ[178], GIT-1[178] und der leichten Kette des Myosins[145]. Allerdings ist zu beachten, dass sich VSMC in ihrer Ausstattung mit z.B. Rezeptoren oder Ionenkanälen unterscheiden, unter anderem abhängig von dem Kreislaufabschnitt aus dem sie stammen. So kann z.B. in VSMC von Widerstandsgefäßen eine Dehnungs-abhängige Vasokonstriktion induziert werden nicht jedoch in den großen Leitarterien. Dem Unterschied der VSMC abhängig von ihrem Herkunftsort wurde bisher wenig Beachtung geschenkt.

Transaktivierung

Zu den Substanzen die den EGFR in VSMC transaktivieren können gehören unter anderem Angiotensin II[46,177,179], Aldosteron[180], adrenerge Agonisten[153,154], Endothelin-1[181-183], ATP[184], Thrombin, Urokinase-Plasminogen-Aktivator[185], Oxyhämoglobin[186], Lipopolysaccharide[187], Leptin[188], Oxysterole[189], Lipoproteine, Ouabain[190], Sauerstoff[191] und Dehnung[51].

Sowohl die Liganden-abhängige als auch die Liganden-unabhängige Transaktivierung sind in VSMC beschrieben, nicht selten auch beide Wege für dieselbe Substanz. So soll Angiotensin II den EGFR zum einen Liganden-abhängig[179] aber auch Liganden-unabhängig[46,177] aktivieren. Ob die Aktivierung des EGFR in VSMC unterschiedlicher Arterien mit Hilfe des gleichen Mechanismus erfolgt ist bisher noch nicht untersucht worden. Der Ligand der die Transaktivierung des EGFR in den meisten Fällen vermittelt ist HB-EGF[153,154,185,186]. Aber auch die Transaktivierung des EGFR durch TGF-α[187] ist beschrieben worden. Freigesetzt werden die Liganden durch verschiedene MMPs oder ADAMs. Zum Beispiel aktiviert Urokinase-Plasminogen-Aktivator über $G_{\beta\gamma}$-Untereinheiten die ADAMs-9 und -10, die dann HB-EGF freisetzen[185]. Lipopolysaccharide hingegen fördern die Freisetzung von TGF-α durch die Aktivierung von ADAM-17[187]. Vermittelt werden die Effekte des transaktivierten EGFR in VSMC hauptsächlich durch ERK 1/2[153,154,189], Akt[153,154,189] und p38[153,154].

Neben den bereits beschriebenen Signalwegen der EGFR Transaktivierung durch die Aktivierung von c-Src oder Jak ist in VSMC auch die Aktivierung des EGFR durch die Veränderung von Ionenströmen beschrieben[51,181,182], wobei nicht-spannungsabhängige Ca^{2+}-Kanäle die entscheidende Rolle spielen. So führt mechanische Dehnung in VSMC zur Öffnung von Dehnungs-abhängigen Ca^{2+}-Kanälen und darüber zur Aktivierung des EGFR und ERK 1/2[51]. Auch Endothelin-1 induziert über die Aktivierung von Ca^{2+}-Kanälen eine EGFR-Transaktivierung[181,182].

Proliferation

Die Bedeutung des EGFR für die Proliferation der VSMC wurde bereits mehrfach beschrieben. Allerdings sind auch hier die zugrunde liegenden Signalwege noch nicht vollständig aufgeklärt. Der EGFR vermittelt neben anderen Rezeptortyrosinkinasen die Proliferation von VSMC der Pulmonalarterien[192] und könnte damit in die Entstehung des pulmonalen Bluthochdrucks involviert sein. Die Interpretation der publizierten Daten ist leider in vielen Fällen schwierig, da häufig die DNA-Synthese als Parameter für die Proliferation betrachtet wurde, nicht die Zellzahl selber.

Auch der EGFR-Ligand EGF stimuliert in VSMC der Ratte die Proliferation. Biochemisch erfolgt die Steigerung der Proliferation vermutlich durch die Synthese von Puterescine, Spermidine und Spermin, die für die Genexpression, Zellproliferation und weitere biologische Zellfunktionen eine entscheidende Bedeutung haben[193].

Der EGFR vermittelt auch die mitogene Wirkung von Angiotensin II[46,175], Endothelin-1[175], Oxysterolen[189], Lipoproteinen und Dehnung[51]. Ob der EGFR durch den „Triple-Membrane-Spanning"-Mechanismus[189] oder durch intrazellulärer Signalkaskaden[51,175] aktiviert wird ist noch nicht ausreichend untersucht. Auch die Frage, ob der Mechanismus der Transaktivierung abhängig von der stimulierenden Substanz ist, ist noch nicht beantwortet. Dem EGFR nachgeschaltet ist vermutlich ERK 1/2 sowie die PI3-Kinase, verantwortlich für die Induktion der Proliferation[46,51,175,189].

Migration

Die verstärkte Migration von VSMC ist ein Zeichen für die Änderung des Phänotyps von einer kontraktilen Gefäßmuskelzelle hin zu einer vermehrt synthetisierenden Gefäßmuskelzelle. Diese mesenchymale Transition ist in die Entstehung der Intimahyperplasie involviert. Es konnte gezeigt werden, dass ein Teil der in diesen Bereichen vorhandenen Schaumzellen nicht, wie lange angenommen, von Makrophagen abstammen, sondern tatsächlich von aus der Media eingewanderten VSMC[194]. Die Bedeutung des EGFR *per se* für die Migration von VSMC wurde bisher noch nicht untersucht. Die EGFR-Liganden HB-EGF, Epiregulin und Betacellulin, die in VSMC exprimiert werden, haben jedoch einen starken mitogenen und chemotaktischen Effekt[195]. Besser untersucht ist die Bedeutung des EGFR für die Förderung der Migration durch andere Substanzen. So konnte gezeigt werden, dass die Oxysterole 7-Ketocholesterol und Cholesterol-5α,6α-Epoxid die Einzelzellmigration von VSMC durch die Aktivierung des EGFR fördern[189]. Oxysterole erhöhen auch die Migration im Wundheilungsassay, ob dieser Effekt durch den EGFR vermittelt wird wurde jedoch nicht untersucht. Dies ist in sofern von Bedeutung, da die Einzelzellmigration und die Migration im Zellverbund von unterschiedlichen Signalwegen abhängig sind. Neben den Oxysterolen fördert auch Urokinase-Plasminogen-Aktivator, über Bindung an seinen Rezeptor und die Liganden-abhängige Transaktivierung des EGFR die Einzelzellmigration[185]. Interessanterweise scheinen hier die βγ-Untereinheiten der GPCRs die Aktivierung der MMPs zu vermitteln.

Kontraktion

Die Bedeutung des EGFR in VSMC für die Kontraktion wurde hauptsächlich *in vivo* bzw. *ex vivo* untersucht. Dies ist unter anderem darin begründet, dass a) die Messung der Kontraktion von einzelnen VSMC kaum möglich ist und b) VSMC in der Zellkultur ihren Phänotyp ändern können, dies könnte die gegensätzlichen Befunde erklären.

In VSMC kann der EGFR, neben anderen Signalwegen, auch die Phosphorylierung der leichten Kette des Myosins induzieren, was zu einer verstärkten Kontraktion führen könnte[145]. Auch scheint die Bindung von EGF, TGF-α oder HB-EGF an den EGFR die Adenylatzyklase-5 zu aktivieren und damit den Calcium-abhängigen Big K^+-Kanal. Dies bewirkt die Hyperpolarisation der VSMC[150], was die Kontraktion eher reduzieren würde.

Eine interessante Studie hat die Bedeutung des EGFR für die Kontraktion des Ductus arteriosus Botalli untersucht. Diese wird unter der Geburt durch die erhöhte Sauerstoffspannung im Blut ausgelöst. *In vitro* konnte gezeigt werden, dass die Kontraktion zumindest teilweise durch die Transaktivierung des EGFR über mitochondrial synthetisiertes Wasserstoffperoxid erfolgt[191].

Entzündung

Da der EGFR in Prozesse die das Zellüberleben beeinflussen involviert ist, sowie in Prozesse die die Fibrose fördern, liegt es nahe, dass seine Aktivierung auch Entzündungsprozesse beeinflussen könnte. Auf die Modulation von Immunzellen durch den EGFR soll in dieser Arbeit nicht näher eingegangen werden. Über seine Rolle in glatten Muskelzellen im Rahmen von Entzündungsprozessen ist wenig bekannt. So konnte in glatten Muskelzellen der Trachea durch Lipopolysaccharide eine ADAM-17/TGF-α/EGFR-abhängige Freisetzung von IL-8 nachgewiesen werden[187]. Im Rahmen einer Säure-induzierten Entzündung der Lunge hingegen führt die Aktivierung von ADAM-17 zur Freisetzung von Neuregulinen und damit zur Aktivierung des ErbB4-Signalweges[187].
Auch ein Einfluss der Transaktivierung des EGFR auf entzündliche Veränderungen in VSMC ist bereits beschrieben. So fördert der Mineralocorticoidrezeptor in VSMC die Expression von proinflammatorischen und profibrotischen Genen durch den Vermittlung des EGFR[137].

Arteriosklerose

In vivo ist bisher noch wenig bekannt über die Bedeutung des EGFR für die Entstehung und die Aufrechterhaltung der Arteriosklerose. Wie oben bereits zusammengefasst, konnte in einigen Modellen gezeigt werden, dass die Hemmung des EGFR *in vivo* zu einer Reduktion des Umbaus nach Gefäßwandschädigung führt. *In vitro* zeigte sich, dass die Oxysterole 7-Ketocholesterol und Cholesterol-5α,6α-Epoxid den EGFR in VSMC der Ratte durch die Freisetzung von Liganden für den EGFR transaktivieren[189]. Die Freisetzung der Liganden wird begleitet durch eine erhöhte Expression von MMP-2 und MMP-9. Durch die Aktivierung des EGFR kommt es zu einer verstärkten Proliferation und Migration der VSMC. Llao *et al*[189] beschreiben zwei Arten von Signalweiterleitung durch den EGFR, zum einen eine schnelle, kurz anhaltende Aktivierung des MAPK-Signalweg mit Phosphorylierung von ERK 1/2 und eine langsam einsetzende, länger anhaltende Aktivierung des PI3-Kinase- und mTOR-Signalweges. Welcher der Signalwege für die Veränderungen der VSMC verantwortlich ist, wird leider nicht weiter untersucht.

2.5. Zusammenfassende Betrachtung der Erkenntnisse zu der Bedeutung des EGFR im kardiovaskulären System.

Betrachtet man die hier zusammengetragenen Erkenntnisse, so ist das Wissen über die Funktion des EGFR im kardiovaskulären System noch unvollständig (s. **Abbildung 6**). So konnte im ganzen Herzen eine Reduktion der Apoptose durch die Aktivierung des EGFR beobachtet werden, betrachtet man aber Kardiomyozyten isoliert, so reduziert der EGFR das Zellüberleben. Auch der Beitrag des EGFR zur Entstehung einer Herzhypertrophie ist noch nicht vollständig geklärt. So kann bei einigen Mauslinien eine Hypertrophie des Herzens auf Grund morphologischer Veränderungen der Herzklappen beobachtet werden, bei anderen Mauslinien konnte dies nicht bestätigt werden. Wenig bekannt ist bisher auch über die Funktion des EGFR im Herzen unter pathophysiologischen Bedingungen, z.B. in Situationen mit Ischämie/Reperfusion oder mit einer erhöhten Aktivierung des Renin-Angiotensin-Aldosteron-Systems. Einzelne Studien haben sich zwar bereits mit diesen Fragestellungen beschäftigt, aber bisher ist noch keine Betrachtung der involvierten Zelltypen vorgenommen. Ähnliche Feststellungen lassen sich auch für die Blutgefäße treffen. Hier ist noch nicht ausreichend untersucht welche Bedeutung der EGFR für die morphologische und funktionelle Homöostase der Blutgefäße bzw. des daraus resultierenden Blutdruckes und Endorganschadens hat. Ein wenig besser untersucht ist die Bedeutung des EGFR unter Bedingungen eines Bluthochdruckes. Hier kann davon ausgegangen werden, dass er die Wirkung einzelner vasoaktiver Substanzen fördert. Welcher Zelltyp letztendlich für die Effekt verantwortlich ist konnte jedoch bisher noch nicht untersucht werden.

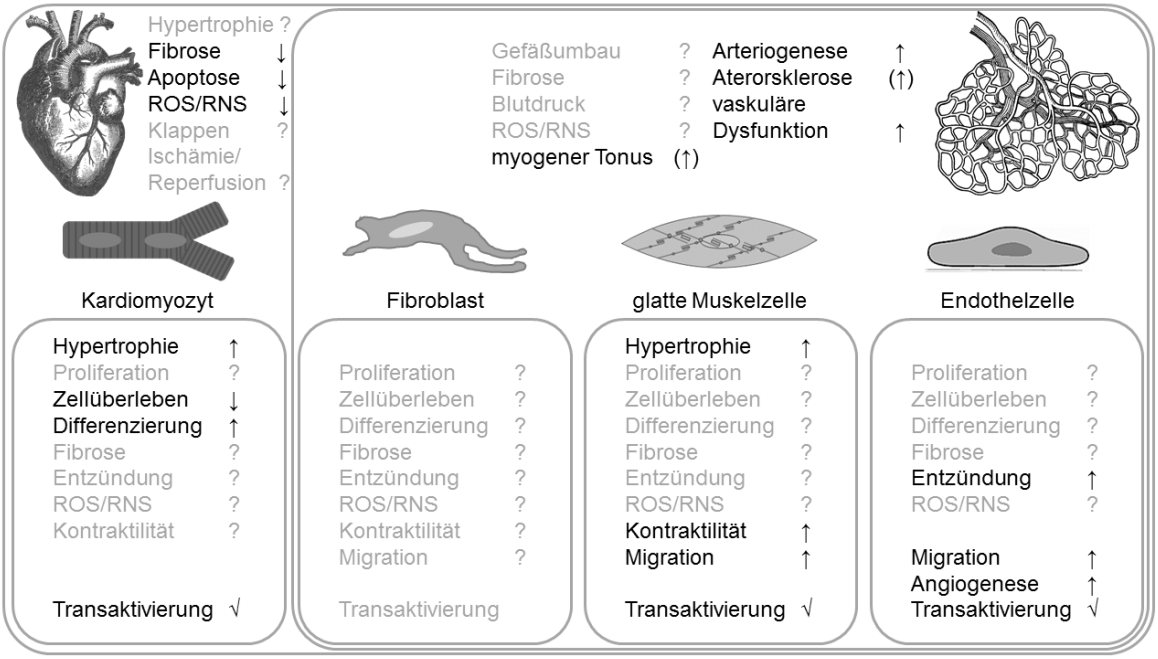

Abbildung 6: Physiologische und pathophysiologische Bedeutung des Epidermalen Wachstumsfaktorrezeptors für das Herzkreislaufsystem.

3. Zielstellung

Zusammenfassend ergeben sich die folgenden Fragestellungen für die hier vorliegende Arbeit (s.a. **Abbildung 6**):

I. Beeinflusst der EGFR in Kardiomyozyten die Funktion und die Morphologie des Herzens? Fördert er die Hypertrophie des Herzens? Kommt es durch Deletion des EGFR in Kardiomyozyten zu einer konzentrischen oder zu einer extzentrischen Herzhypertrophie?

II. Vermittelt der EGFR in Kardiomyozyten die durch Aldosteron induzierten pathophysiologischen Umbauprozesse im Herzen?

III. Welche Bedeutung hat der EGFR in VSMC für die physiologische Blutdruckhomöostase?

IV. Welche Bedeutung hat der EGFR für die blutdrucksteigernde Wirkung von Angiotensin II, Aldosteron, Endothelin-1, Adrenalin und Noradrenalin?

V. Vermittelt der EGFR in VSMC einen Teil der akuten Wirkung der oben genannten Substanzen auf die Blutgefäße? Fördert der EGFR die Kontraktilität der Blutgefäße und trägt damit z.B. zu akuten Blutdruckspitzen bei?

VI. Vermittelt der EGFR in VSMC einen Teil der chronischen Wirkung der oben genannten Substanzen auf die Blutgefäße? Verändert der EGFR die Zusammensetzung der Blutgefäße und damit z.B. deren Steifigkeit?

VII. Über welchen Mechanismus vermittelt der EGFR die veränderte Gefäßreaktivität?

VIII. Welche zellulären Veränderungen bewirkt der EGFR in VSMC? Bewirkt er eine vermehrte Proliferation? Verändert er die Anzahl der Zellen die sterben? Fördert er die Migration der VSMC? Wenn er die Migration der VSMC verändert, hat er hauptsächlich einen Einfluss auf die Einzelzellmigration oder doch eher auf die Migration im Zellverbund?

4. Zusammenfassung der eigenen Arbeiten

Ziel unserer Untersuchungen ist es die Bedeutung des Epidermalen Wachstumsfaktorezeptor im kardiovaskulären System sowohl *in vivo*, an der lebenden Maus, als auch *ex vivo*, an isolierten Geweben von der Maus, hier besonders an Blutgefäßen, und *in vitro*, in primären Zellen der Maus zu analysieren. Schwerpunkt war hierbei die Untersuchung der Auswirkungen der Deletion des EGFR in VSMC. Um dieses Ziel zu erreichen, wurden zwei Mausmodelle generiert bei denen der EGFR in VSMC und zum anderen in Kardiomyozyten konditional ausgeschaltet wurde. Hierfür haben wir das *Cre/LoxP*-System verwendet. Die konditionale Deletion des EGFR erfolgte in dem Mäuse,

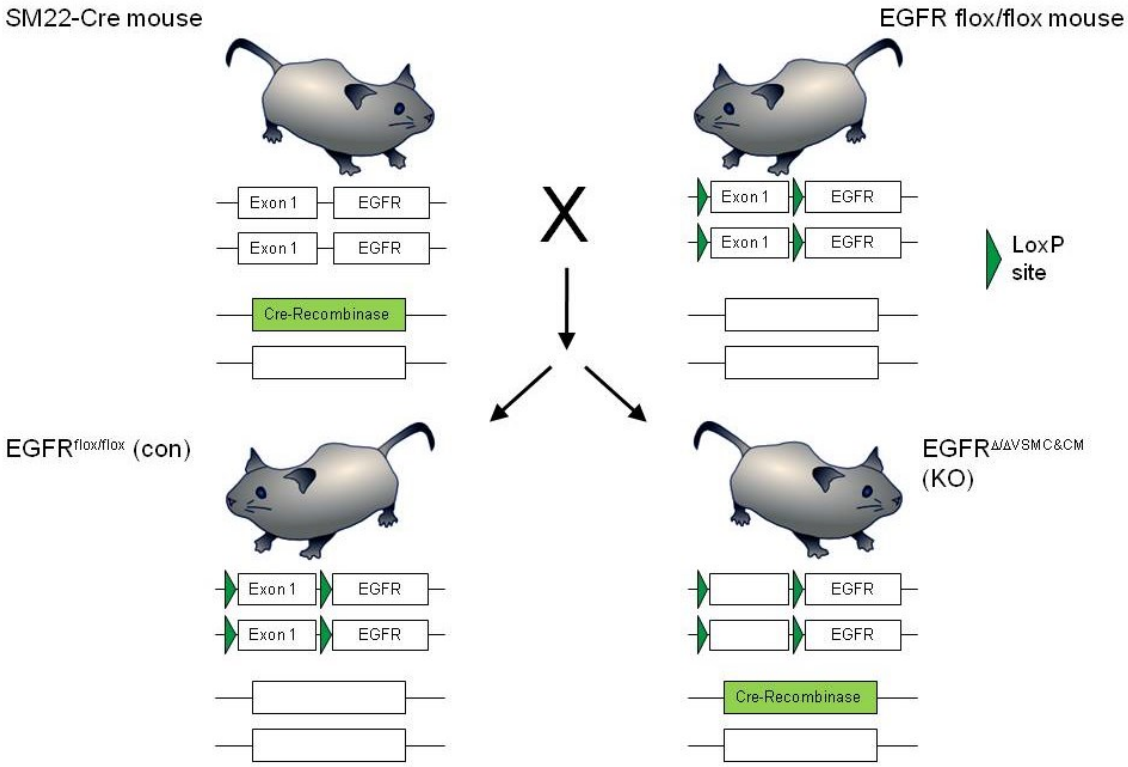

Abbildung 7: Verpaarungsschema für die EGFR$^{\Delta/\Delta VSMC\&CM}$-Maus. Für diese Arbeit wurden zwei Mauslinien generiert mit Deletion des EGFR in VSMC und starker Reduktion in Kardiomyozyten[1] und zum anderen eine Mauslinie mit induzierbarer Deletion des EGFR in VSMC (EGFR$^{\Delta/\Delta VSMC}$)[2]. Erreicht wurde dieses Ziel mit Hilfe des Cre/LoxP-Systems. Die Abbildung wurde Schreier *et al*[1] entnommen.

die die Cre-Rekombinase unter Kontrolle des SM22-Promotors tragen mit Mäusen verpaart wurden, bei denen auf DNA-Ebene das Exon 1 des EGFR von *LoxP*-sites flankiert wird (**Abbildung 7**). Die Deletion des EGFR erfolgt bereits während der Embryonalentwicklung. In dieser Zeit ist der Promotor auch im Herzen aktiv, so dass daraus neben der Deletion in den Blutgefäßen auch eine Deletion des EGFR im Herzen

resultiert. Mäuse dieser Linie werden im Folgenden als $EGFR^{\Delta/\Delta VSMC\&CM}$ bezeichnet. Glatte, aortale Gefäßmuskelzellen die aus diesen Tieren gewonnen wurden werden mit $VSMC^{\Delta/\Delta EGFR}$, bei Deletion des EGFR, oder $VSMC^{+/+EGFR}$, ohne Deletion des EGFR, bezeichnet. Für das zweite Mausmodell wurde eine induzierbare *Cre*-Rekombinase gewählt. Hier steht die *Cre*-Rekombinase unter Kontrolle des Smooth Muscle Aktin-Promotors. Zusätzlich wird die *Cre*-Rekombinase noch von zwei modifizierten Östrogenrezeptorbindungsdomänen flankiert. Damit erfolgt eine Translokation der *Cre*-Rekombinase in den Zellkern erst, wenn den Mäusen Tamoxifen zugeführt wird. Diese Mäuse werden im Folgenden als $EGFR^{\Delta/\Delta VSMC}$ bezeichnet. Der Zeitpunkt der Induktion ist bei den Mäusen frei wählbar. Wir haben damit ein Modell für die Deletion des EGFR generiert, das die embryonale Entwicklung mit umfaßt und ein Modell, bei dem eine Deletion erst im Adulten erfolgt. Letzteres würde der Situation eines Patienten entsprechen, der im Rahmen z.B. einer Tumortherapie einen EGFR-Inhibitor erhält. Die Ergebnisse *in vivo*, *ex vivo* und *in vitro* aus diesen zwei Modellen werden hier zusammenfassend präsentiert. Die Publikationen werden entsprechend der Reihenfolge *in vivo, ex vivo* und *in vitro* kurz besprochen.

A) Barbara Schreier, Sindy Rabe, Bettina Schneider, Maria Bretschneider, Sebastian Rupp, Stefanie Ruhs, Joachim Neumann, Uwe Rueckschloss, Maria Sibilia, Michael Gotthardt, Claudia Grossmann, Michael Gekle (2013) Loss of Epidermal Growth Factor Receptor in Vascular Smooth Muscle Cells and Cardiomyocytes Causes Arterial Hypotension and Cardiac Hypertrophy. Hypertension 61(2): 333-340.

In dieser Arbeit, die 2013 in Hypertension erschien, konnten wir zeigen, dass die Reduktion des EGFR im Herzen während der Embryonalentwicklung nicht zu einer fehlerhaften embryonalen Herzentwicklung oder -funktion führte und dass die Deletion des EGFR in VSMC keine Fehlfunktion der Plazenta erzeugte. Mäuse beider Genotypen wurden mit gleichem Gewicht und Größe geboren. Wir konnten zudem bestätigen, dass der EGFR für die Aufrechterhaltung der Herzfunktion und -gewebshomöostase notwendig war. Der partielle Verlust des EGFR in Kardiomyozyten führte zu einer exzentrischen Herzhypertrophie mit erhaltener systolischer Funktion in Abwesenheit von arterieller Hypertonie oder makroskopischer, kardialer Fibrose. Die fraktionelle Verkürzung, das Schlagvolumen und das Herzzeitvolumen waren bei den Knockout Tieren erhalten.
Die meisten KO-Tiere starben innerhalb der ersten 6 Monate, vermutlich weil die massive Herzhypertrophie die Entstehung von fatalen Herzrhythmusstörungen förderte. Im Gegensatz zu dem bei *Wa-2* Mäusen beobachteten Phänotyp konnten wir keine funktionell relevante Stenose der Aortenklappe beobachten. Dies schien darauf hinzudeuten, dass die Herzhypertrophie durch eine leichte tonische Dysbalance der kardialen ROS-Homöostase hervorgerufen wurde. Wir konnten eine Erhöhung der NOX-4 mRNA in den Herzen von KO-Tieren beobachten, genauso wie eine erhöhte, NADPH-Oxidasen-abhängige ROS-Produktion.
Der Blutdruck der Mäuse wurde sowohl mit Hilfe von Tailcuff-Messungen als auch invasisv mit Hilfe von Millar-Katheter-Messungen bestimmt. Während der systolische Blutdruck bei den Tieren unverändert war, wiesen die $EGFR^{\Delta/\Delta VSMC\&CM}$ Mäuse eine leichte, aber signifikante Reduktion des diastolischen Blutdruckes und des mittleren arteriellen Blutdruckes auf. Der reduzierte mittlere arterielle Blutdruck führte aber nicht zu einer Minderperfusion der Gewebe, zumindest im Herzen konnte keine gesteigerte Expression von HIF1-α oder dem Glukosetransporter 1 nachgewiesen werden. Morphologisch zeigten sich eine reduzierte Dicke der Media der Aorta und ein erhöhter Lumendurchmesser intramyocardialer Blutgefäße. Dies wies auf einen reduzierten vaskulären Tonus bei Deletion des EGFR in VSMC hin. Bei Infusion von Angiotensin II in narkotisierte Mäuse konnte ein reduzierter akuter Anstieg des intravaskulären, mittleren arteriellen Blutdruckes beobachtet werden. Zudem wiesen die Knockout Tiere ebenfalls eine verkürzte Dauer des Angiotensin II induzierten Blutdruckanstiegs auf. Dies würde zu einer geringeren Belastung des Herzkreislaufsystems durch Angiotensin II führen. In den Aorten von KO Tieren war die Expression von Markern für Fibrose und Inflammation erhöht. Somit war

der EGFR für die physiologische Entwicklung der Blutgefäßstruktur von Bedeutung und verhinderte proinflammatorische Veränderungen. Damit reduzierte der EGFR *in vivo* die Breite der physiologischen Blutdruckregulation bei kurzfristiger Applikation von Angiotensin II und bei langfristig erhöhten Angiotensin II Plasmaspiegeln förderte er den vaskulären Gefäßumbau. Zusammengenommen erklärten die Veränderungen in der Gefäßmorphologie den verringerten diastolischen und mittleren arteriellen Blutdruck. Durch das erhöhte Schlagvolumen wurde gleichzeitig der systolische Blutdruck aufrechterhalten.

Synopse: Die Daten in dieser Arbeit zeigten, dass der EGFR in Kardiomyozyten das exzessive Wachstum des Herzens verhindert, vermutlich über seine Wirkung auf die ROS Homöostase. In VSMC war der EGFR an der Entwicklung der physiologischen, angepassten Entstehung der Wandstruktur und Reaktivität der Blutgefäße beteiligt. Allerdings wirkte der EGFR auch als Verstärker von vaskulären Stimuli, wie Angiotensin II, die den pathophysiologischen Umbau der Blutgefäße fördern.

B) Barbara Schreier, Mirja Hünerberg, Sindy Rabe, Sigrid Mildenberger, Daniel Bethmann, Christian Heise, Maria Sibilia, Stefan Offermanns and Michael Gekle (2016) Consequences of postnatal vascular smooth muscle EGF-receptor deletion on acute angiotensin II action. Clin Sci (Lond). 2016 Jan;130(1):19-33.

Da in den *EGFR*$^{\Delta/\Delta VSMC\&CM}$ Mäusen nicht nur eine Deletion des EGFR in VSMC zu beobachten war, sondern auch in den Kardiomyozyten eine starke Reduktion des EGFR, konnte die Frage nach der Bedeutung des EGFR in VSMC *in vivo* an diesem Modell nicht abschliessend geklärt werden. Zu dem zeigten sich adaptive Effekte, wie sie bei einer kurzfristigen Inhibition des EGFR (zum Beispiel während einer Tumortherapie), nicht zu beobachten waren. Daher wurde ein zweites Mausmodell generiert, um nach Induktion des Knockouts die Bedeutung des EGFR in VSMC im adulten Tier zu untersuchen.
iEGFR$^{\Delta/\Delta VSMC}$ Mäuse wiesen keine morphologischen Veränderungen in der Aorta oder intrarenalen Arterien auf. Jedoch war das Wand/Lumen-Verhältnis von intramyocardialen Arterien erhöht, wobei die Wanddicke nahezu gleich blieb. Dies deutete daraufhin, dass die kurzfristige, postnatale Deletion des EGFR in VSMC zu einem leicht dilatativen Phänotyp der Blutgefäße führte. Bei den *iEGFR*$^{\Delta/\Delta VSMC}$ Mäusen konnten keine Zeichen für eine Herzhypertrophie festgestellt werden. Damit lag der Schluss nahe, dass bei den *EGFR*$^{\Delta/\Delta VSMC\&CM}$ Mäusen die Herzhypertrophie durch die Deletion des EGFR in den Kardiomyozyten induziert wurde.
Funktionell war bei den induzierbaren *VSMC-EGFR KO* Mäusen der systolische, diastolische und mittlere arterielle Blutdruck reduziert. Dies unterstützte die Hypothese, dass der *VSMC-EGFR* für die Aufrechterhaltung des physiologischen, vaskulären Tonus notwendig war und dass die Reduktion des peripheren Widerstands in der Konsequenz eine Reduktion des Blutdruckes bedingte. Wir gehen davon aus, dass in den konstitutiven KO-Tieren die Herzhypertrophie mit dem erhöhten Schlagvolumen den reduzierten peripheren Widerstand kompensierte, so dass der systolische Blutdruck im physiologischen Bereich blieb.
Bei der Analyse der Blutgefäßfunktion konnte gezeigt werden, dass weder bei den konstitutiven KO Mäusen noch bei den induzierbaren KO Mäusen ein Unterschied in der Blutgefäßkontraktion bei Applikation von Kaliumchlorid oder Serotonin erfolgt. Auch die Vasorelaxation bei Applikation von Carbachol oder dem NO-Donor SNAP war nicht unterschiedlich zwischen Wildtyp und KO-Tieren. Dies liess folgende Schlussfolgerungen zu: a) die Deletion des EGFR in glatten Muskelzellen störte die Vasodilatation nicht, b) die Grundkomponenten der pharmakomechanischen und elektromechanischen Kopplung in den glatten Muskelzellen war auch nach Deletion des EGFR intakt und c) die Kraftentwicklung der Blutgefäße war in Abwesenheit des EGFR nicht gestört. Damit wurde die Grundlage geschaffen, um weitere vasoaktive Substanzen zu vergleichen. Obwohl die Transaktivierung des EGFR in VSMC durch Endothelin-1 bereits beschrieben wurde[147,175,196], konnten wir in der Mulvany-Myographie keine unterschiedliche

Kraftentwicklung zwischen Wildtyp- und Knockout-Tieren beobachten. Im Gegensatz dazu war die Kraftentwicklung durch Angiotensin II abhängig von der Transaktivierung des EGFR. Bei der kumulativen Dosis-Wirkungskurve von Angiotensin II zeigte sich eine reduzierte Effizienz von Angiotensin II (dFmax war reduziert.). Zwischen den Genotypen war kein Unterschied des mRNA-Gehaltes für die Angiotensin II-Rezeptoren zwischen den Genotypen feststellbar. Allerdings zeigte sich bei wiederholter Applikation von Angiotensin II eine erhöhte homologe Desensitivierung des Angiotensin II-Rezeptors bei Fehlen des VSMC-EGFR. Damit konnten wir zeigen, dass der VSMC-EGFR nicht nur die vasokonstriktorische Aktivität des Angiotensin II durch Vermittlung der ERK 1/2-Phosphorylierung unterstützte, sondern auch durch eine Veränderung der Rezeptordynamik.

Es konnte somit gezeigt werden, dass sowohl *in vivo* als auch *ex vivo* der EGFR in VSMC nicht nur die langfristige Wirkung von Angiotensin II förderte sondern auch die kurzfristige. Auch in den *iEGFR*$^{\Delta/\Delta VSMC}$ Tieren liess sich belegen, dass der EGFR in VSMC für die Generierung des physiologischen Blutdruckanstiegs und für dessen Aufrechterhaltung notwendig war. Damit trug der EGFR nicht nur zu der Blutdrucklast unter Ruhebedingungen, sondern auch unter Bedingungen eines aktivierten Renin-Angiotensin-Systems bei. Eine zentrale Erkenntnis unserer Untersuchungen war auch, dass der VSMC-EGFR bezüglich des vaskulären Tonus eine unterschiedliche Rolle einnimmt, abhängig davon, welche Substanz ihn transaktiviert. Die Transaktivierung des EGFR durch eine bestimmte vasoaktive Substanz bedeutete nicht, dass durch diese Substanz auch die akute Kontraktion der Blutgefäße gefördert wurde. So sahen wir eine erhöhte Kraftentwicklung bei der Mulvany-Myographie durch Angiotensin in Anwesenheit des EGFR, aber nicht durch Endothelin-1, obwohl beide Substanzen zu einer erhöhten EGFR-abhängigen Phosphorylierung von ERK 1/2 führten.

Zusätzlich zu den Untersuchungen an jungen Tieren konnte auch die Bedeutung des VSMC-EGFR in 10 Monate alten Tieren analysiert werden. Hier zeigte sich, dass die Deletion des EGFR in VSMC die erhöhte Expression von Ccl-2 in der Aorta reduzierte und damit der Alters-abhängige proinflammatorische Phänotyp der Blutgefäße abgemildert wurde. Durch die Deletion des EGFR in VSMC konnte auch die altersabhängige Zunahme des Herzgewichtes reduziert werden, ebenso wie die kardiale Expression von Serpine-1. Dieser Phänotyp resultierte höchstwahrscheinlich von dem dauerhaft reduzierten Blutdruck.

Synopse: Der VSMC-EGFR ist in die basale Blutdruckhomöostase involviert und vermittelt, abhängig von der Substanz, die zur Transaktivierung führt, auch einen Teil der akuten, vasokonstriktorischen Effekte. Die Morphologie der Blutgefäße wird durch den VSMC-EGFR wenig beeinflusst. Der VSMC-EGFR trägt zu der durch akute Angiotensin II-Gabe induzierten Blutdruckbelastung bei vier und zehn Monate alten Tieren bei und damit

zu der lebenslangen Druckbelastung, die zu einem pathologisch relevanten Umbau von Herz und Gefäßen führt.

C) Barbara Schreier, Sindy Rabe, Sabrina Winter, Stefanie Ruhs, Sigrid Mildenberger, Bettina Schneider, Maria Sibilia, Michael Gotthardt, Sabine Kempe, Karsten Mäder, Claudia Grossmann & Michael Gekle (2014) Moderate inappropriately high aldosterone/NaCl constellation in mice: cardiovascular effects and the role of cardiovascular epidermal growth factor receptor, Scientific reports 4:7430, DOI: 10.1038/srep07430

In früheren Studien konnte gezeigt werden, das die nicht-physiologische Aktivierung des Mineralocorticoidrezeptors durch Aldosteron zusammen mit der erhöhten Salzaufnahme zu der Entwicklung von kardiovaskulären Schäden bei trug[197]. Einen Teil der Effekte des Mineralocorticoidrezeptors, auf die Veränderungen im Herzkreislaufsystem, vermittelte der EGFR. Daher wurden *EGFR*$^{\Delta/\Delta VSMC\&CM}$ und entsprechende Wildtyp-Kontrollen ein Aldosteron-freisetzendes Pellet subkutan für vier Wochen implantiert. Die Dosis war so berechnet, dass der physiologische Aldosteron-Plasmaspiegel nicht überschritten wurde. Zusätzlich bekamen die Mäuse noch 1%ige NaCl-Lösung zu trinken. Die erhöhte Salzaufnahme führte physiologisch zu einer reduzierten Aldosteronsynthese in der Nebenniere. Da in diesem Modell die physiologische Reduktion des Aldosterons durch die implantierten Pellets verhindert wurde, entstand ein Modell eines unverhältnismäßig hohen Aldosteronplasmaspiegels bei erhöhter Salzzufuhr. Die Kontrolltiere erhielten normales Leitungswasser und kein Aldosteronpellet. Auf die Entfernung der Niere wurde verzichtet, da nur die Auswirkungen von Aldosterone/NaCl in Abhängigkeit des EGFR untersucht und auch eine Erhöhung des Blutdruckes durch die Nephrektomie vermieden werden sollte. Das hier angewendete Modell entspricht der Situation wie sie auch in Menschen beobachtet wird und zu einer erhöhten kardiovaskulären Morbidität und Mortalität führt[198].
Wie erwartet konnten wir unter Aldosteron/NaCl-Behandlung keine Erhöhung des Blutdruckes bei den Mäusen beobachten. Die unverhältnismäßig hohe Aldosteron/NaCl-Konstellation führte in Wildtypmäusen zu einem milden, profibrotischen Phänotyp der Herzen mit einer reduzierten atrioventrikulären Erregungsweiterleitung bei männlichen Tieren. Bei den Weibchen traten keinerlei Veränderungen durch die Aldosteron/NaCl-Behandlung auf. Auch bei den Männchen waren lediglich Veränderungen am Herzen, nicht aber an der Aorta feststellbar. Die hier beschriebene Verlängerung der Überleitungszeit wurde bereits in anderen Aldosteron-Modellen beobachtet[199,200], genauso wie ein pro-arhythmogener Effekt von Aldosteron[201,202], allerdings handelte es sich immer um Modelle mit pathologisch erhöhten Aldosteronplasmaspiegeln. Da wir deutliche Unterschiede bezüglich der kardialen Veränderungen durch Aldosteron zwischen männlichen und weiblichen Mäusen beobachten konnten, wurde untersucht, ob die Deletion unterschiedliche Veränderungen im Herzen abhängig vom Geschlecht bedingte. Dabei zeigte sich, dass weibliche EGFR KO Mäuse eine deutlich geringere Herzhypertrophie entwickelten und auch die kardiale und aortale Aktivierung von proinflammatorischen Genen geringer war als bei den entsprechenden Männchen. Bei

neugeborenen EGFR KO Mäusen konnte zwar bereits eine signifikante Erhöhung des Herzgewichts gegenüber den Kontrollen festgestellt werden, aber es war noch kein Effekt des Geschlechts zu beobachten. Während die Zunahme des Herzgewichts bei Neugeborenen etwa 25% betrug, war bei den adulten Männchen eine Erhöhung des Herzgewichtes im Verhältnis zur Tibialänge auf etwa 300% des Herzgewichtes von Wildtyptieren zu beobachten, bei Weibchen war es „lediglich“ eine Erhöhung auf 200% des Herzgewichtes. Auf Grund dieser Befunde vermuteten wir, dass pränatal ein geschlechts-unabhängiger Effekt zu der Herzhypertrophie bei Deletion des EGFR führte, postnatal dann ein zweiter, geschlechtsabhängiger Effekt hinzukam. Wie bereits oben erwähnt war in den Herzen der adulten Tiere die NOX-4 Expression und die ROS-Produktion erhöht, wir konnten feststellen, dass die Deletion des EGFR bei Männchen zu einer stärkeren Zunahme der NOX-4 Expression führt (ca. 300%). In den Herzen der Weibchen stieg die NOX-4-Expression nur auf ca. 175%. In den Herzen der neonatalen Mäuse ließ sich keine erhöhte Expression der NOX-4 beobachten. Zusammen mit dem erhöhten Herzgewicht kam es bei den männlichen Knockout-Tieren zu einer Verlängerung des P-Intervalls, wie auch zu einer Verbreiterung des QRS-Komplexes und einer verlängerten QTc-Zeit. Diese Kombination stellt eine hochriskante Konstellation für das Auftreten von Reentry-Arrhythmien dar. Dies könnte die erhöhte Mortalität der Tiere unter basalen Bedingungen erklären. In männlichen KO Tieren führte die Behandlung mit Aldosteron/Salz zu einer i) partiellen Verbesserung der Herzhypertrophie mit einer Veränderung der intraatrialen und intraventrikulären Erregungsweiterleitung, ii) einer Erhöhung der Herzfrequenzvariabilität, iii) aber nicht zu einer Verbesserung des profibrotischen/proinflammatorischen Phänotyps. Dies lässt die Schlussfolgerung zu, dass die schädigenden Effekte von Aldosteron/NaCl im Herzen zumindest teilweise abhängig waren vom EGFR. Die positiven Effekte von Aldosteron/NaCl hingegen schienen unabhängig vom EGFR zu sein.

Synopse: Der EGFR und eine unverhältnismäßig hohe Aldosteronplasmakonzentration bei erhöhter Salzaufnahme übten am Herzen einen Ying-Yang Effekt aus. Während der EGFR schützend für die physiologische Gewebshomöostase des Herzens und der Aorta war, vermittelte er zumindest einen Teil der schädigenden Wirkung von Aldosteron/NaCl bei männlichen Tieren. Andererseits übte Aldosteron/NaCl am Herzen auch einen protektiven Effekt aus, dieser wurde aber vermutlich nicht durch den EGFR vermittelt.

D) Barbara Schreier, Maria Döhler, Sindy Rabe, Bettina Schneider, Gerald Schwerdt, Stefanie Ruhs, Maria Sibilia, Michael Gotthardt, Michael Gekle, Claudia Grossmann (2011) Consequences of Epidermal Growth Factor Receptor (ErbB1) Loss for Vascular Smooth Muscle Cells From Mice With Targeted Deletion of ErbB1, Arterioscler Thromb Vasc Biol. 31:1643-1652.

Die pathophysiologischen Effekte des EGFR umfassen unter anderem den vaskulären Gefäßumbau. Die Transaktivierung des EGFR in VSMC soll signifikant zur heterologen Signaltransduktion und dem Gefäßumbau beitragen. Deswegen wurden primäre VSMC aus Aorten von Mäusen mit Deletion des EGFR und von deren wildtypischen Geschwistertieren isoliert und ihre Funktion, unter besonderer Berücksichtigung des Zellüberlebens, des Pentosephosphatweges, der Matrixhomöostase, der ERK 1/2 Phosphorylierung und der Ca^{2+}-Homöostase, analysiert. Zellen ohne Deletion des EGFR ($VSMC^{+/+EGFR}$) wiesen durch EGF eine deutlich Phosphorylierung der ERK 1/2 Kinase und eine Erhöhung der intrazellulären Calcium-Konzentration auf. In $VSMC^{-/-EGFR}$ Zellen war die EGF induzierte Zellantwort nicht auslösbar. Bereits unter basalen Bedingungen war die Netto-Proliferation bei $VSMC^{-/-EGFR}$ im Vergleich zu den $VSMC^{+/+EGFR}$ Zellen deutlich reduziert. Die Zellgröße war aber zwischen $VSMC^{+/+EGFR}$ und $VSCM^{-/-EGFR}$ nicht unterschiedlich. Unter Ruhebedingungen (Inkubation ohne Wachstumsfaktoren) war die LDH-Freisetzung (Nekrose) und die Caspase-3 Aktivität (Apoptose) bei $VSMC^{-/-EGFR}$ höher als bei $VSMC^{+/+EGFR}$. Dies deutete daraufhin, dass der EGFR eine zytoprotektive Wirkung in VSMC besitzt. Zellen mit einer Deletion des EGFR zeichneten sich durch eine reduzierte Aktivität des Pentosephosphatzykluses aus. Der Pentosephosphatweg ist für die Versorgung der Zellen mit NADPH notwendig. Dieses wiederum schützt die Zellen vor oxidativem Stress und wirkt als wichtiger Co-Faktor für die NADPH-Oxidasen sowie für die Lipogenese und die Nukleotidsynthese. Es wurde daher postuliert, dass der reduzierte Schutz vor oxidativen Schädigungen zu der erhöhten Sterblichkeit der $VSMC^{-/-EGFR}$ führte.
Bereits unter basalen Bedingungen synthetisierten und sezernierten die $VSMC^{-/-EGFR}$-Zellen vermehrt Kollagen III und Fibronektin-1 Protein. Dies ließ die Schlussfolgerung zu, dass unter basalen Bedingungen der EGFR die exzessive Synthese von extrazellulärer Matrix verhindert. Die Stimulation mit EGF hemmte die Synthese von Kollagen III und Fibronectin 1. Dies würde die Hypothese unterstützen, dass der EGFR in der Gefäßwand eine protektive Funktion besitzt. Eventuell lag dem Geschehen ein biphasischer Effekt zu Grunde, da wir feststellen konnten, dass die Sekretion von Kollagen III in $VSMC^{+/-EGFR}$ deutlich stärker durch EGF stimuliert wurde als in $VSMC^{+/+EGFR}$.
Bezüglich der ERK 1/2-Phosphorylierung konnte gezeigt werden, dass der EGFR in VSMC maßgeblich zu den Effekten von Endothelin-1, Phenylephrin, ATP und Wasserstoffperoxid beitrug. Die durch Endothelin-1 und Phenylephrin induzierte Proliferation von VSMC war in Zellen mit EGFR-Deletion vollständig aufgehoben. Bezüglich seiner Bedeutung für die Änderung der intrazellulären Ca^{2+}-Ionenkonzentration konnten unterschiedliche Effekte

beobachtet werden. Die Deletion des EGFR steigert den intrazellulären Ca^{2+}-Anstieg durch ATP bzw. Wasserstoffperoxid. Im Gegensatz dazu reduzierte die Deletion des EGFR den Anteil der Zellen, die bei Stimulation mit Endothelin-1 oder Phenylephrin eine Erhöhung des intrazellulären Ca^{2+} aufwiesen. Während jedoch der Anstieg des intrazellulären Ca^{2+} in Endothelin-1 Responderzellen gleich blieb, war der Ca^{2+}-Anstieg durch Phenylephrin in Responderzellen erhöht. Bisher konnten wir noch keine Erklärung für diese Unterschiede geben. Da durch die akute Gabe von AG1478 die Anzahl der Zellen die auf die Stimuli mit einem intrazellulären Ca^{2+}-Anstieg reagierten nicht verändert wurde, muss davon ausgegangen werden, dass es sich hierbei vermutlich um adaptive Prozesse als Antwort auf die Deletion des EGFR handelte und weniger um Veränderungen in der akuten Transaktivierung des EGFR.

Synopse: Der EGFR in VSMC ist wichtig für die basale Homöostase der Zellen und die ERK 1/2 Aktivierung durch Endothelin-1, Phenylephrin, ATP und Wasserstoffperoxid. Die intrazelluläre Ca^{2+}-Freisetzung bei Stimulation mit den unterschiedlichen Substanzen wird unterschiedlich durch den EGFR beeinflusst, besonders wenn man den Anteil der reagierenden Zellen und die Höhe des intrazellulären Ca^{2+}-Anstiegs betrachtet. Zusammengenommen kann mit dieser Arbeit gezeigt werden, dass der EGFR sowohl schützend als auch schädigend in den Gefäßen wirken kann.

E) Barbara Schreier, Gerald Schwerdt, Christian Heise, Daniel Bethmann, Sindy Rabe, Sigrid Mildenberger, Michael Gekle (2016) Substance-specific importance of EGFR for vascular smooth muscle cells motility in primary culture. Biochimica et Biophysica Acta – Molecular Cell Research 1863: 1519–1533

Neben ihrer bedeutenden Rolle in der Regulation des vaskulären Tonus sind VSMC auch an der Entwicklung von pathophysiologischen Gefäßveränderungen beteiligt, wie z.B. der erhöhten Synthese von Matrixproteinen oder der Bildung von atherosklerotischen Plaques. Verschiedene Liganden von GPCRs sind an der Entstehung und Aufrechterhaltung der vaskulären Dysfunktion und dem pathophysiologischen Gefäßumbau beteiligt, vermutlich durch die Transaktivierung des EGFR. Das Ziel dieser Studie war es die Bedeutung des EGFR in VSMC für die Proliferation, Migration und die Expression von Markergenen der Entzündung, Fibrose und Bildung reaktiver Sauerstoffspezies zu charakterisieren. Zusätzlich sollte die Hypothese geprüft werden, dass die Effekte, welche die unterschiedlichen vasoaktiven Substanzen in VSMC induzieren, in unterschiedlicher Weise von der Transaktivierung des EGFR abhängen. Hierfür wurden VSMC mit oder ohne genetische Deletion des EGFR bei Applikation von Endothelin-1, Phenylephrin, Thrombin, antidiuretischem Hormon oder ATP bezüglich der obengenannten Parameter untersucht. Unter normalen Zellkulturbedingungen, d.h. bei Zugabe von fetalem Kälberserum im Medium, spielte die Deletion des EGFR in VSMC bezüglich Proliferation, Migration im Zellverband (Gap-closure-assay) oder Einzelzellmigration (Boyden Chamber, Single-Cell-Tracking) eine untergeordnete Rolle. Das heißt der EGFR war nicht essentiell für diese Prozesse in VSMC. Aber da der EGFR als Signalplattform fungiert, also durch z.B. c-Src phosphoryliert wird und damit auch ohne Aktivierung durch einen Liganden Signale weiterleiten kann, ist es von Interesse zu untersuchen, ob der EGFR *per se* zu Veränderungen in der Proliferation bzw. Migration von Zellen führt. Dafür wurde das fetale Kälberserum aus dem Medium entfernt, da es eine breite Palette von Wachstumsfaktoren enthält und es somit bereits durch fetales Kälberserum zu einer Stimulation des EGFR kommen könnte. Die Proliferation konnte unter diesen Bedingungen nicht beurteilt werden, da weder die Zellen mit noch ohne EGFR proliferierten. Die Einzelzellmigration zwischen den beiden Zelltypen wies keinen Unterschied auf. In Single-Cell-Tracking Experimenten konnten wir feststellen, dass Zellen mit Deletion des EGFR eine reduzierte Migrationswahrscheinlichkeit aufwiesen, dafür aber eine leicht größere Migrationsgeschwindigkeit. Die reduzierte Migrationswahrscheinlichkeit könnte auf eine verlängerte Art von Taumelperiode (Tumbling period) wie in Primordialzellen der Fruchtfliege hindeuten[203]. Diese Zeit dient den Primordialzellen sich entlang eines chemischen Gradienten neu zu orientieren. Damit würde der EGFR unter anderem während der gerichteten Migration eine Bedeutung haben. Im Gegensatz zu der Einzelzellmigration war die kollektive Migration im Gap-Closure-Assay durch die Deletion des EGFR signifikant reduziert. Für die Grenzzellen von Fruchtfliegen konnte bereits

gezeigt werden, dass ein auf der Zelloberfläche gleichmäßig aktivierter EGFR die Migration der Zellen hemmt[204,205]. Es wurde vermutet, dass durch die künstliche Erstellung einer Lücke im Zellrasen an den Rändern der Lücke die einheitliche Verteilung der autokrin freigesetzten Liganden gestört wurde und damit die Migration durch den EGFR gefördert. Die Expression von Markergenen für Fibrose, Inflammation und ROS-Homöostase war durch die Deletion des EGFR unter unstimulierten Bedingungen nicht beeinflusst.

EGF selber stimulierte EGFR abhängig die Proliferation, Einzelzellmigration, Chemotaxis und Migration im Zellverband. Zudem konnte gezeigt werden, dass EGF die Expression von Osteopontin und MCP-1 (Monocyte chemoattractant protein-1) abhängig vom EGFR förderte. Interessanterweise wurde die Expression von TGF-β in VSMC durch die Deletion des EGFR gehemmt, während in den Wildtyp-VSMC kein Effekt auf die Expression von TGF-β zu beobachten war. Dies könnte darauf hinweisen, dass der EGFR die Expression von TGF-β in VSMC förderte, dies aber unter normalen Bedingungen durch andere Signalwege kompensiert wurde. Es wäre hier an andere ErbB-Heterodimere zu denken, die ebenfalls EGF binden können.

Die Bedeutung des EGFR in VSMC für die pathophysiologischen Effekt verschiedener vasoaktiver Substanzen wurde anhand von Endothelin-1, Phenylephrin, Thrombin, antidiuretischem Hormon oder ATP getestet. Wir konnten zeigen, dass der EGFR in VSMC notwendig für die Thrombin induzierte Proliferation und Migration im Zellverbund war. Thrombin selber hatte keinen Effekt auf die Einzelzellmigration in der *Boyden Chamber*. Es stimulierte jedoch in den untersuchten Zellen die Expression von Markergenen für Inflammation und Fibrose. Aber nur der Effekt auf die MCP-1 Expression war abhängig vom EGFR.

Das antidiuretische Hormon hatte keinen Effekt auf die Zellproliferation im untersuchten Modell, obwohl es zu einer EGFR abhängigen ERK 1/2-Phosphorylierung und Ca^{2+}-Antwort führte. Das antidiuretische Hormon förderte die Migration im Zellverbund abhängig vom EGFR aber nicht die Einzelzellmigration. Die Expression der von uns untersuchten Markergene der Inflammation und Fibrose wurde durch das antidiuretische Hormon nicht beeinflusst.

Endothelin-1 erhöhte die ERK 1/2-Phosphorylierung abhängig vom EGFR, hatte aber keinen Effekt auf die VSMC-Proliferation oder -Migration. Auch die Effekte von Endothelin-1 auf die untersuchte Markergen-Expression waren gering. Weder die Proliferation noch die Migration wurde durch Inkubation mit Phenylephrin stimuliert, allerdings wurde die Expression von c-Jun durch Phenylephrin EGFR-abhängig reguliert. ATP hatte in den Experimenten keinen Einfluss auf die Proliferation oder Migration von VSMC im Verbund. Es erhöhte jedoch die Einzelzellmigration in der Boyden Kammer und somit die Chemotaxis. ATP induzierte eine leichte Abnahme der Expression von c-Jun in VSMC mit Deletion des EGFR. Der zugrundeliegende Mechanismus muss noch untersucht werden.

Synopse: In dieser Arbeit konnte gezeigt werden, dass 1) der EGFR ohne weitere Aktivierung notwendig ist für die Aufrechterhaltung des Phänotyps der VSMC, da die VSMC in Abwesenheit einen eher kontraktilen Phänotyp annahmen. 2) Wird der EGFR durch seinen Liganden EGF aktiviert, fördert dies die Proliferation, Einzelzellmigration und Migration im Zellverbund sowie die Expression von proinflammatorischen Genen in VSMC. Dies könnte zu der Entstehung von vaskulären Veränderungen beitragen. 3) Des Weiteren konnte gezeigt werden, dass der Beitrag des EGFR zu den unterschiedlichen phänotypischen Veränderungen, die durch die unterschiedlichen vasoaktiven Substanzen hervorgerufen werden, variiert. Dabei vermittelt der EGFR hauptsächlich die Förderung der Migration im Zellverbund, weniger die Veränderungen in der Genexpression von Markergenen der Fibrose oder Inflammation. Nahezu keinen Einfluss scheint der EGFR auf die Expression von Genen zu besitzen, die in die Regulation der ROS-Homöostase involviert sind.

Gesamtfazit:

Zusammenfassend konnten wir zeigen dass der EGFR in Kardiomyozyten und in VSMC für die Gewebshomöostase notwendig ist, die Hypertrophie des Herzens verhindert sowie den basalen, vaskulären Tonus aufrecht erhält. Andererseits verstärkt er die akute Wirkung von Angiotensin II auf die Vasokonstriktion der Blutgefäße und das vaskuläre Remodeling. Zudem vermittelt er bei männlichen Versuchstieren einen Teil der kardiovaskulären Effekte, die im Rahmen von inadäquat erhöhten Aldosteronspiegeln im Verhältnis zur gesteigerten Salzaufnahme hervorgerufen werden. Damit fungiert der EGFR als wichtiger Vermittler der Herz- und Gefäß-schädigenden Wirkungen des Renin-Angiotensin-Aldosteron-Systems. Des Weiteren ergaben sich Hinweise darauf, dass der EGFR zu den kardiovaskulären Veränderungen beiträgt die mit steigendem Lebensalter auftreten. Die Transaktivierung des EGFR führt abhängig von der ihn aktivierenden Substanz zu unterschiedlichen Veränderungen in VSMC. So konnten wir zeigen, dass der EGFR grundsätzlich sowohl an der Aktivierung des ERK 1/2-Signalweges, der intrazellulären Freisetzung von Ca^{2+} und der Förderung der Migration im Zellverbund beteiligt ist. Für ein umfassendes Verständnis der regulierenden Prozesse des EGFR müssen sich weitere Untersuchungen anschließen, wie z. B. die Identifizierung der relevanten Phosphorylierungsstellen des EGFR und der sich daraus ergebenden Identifizierung der verantwortlichen Signalwege sowie den entsprechenden phänotypischen Veränderungen.

In **Abbildung 8** sind die gewonnenen Erkenntnisse entsprechend der Fragen auf Seite 32 sowie der **Abbildung 6** aufgelistet und zusammengefasst.

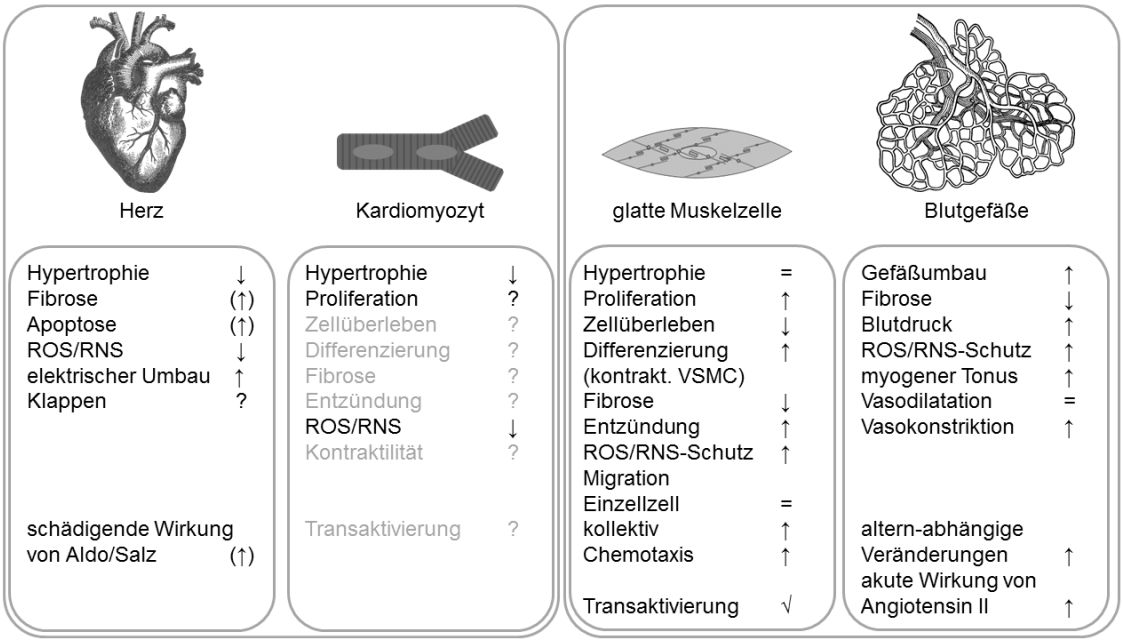

Abbildung 8: Zusammenfassung der Bedeutung des EGFR für das kardiovaskuläre System wie sie sich nach unseren Untersuchungen darstellen.

5. Literaturverzeichnis

1. Schreier B et al. Loss of Epidermal Growth Factor Receptor in Vascular Smooth Muscle Cells and Cardiomyocytes Causes Arterial Hypotension and Cardiac Hypertrophy. Hypertension 2013;61:333-40.
2. Schreier B et al. Consequences of Epidermal Growth Factor Receptor (ErbB1) Loss for Vascular Smooth Muscle Cells From Mice With Targeted Deletion of ErbB1. Arterioscler Thromb Vasc Biol 2011;31:1643-53.
3. Schreier B et al. Role of epidermal growth factor receptor in vascular structure and function. Curr Opin Nephrol Hypertens 2014;23:113-21.
4. Nyati MK et al. Integration of EGFR inhibitors with radiochemotherapy. Nat Rev Cancer 2006;6:876-85.
5. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127-37.
6. Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. Annu Rev Pharmacol Toxicol 2004;44:195-217.
7. Bessman N et al. Complex Relationship between Ligand Binding and Dimerization in the Epidermal Growth Factor Receptor. Cell Reports 2014;9:1306-17.
8. Amin DN et al. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. Cancer Res 2006;66:2173-80.
9. Sweeney WE et al. Treatment of polycystic kidney disease with a novel tyrosine kinase inhibitor. Kidney Int 2000;57:33-40.
10. Elder JT et al. Overexpression of transforming growth factor alpha in psoriatic epidermis. Science 1989;243:811.
11. Cook PW et al. Transgenic expression of the human amphiregulin gene induces a psoriasis-like phenotype. J Clin Invest 1997;100:2286-94.
12. Davies DE et al. The epidermal growth factor receptor and its ligand family: their potential role in repair and remodelling in asthma. Allergy 1999;54:771-83.
13. Barrick CJ et al. Chronic pharmacologic inhibition of EGFR leads to cardiac dysfunction in C57BL/6J mice. Toxicol Appl Pharmacol 2008;228:315-25.
14. Gill GN et al. Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. Journal of Biological Chemistry 1984;259:7755-60.
15. Masui H et al. Growth Inhibition of Human Tumor Cells in Athymic Mice by Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies. Cancer Res 1984;44:1002-7.
16. Miettinen PJ et al. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature 1995;376:337-41.

17. Seidman A et al. Cardiac Dysfunction in the Trastuzumab Clinical Trials Experience. JCO 2002;20:1215-21.
18. Grossmann C, Gekle M. Non-classical actions of the mineralocorticoid receptor: misuse of EGF receptors? Mol Cell Endocrinol 2007;277:6-12.
19. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nature Reviews Molecular Cell Biology 2006;7:505-16.
20. Ullrich A et al. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 1984;309:418-25.
21. Burgess AW et al. An Open-and-Shut Case? Recent Insights into the Activation of EGF/ErbB Receptors. Molecular Cell 2003;12:541-52.
22. Klapper LN et al. The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. Proceedings of the National Academy of Sciences 1999;96:4995-5000.
23. Guy PM et al. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proceedings of the National Academy of Sciences of the United States of America 1994;91:8132-6.
24. Ferguson KM et al. EGF Activates Its Receptor by Removing Interactions that Autoinhibit Ectodomain Dimerization. Molecular Cell 2003;11:507-17.
25. Cho HS, Leahy DJ. Structure of the Extracellular Region of HER3 Reveals an Interdomain Tether. Science 2002;297:1330-3.
26. Garrett TPJ et al. The Crystal Structure of a Truncated ErbB2 Ectodomain Reveals an Active Conformation, Poised to Interact with Other ErbB Receptors. Molecular Cell 2003;11:495-505.
27. Yaffe MB. Phosphotyrosine-binding domains in signal transduction. Nat Rev Mol Cell Biol 2002;3:177-86.
28. Jones RB et al. A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature 2006;439:168-74.
29. Jones JT et al. Binding specificities and affinities of egf domains for ErbB receptors. Febs Letters 1999;447:227-31.
30. Olayioye MA et al. The ErbB signaling network: receptor heterodimerization in development and cancer. The EMBO Journal 2000;19:3159-67.
31. Soltoff SP, Cantley LC. p120 Is a Cytosolic Adapter Protein That Associates with Phosphoinositide 3-Kinase in Response to Epidermal Growth Factor in PC12 and Other Cells. Journal of Biological Chemistry 1996;271:563-7.
32. Jorissen RN et al. Epidermal growth factor receptor: mechanisms of activation and signalling. Experimental Cell Research 2003;284:31-53.
33. Slieker LJ et al. Synthesis of epidermal growth factor receptor in human A431 cells. Glycosylation-dependent acquisition of ligand binding activity occurs post-

translationally in the endoplasmic reticulum. Journal of Biological Chemistry 1986;261:15233-41.
34. Harris RC et al. EGF receptor ligands. Exp Cell Res 2003;284:2-13.
35. Yamabhai M, Anderson RGW. Second Cysteine-rich Region of Epidermal Growth Factor Receptor Contains Targeting Information for Caveolae/Rafts. Journal of Biological Chemistry 2002;277:24843-6.
36. Callera GE et al. Vascular signaling through cholesterol-rich domains: implications in hypertension. Current Opinion in Nephrology and Hypertension 2007;16:90-104.
37. Makki N et al. The epidermal growth factor receptor and its ligands in cardiovascular disease. Int J Mol Sci 2013;14:20597-613.
38. den Hartigh JC et al. The EGF receptor is an actin-binding protein. The Journal of Cell Biology 1992;119:349-55.
39. Krall JA et al. High- and Low-Affinity Epidermal Growth Factor Receptor-Ligand Interactions Activate Distinct Signaling Pathways. Plos One 2011;6:e15945.
40. Morandell S et al. Quantitative proteomics and phosphoproteomics reveal novel insights into complexity and dynamics of the EGFR signaling network. Proteomics 2008;8:4383-401.
41. Sachsenmaier C et al. Involvement of growth factor receptors in the mammalian UVC response. Cell 1994;78:963-72.
42. Kodama H et al. Role of EGF Receptor and Pyk2 in Endothelin-1-induced ERK Activation in Rat Cardiomyocytes. Journal of Molecular and Cellular Cardiology 2002;34:139-50.
43. Hsieh HL et al. c-Src-dependent EGF receptor transactivation contributes to ET-1-induced COX-2 expression in brain microvascular endothelial cells. Journal of Neuroinflammation 2012;9:152.
44. Daub H et al. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 1996;379:557-60.
45. Buchanan FG et al. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. J Biol Chem 2003;278:35451-7.
46. Bokemeyer D et al. Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor. Kidney Int 2000;58:549-58.
47. Akhtar S et al. Angiotensin-(1-7) inhibits epidermal growth factor receptor transactivation via a Mas receptor-dependent pathway. Br J Pharmacol 2012;165:1390-400.
48. Chen K et al. c-Jun N-terminal Kinase Activation by Hydrogen Peroxide in Endothelial Cells Involves Src-dependent Epidermal Growth Factor Receptor Transactivation. Journal of Biological Chemistry 2001;276:16045-50.

49. Lee S et al. Metalloproteinase Processing of HBEGF Is a Proximal Event in the Response of Human Aortic Endothelial Cells to Oxidized Phospholipids. Arteriosclerosis, Thrombosis, and Vascular Biology 2012;32:1246-54.
50. Lezama R et al. Epidermal growth factor receptor is a common element in the signaling pathways activated by cell volume changes in isosmotic, hyposmotic or hyperosmotic conditions. Neurochem Res 2005;30:1589-97.
51. Iwasaki H et al. Mechanical stretch stimulates growth of vascular smooth muscle cells via epidermal growth factor receptor. Am J Physiol Heart Circ Physiol 2000;278:H521-H529.
52. Yamauchi T et al. Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. Nature 1997;390:91-6.
53. Yamauchi T et al. Constitutive Tyrosine Phosphorylation of ErbB-2 via Jak2 by Autocrine Secretion of Prolactin in Human Breast Cancer. Journal of Biological Chemistry 2000;275:33937-44.
54. Prenzel N et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 1999;402:884-8.
55. Stover DR et al. Src Phosphorylation of the Epidermal Growth Factor Receptor at Novel Sites Mediates Receptor Interaction with Src and P85 alpha. Journal of Biological Chemistry 1995;270:15591-7.
56. Biscardi JS et al. c-Src-mediated Phosphorylation of the Epidermal Growth Factor Receptor on Tyr845 and Tyr1101 Is Associated with Modulation of Receptor Function. Journal of Biological Chemistry 1999;274:8335-43.
57. Huang Y et al. Growth hormone-induced phosphorylation of epidermal growth factor (EGF) receptor in 3T3-F442A cells. Modulation of EGF-induced trafficking and signaling. J Biol Chem 2003;278:18902-13.
58. Noma T et al. Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. J Clin Invest 2007;117:2445-58.
59. Kim J et al. Independent beta-Arrestin2 and G(q)/Protein Kinase C-zeta Pathways for ERK Stimulated by Angiotensin Type 1A Receptors in Vascular Smooth Muscle Cells Converge on Transactivation of the Epidermal Growth Factor Receptor. The Journal of Biological Chemistry 2009;284:11953-62.
60. Overland AC, Insel PA. Heterotrimeric G proteins directly regulate MMP14/membrane type-1 matrix metalloprotease: a novel mechanism for GPCR-EGFR transactivation. J Biol Chem 2015;290:9941-7.
61. Amorino GP et al. Neurotensin stimulates mitogenesis of prostate cancer cells through a novel c-Src//Stat5b pathway. Oncogene 2006;26:745-56.
62. Barton M, Meyer MR. Nicolaus Copernicus and the rapid vascular responses to aldosterone. Trends in Endocrinology & Metabolism 2015;26:396-8.
63. Carpenter G. EGF Receptor Transactivation Mediated by the Proteolytic Production of EGF-like Agonists. Sci STKE 2000;2000:e1.

64. Gschwind A et al. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. Oncogene 2001;20:1594-600.
65. Gschwind A et al. Lysophosphatidic Acid-induced Squamous Cell Carcinoma Cell Proliferation and Motility Involves Epidermal Growth Factor Receptor Signal Transactivation. Cancer Res 2002;62:6329.
66. McCole DF et al. Transactivation of the Epidermal Growth Factor Receptor in Colonic Epithelial Cells by Carbachol Requires Extracellular Release of Transforming Growth Factor-alpha. Journal of Biological Chemistry 2002;277:42603-12.
67. Daub H et al. Signal characteristics of G protein-transactivated EGF receptor. Embo Journal 1997;16:7032-44.
68. Eguchi S, Inagami T. Signal transduction of angiotensin II type 1 receptor through receptor tyrosine kinase. Regulatory Peptides 2000;91:13-20.
69. Teixeira AL et al. EGFR signaling pathway and related-miRNAs in age-related diseases: the example of miR-221 and miR-222. Front Genet 2012;3:286. doi: 10.3389/fgene.2012.00286.:286.
70. Luttrell LM et al. Gbeta-gamma Subunits Mediate Src-dependent Phosphorylation of the Epidermal Growth Factor Receptor: A scaffold for G protein-coupled receptor-mediated ras activation. Journal of Biological Chemistry 1997;272:4637-44.
71. Iwamoto R et al. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. Proc Natl Acad Sci U S A 2003;100:3221-6.
72. Goishi K et al. Inhibition of zebrafish epidermal growth factor receptor activity results in cardiovascular defects. Mech Dev 2003;120:811-22.
73. Chen B et al. Mice mutant for Egfr and Shp2 have defective cardiac semilunar valvulogenesis. Nat Genet 2000;24:296-9.
74. Gassmann M et al. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 1995;378:390-4.
75. Lee KF et al. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 1995;378:394-8.
76. Erickson SL et al. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2- and heregulin-deficient mice. Development 1997;124:4999-5011.
77. Iwamoto R, Mekada E. ErbB and HB-EGF signaling in heart development and function. Cell Struct Funct 2006;31:1-14.
78. Jackson LF et al. Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. EMBO J 2003;22:2704-16.
79. Negro A et al. erbB2 is required for G protein-coupled receptor signaling in the heart. Proceedings of the National Academy of Sciences of the United States of America 2006;103:15889-93.

80. Negro A et al. Essential roles of Her2/erbB2 in cardiac development and function. Recent Progress in Hormone Research, Vol 59 2004;59:1-12.
81. Threadgill DW et al. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 1995;269:230-4.
82. Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 1995;269:234-8.
83. Keeler CE. A second rexoid coat character in the house mouse. Journal of Heredity 1935;26:189-91.
84. Luetteke NC et al. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev 1994;8:399-413.
85. Fowler KJ et al. A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. Proc Natl Acad Sci U S A 1995;92:1465-9.
86. Wang DZ et al. Mutation in Sos1 dominantly enhances a weak allele of the EGFR, demonstrating a requirement for Sos1 in EGFR signaling and development. Genes & Development 1997;11:309-20.
87. Fitch KR et al. Genetics of dark skin in mice. Genes & Development 2003;17:214-28.
88. Thaung C et al. Novel ENU-induced eye mutations in the mouse: models for human eye disease. Human Molecular Genetics 2002;11:755-67.
89. Lee D et al. Wa5 is a novel ENU-induced antimorphic allele of the epidermal growth factor receptor. Mammalian Genome 2004;15:525-36.
90. Zhao Yy et al. Neuregulin Signaling in the Heart: Dynamic Targeting of erbB4 to Caveolar Microdomains in Cardiac Myocytes. Circulation Research 1999;84:1380-7.
91. Zhao Yy et al. Neuregulins Promote Survival and Growth of Cardiac Myocytes: Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. Journal of Biological Chemistry 1998;273:10261-9.
92. Srinivasan R et al. Expression of the c-erbB-4/HER4 protein and mRNA in normal human fetal and adult tissues and in a survey of nine solid tumour types. J Pathol 1998;185:236-45.
93. Ulu N et al. Epidermal growth factor receptor inhibitor PKI-166 governs cardiovascular protection without beneficial effects on the kidney in hypertensive 5/6 nephrectomized rats. J Pharmacol Exp Ther 2013;345:393-403.
94. Kagiyama S et al. Antisense to epidermal growth factor receptor prevents the development of left ventricular hypertrophy. Hypertension 2003;41:824-9.
95. Pareja M et al. Activated epidermal growth factor receptor (ErbB1) protects the heart against stress-induced injury in mice. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 2003;285:R455-R462.

96. Howes AL et al. G alpha q expression activates EGFR and induces Akt mediated cardiomyocyte survival: dissociation from G alpha q mediated hypertrophy. Journal of Molecular and Cellular Cardiology 2006;40:597-604.
97. Grisanti LA et al. b-Adrenergic receptor-mediated transactivation of epidermal growth factor receptor decreases cardiomyocyte apoptosis through differential subcellular activation of ERK1/2 and Akt. Journal of Molecular and Cellular Cardiology 2014;72:39-51
98. Liang D et al. EGFR inhibition protects cardiac damage and remodeling through attenuating oxidative stress in STZ-induced diabetic mouse model. Journal of Molecular and Cellular Cardiology 2015;82:63-74.
99. Galan M et al. A Novel Role for Epidermal Growth Factor Receptor Tyrosine Kinase and Its Downstream Endoplasmic Reticulum Stress in Cardiac Damage and Microvascular Dysfunction in Type 1 Diabetes Mellitus. Hypertension 2012;60:71-80.
100. Shah BH, Catt KJ. A central role of EGF receptor transactivation in angiotensin II - induced cardiac hypertrophy. Trends Pharmacol Sci 2003;24:239-44.
101. Akhtar S et al. Activation of EGFR/ERBB2 via Pathways Involving ERK1/2, P38 MAPK, AKT and FOXO Enhances Recovery of Diabetic Hearts from Ischemia-Reperfusion Injury. Plos One 2012;7:e39066.
102. Liao JK. Shedding growth factors in cardiac hypertrophy. Nature Medicine 2002;8:20-1.
103. Barrick CJ et al. Reduced EGFR causes abnormal valvular differentiation leading to calcific aortic stenosis and left ventricular hypertrophy in C57BL/6J but not 129S1/SvImJ mice. Am J Physiol Heart Circ Physiol 2009;297:H65-H75.
104. Griol-Charhbili V et al. Epidermal growth factor receptor mediates the vascular dysfunction but not the remodeling induced by aldosterone/salt. Hypertension 2011;57:238-44.
105. Grisanti LA et al. Temporal and gefitinib-sensitive regulation of cardiac cytokine expression via chronic beta-adrenergic receptor stimulation. Am J Physiol Heart Circ Physiol 2015;308:H316-H330.
106. Thomas WG et al. Adenoviral-directed expression of the type 1A angiotensin receptor promotes cardiomyocyte hypertrophy via transactivation of the epidermal growth factor receptor. Circ Res 2002;90:135-42.
107. Stern LE et al. Salivary epidermal growth factor and intestinal adaptation in male and female mice. Am J Physiol Gastrointest Liver Physiol 2000;278:G871.
108. Lazar LM, Blum M. Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-alpha mRNA in mouse brain by a quantitative nuclease protection assay. J Neurosci 1992;12:1688-97.

109. Fukazawa R et al. Neuregulin-1 protects ventricular myocytes from anthracycline-induced apoptosis via erbB4-dependent activation of PI3-kinase/Akt. Journal of Molecular and Cellular Cardiology 2003;35:1473-9.
110. Chen H et al. beta2-Adrenergic receptor-induced transactivation of epidermal growth factor receptor and platelet-derived growth factor receptor via Src kinase promotes rat cardiomyocyte survival. Cell Biol Int 2012;36:237-44.
111. Tsutsui H et al. Oxidative stress and heart failure. American Journal of Physiology-Heart and Circulatory Physiology 2011;301:H2181-H2190.
112. Krieg T et al. Mitochondrial ROS generation following acetylcholine-induced EGF receptor transactivation requires metalloproteinase cleavage of proHB-EGF. J Mol Cell Cardiol 2004;36:435-43.
113. Armstrong EJ, Bischoff J. Heart valve development: endothelial cell signaling and differentiation. Circ Res 2004;95:459-70.
114. Schroeder JA et al. Form and function of developing heart valves: coordination by extracellular matrix and growth factor signaling. Journal of Molecular Medicine-Jmm 2003;81:392-403.
115. Sun L et al. Grb2-associated binder 1 is essential for cardioprotection against ischemia/reperfusion injury. Basic Res Cardiol 2014;109:420-0420.
116. Methner C et al. Cardioprotection of bradykinin at reperfusion involves transactivation of the epidermal growth factor receptor via matrix metalloproteinase-8. Acta Physiol (Oxf) 2009;197:265-71.
117. Hausenloy DJ et al. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am J Physiol Heart Circ Physiol 2005;288:H971.
118. Forster K et al. The delta-opioid receptor agonist DADLE at reperfusion protects the heart through activation of pro-survival kinases via EGF receptor transactivation. Am J Physiol Heart Circ Physiol 2007;293:H1604-H1608.
119. Fujiu K, Nagai R. Fibroblast-mediated pathways in cardiac hypertrophy. Journal of Molecular and Cellular Cardiology 2014; 70:64-73.
120. Correa MV et al. Endogenous endothelin 1 mediates angiotensin II-induced hypertrophy in electrically paced cardiac myocytes through EGFR transactivation, reactive oxygen species and NHE-1. Pflugers Arch 2014;466:1819-30.
121. Zeng SY et al. Upregulation of Nox4 Promotes Angiotensin II-Induced Epidermal Growth Factor Receptor Activation and Subsequent Cardiac Hypertrophy by Increasing ADAM17 Expression. Canadian Journal of Cardiology 2013;29:1310-9.
122. Kleine-Brueggeney M et al. Alpha1a-adrenoceptor genetic variant induces cardiomyoblast-to-fibroblast-like cell transition via distinct signaling pathways. Cellular Signalling 2014; 6(9):1985-97.
123. Xu X et al. Growth Differentiation Factor (GDF)-15 Blocks Norepinephrine-induced Myocardial Hypertrophy via a Novel Pathway Involving Inhibition of Epidermal

Growth Factor Receptor Transactivation. Journal of Biological Chemistry 2014;289:10084-94.
124. Anderson HD et al. Role of the epidermal growth factor receptor in signaling strain-dependent activation of the brain natriuretic peptide gene. J Biol Chem 2004;279:9287-97.
125. Miao Y et al. Acetylcholine inhibits tumor necrosis factor alpha activated endoplasmic reticulum apoptotic pathway via EGFR-PI3K signaling in cardiomyocytes. J Cell Physiol 2015;230:767-74.
126. Xu Z, Cai L. Diabetic cardiomyopathy: Role of epidermal growth factor receptor tyrosine kinase. Journal of Molecular and Cellular Cardiology;84:10-2.
127. Kodama H et al. Selective Involvement of p130Cas/Crk/Pyk2/c-Src in Endothelin-1-Induced JNK Activation. Hypertension 2003;41:1372-9.
128. Chan HW et al. Effect of Dominant-Negative Epidermal Growth Factor Receptors on Cardiomyocyte Hypertrophy. Journal of Receptors and Signal Transduction 2006;26:659-77.
129. Li Y et al. Transactivated EGFR mediates alpha(1)-AR-induced STAT3 activation and cardiac hypertrophy. American Journal of Physiology-Heart and Circulatory Physiology 2011;301:H1941-H1951.
130. Chen M et al. Time-dependent regulation of neuregulin-1b/ErbB/ERK pathways in cardiac differentiation of mouse embryonic stem cells. Mol Cell Biochem 2013;380:67-72.
131. Mann DL. Mechanisms and Models in Heart Failure. Circulation 1999;100:999.
132. Chen CH et al. Reactive oxygen species generation is involved in epidermal growth factor receptor transactivation through the transient oxidization of Src homology 2-containing tyrosine phosphatase in endothelin-1 signaling pathway in rat cardiac fibroblasts. Mol Pharmacol 2006;69:1347-55.
133. Zhang H et al. Heparin-binding epidermal growth factor-like growth factor signaling in flow-induced arterial remodeling. Circ Res 2008;102:1275-85.
134. Chan AK et al. Blockade of the epidermal growth factor receptor decreases intimal hyperplasia in balloon-injured rat carotid artery. J Vasc Surg 2003;37:644-9.
135. Helle F et al. Improvement of renal hemodynamics during hypertension-induced chronic renal disease: role of EGF receptor antagonism. Am J Physiol Renal Physiol 2009;297:F191-F199.
136. Benter IF et al. Early inhibition of EGFR signaling prevents diabetes-induced up-regulation of multiple gene pathways in the mesenteric vasculature. Vascular Pharmacology 2009;51:236-45.
137. Krug AW et al. Elevated mineralocorticoid receptor activity in aged rat vascular smooth muscle cells promotes a proinflammatory phenotype via extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase and epidermal growth factor receptor-dependent pathways. Hypertension 2010;55:1476-83.

138. Chan SL et al. Epidermal Growth Factor Receptor Is Critical For Angiotensin II-Mediated Hypertrophy in Cerebral Arterioles. Hypertension 2015;65:806-12.
139. Kagiyama S et al. Angiotensin II-induced cardiac hypertrophy and hypertension are attenuated by epidermal growth factor receptor antisense. Circulation 2002;106:909-12.
140. Sambhi MP et al. Increased EGF binding and EGFR mRNA expression in rat aorta with chronic administration of pressor angiotensin II. Biochemical Medicine and Metabolic Biology 1992;48:8-18.
141. Benter IF et al. Global upregulation of gene expression associated with renal dysfunction in DOCA-salt-induced hypertensive rats occurs via signaling cascades involving epidermal growth factor receptor: A microarray analysis. Vascular Pharmacology 2009;51:101-9.
142. Jamroz-Wisniewska A et al. Transactivation of epidermal growth factor receptor in vascular and renal systems in rats with experimental hyperleptinemia: role in leptin-induced hypertension. Biochem Pharmacol 2008;75:1623-38.
143. Belmadani S et al. Microvessel vascular smooth muscle cells contribute to collagen type I deposition through ERK1/2 MAP kinase, alphavbeta3-integrin, and TGF-beta1 in response to ANG II and high glucose. Am J Physiol Heart Circ Physiol 2008;295:H69-H76.
144. Lucchesi PA et al. Involvement of Metalloproteinases 2/9 in Epidermal Growth Factor Receptor Transactivation in Pressure-Induced Myogenic Tone in Mouse Mesenteric Resistance Arteries. Circulation 2004;110:3587-93.
145. Amin AH et al. Mechanisms of myogenic tone of coronary arteriole: Role of down stream signaling of the EGFR tyrosine kinase. Microvasc Res 2011;81:135-42.
146. Jang E et al. 3´,4´-Dimethoxythioflavone induces endothelium-dependent vasorelaxation through activation of epidermal growth factor receptor. Naunyn-Schmiedeberg's Arch Pharmacol 2013;386:339-50.
147. Beaucage P, Moreau P. EGF receptor transactivation in angiotensin II and endothelin control of vascular protein synthesis in vivo. J Cardiovasc Pharmacol 2004;44 Suppl 1:S20-3.:S20-S23.
148. Intengan HD, Schiffrin EL. Vascular Remodeling in Hypertension: Roles of Apoptosis, Inflammation, and Fibrosis. Hypertension 2001;38:581-7.
149. Takaguri A et al. A Disintegrin and Metalloprotease 17 Mediates Neointimal Hyperplasia in Vasculature. Hypertension 2011;57:841-5.
150. Ivanov A et al. Adenylate cyclase 5 and KCa1.1 channel are required for EGFR up-regulation of PCNA in native contractile rat basilar artery smooth muscle. J Physiol 2006;570:73-84.
151. Swaminathan N et al. Elevated epidermal growth factor receptor levels in hypertensive Lyon rat kidney and aorta. Clin Exp Pharmacol Physiol 1996;23:793-6.

152. Kim S et al. Cardiovascular effects of combination of perindopril, candesartan, and amlodipine in hypertensive rats. Hypertension 2000;35:769-74.
153. Hao L et al. Vascular responses to alpha1-adrenergic receptors in small rat mesenteric arteries depend on mitochondrial reactive oxygen species. Arterioscler Thromb Vasc Biol 2006;26:819-25.
154. Hao L et al. Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor-receptor pathway. Circ Res 2004;94:68-76.
155. Berk BC et al. Epidermal growth factor, a vascular smooth muscle mitogen, induces rat aortic contraction. J Clin Invest 1985;75:1083-6.
156. Batenburg WW et al. Angiotensin II-aldosterone interaction in human coronary microarteries involves GPR30, EGFR, and endothelial NO synthase. Cardiovascular Research 2012;94:136-43.
157. Grossmann C et al. Aldosterone-induced EGFR expression: interaction between the human mineralocorticoid receptor and the human EGFR promoter. Am J Physiol Endocrinol Metab 2007;292:E1790-E1800.
158. Kim J et al. Epidermal growth factor induces vasoconstriction through the phosphatidylinositol 3-kinase-mediated mitogen-activated protein kinase pathway in hypertensive rats. J Pharmacol Sci 2006;101:135-43.
159. Lorita J et al. Effects of epidermal growth factor on epinephrine-stimulated heart function in rodents. American Journal of Physiology-Heart and Circulatory Physiology 2002;283:H1887-H1895.
160. Krug AW et al. Aldosterone stimulates epidermal growth factor receptor expression. J Biol Chem 2003;278:43060-6.
161. Lemarie CA et al. Transforming growth factor-alpha mediates nuclear factor kappaB activation in strained arteries. Circ Res 2006;99:434-41.
162. Belmadani S et al. Elevated epidermal growth factor receptor phosphorylation induces resistance artery dysfunction in diabetic db/db mice. Diabetes 2008;57:1629-37.
163. Hwa JJ, Bevan JA. Stretch-dependent (myogenic) tone in rabbit ear resistance arteries. Am J Physiol Heart Circ Physiol 1986;250:H87.
164. Matrougui K et al. Impaired Nitric Oxide- and Prostaglandin-Mediated Responses to Flow in Resistance Arteries of Hypertensive Rats. Hypertension 1997;30:942-7.
165. Simons M, Eichmann A. Molecular Controls of Arterial Morphogenesis. Circulation Research 2015;116:1712-24.
166. Chalothorn D et al. Heparin-binding epidermal growth factor-like growth factor, collateral vessel development, and angiogenesis in skeletal muscle ischemia. Arterioscler Thromb Vasc Biol 2005;25:1884-90.

167. Hewing NJ et al. Intravitreal Injection of TIMP3 or the EGFR Inhibitor Erlotinib Offers Protection from Oxygen-Induced Retinopathy in Mice. Invest Ophthalmol Vis Sci 2013;54:864-70.
168. Weskamp G et al. Pathological neovascularization is reduced by inactivation of ADAM17 in endothelial cells but not in pericytes. Circ Res 2010;%19;106:932-40.
169. Stanic B et al. Increased Epidermal Growth Factor-Like Ligands Are Associated With Elevated Vascular Nicotinamide Adenine Dinucleotide Phosphate Oxidase in a Primate Model of Atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology 2012;32:2452-60.
170. Dreux AC et al. The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. Atherosclerosis 2006;186:38-53.
171. Takahashi M et al. Epiregulin as a Major Autocrine/Paracrine Factor Released From ERK- and p38MAPK-Activated Vascular Smooth Muscle Cells. Circulation 2003;108:2524.
172. Gao P et al. Induction of oxidative stress by oxidized LDL via meprin alpha-activated epidermal growth factor receptor in macrophages. Cardiovascular Research 2013;97:533-43.
173. Fuller PJ, Young MJ. Mechanisms of mineralocorticoid action. Hypertension 2005;46:1227-35.
174. Anand-Apte B et al. Betacellulin Induces Increased Retinal Vascular Permeability in Mice. Plos One 2010;5:e13444.
175. Li YA et al. Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR. American Journal of Physiology-Heart and Circulatory Physiology 2010;299:H1959-H1967.
176. Andresen BT et al. Role of EGFR transactivation in angiotensin II signaling to extracellular regulated kinase in preglomerular smooth muscle cells. Hypertension 2003;41:781-6.
177. Lauzier MC et al. Differential Regulation of Hypoxia-Inducible Factor-1 through Receptor Tyrosine Kinase Transactivation in Vascular Smooth Muscle Cells. Endocrinology 2007;148:4023-31.
178. Haendeler J et al. GIT1 mediates Src-dependent activation of phospholipase Cgamma by angiotensin II and epidermal growth factor. J Biol Chem 2003;278:49936-44.
179. Eguchi S et al. Activation of MAPKs by Angiotensin II in Vascular Smooth Muscle Cells: Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. Journal of Biological Chemistry 2001;276:7957-62.

180. Limor R et al. Aldosterone up-regulates 12- and 15-lipoxygenase expression and LDL oxidation in human vascular smooth muscle cells. J Cell Biochem 2009;108:1203-10.
181. Kawanabe Y et al. Involvement of extracellular Ca2+ influx and epidermal growth factor receptor tyrosine kinase transactivation in endothelin-1-induced arachidonic acid release. Br J Pharmacol 2003;139:1516-22.
182. Kawanabe Y et al. Characterization of Ca2+ channels involved in ET-1-induced transactivation of EGF receptors. Am J Physiol Heart Circ Physiol 2002;283:H2671-H2675.
183. Iwasaki H et al. Endothelin-1 stimulates DNA synthesis of vascular smooth-muscle cells through transactivation of epidermal growth factor receptor. J Cardiovasc Pharmacol 1998;31 Suppl 1:S182-S184.
184. Lin CC et al. Transactivation of EGFR/PI3K/Akt involved in ATP-induced inflammatory protein expression and cell motility. J Cell Physiol 2012;227:1628-38.
185. Bakken AM et al. Cell migration in response to the amino-terminal fragment of urokinase requires epidermal growth factor receptor activation through an ADAM-mediated mechanism. Journal of Vascular Surgery 2009;49:1296-303.
186. Koide M et al. Heparin-binding EGF-like growth factor mediates oxyhemoglobin-induced suppression of voltage-dependent potassium channels in rabbit cerebral artery myocytes. Am J Physiol Heart Circ Physiol 2007;293:H1750-H1759.
187. Dreymueller D et al. Smooth Muscle Cells Relay Acute Pulmonary Inflammation via Distinct ADAM17/ErbB Axes. The Journal of Immunology 2014; 192(2):722-31.
188. Beltowski J. Leptin and the Regulation of Renal Sodium Handling and Renal Na-Transporting ATPases: Role in the Pathogenesis of Arterial Hypertension. Curr Cardiol Rev 2010;6:31-40.
189. Liao PL et al. 7-Ketocholesterol and cholesterol-5alpha,6alpha-epoxide induce smooth muscle cell migration and proliferation through the epidermal growth factor receptor/phosphoinositide 3-kinase/Akt signaling pathways. Toxicol Lett 2010;197:88-96.
190. Haas A et al. Src-mediated inter-receptor cross-talk between the Na+/K+-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. Journal of Biological Chemistry 2002;277:18694-702.
191. Hong Z et al. Activation of the EGFR/p38/JNK pathway by mitochondrial-derived hydrogen peroxide contributes to oxygen-induced contraction of ductus arteriosus. J Mol Med (Berl) 2014;92:995-1007.
192. Agbani EO et al. Peroxynitrite stimulates pulmonary artery endothelial and smooth muscle cell proliferation: Involvement of ERK and PKC. Pulmonary Pharmacology & Therapeutics 2011;24:100-9.
193. Miller-Fleming L et al. Remaining Mysteries of Molecular Biology: The Role of Polyamines in the Cell. Journal of Molecular Biology 2015;427:3389-406.

194. Wong MM et al. Sirolimus Stimulates Vascular Stem/Progenitor Cell Migration and Differentiation Into Smooth Muscle Cells via Epidermal Growth Factor Receptor/Extracellular Signal-Regulated Kinase/beta-Catenin Signaling Pathway. Arterioscler Thromb Vasc Biol 2013;33:2397-406.
195. Forrester SJ et al. Epidermal Growth Factor Receptor Transactivation: Mechanisms, Pathophysiology, and Potential Therapies in the Cardiovascular System. Annu Rev Pharmacol Toxicol 2016; 56:627-53.
196. Flamant M et al. Epidermal growth factor receptor trans-activation mediates the tonic and fibrogenic effects of endothelin in the aortic wall of transgenic mice. FASEB J 2003;17:327-9.
197. Catena C et al. Aldosterone, Organ Damage, and Dietary Salt. Clin Exp Pharmacol Physiol 2013;40:922-8.
198. Alderman MH, Cohen HW. Dietary Sodium Intake and Cardiovascular Mortality: Controversy Resolved? American Journal of Hypertension 2012;25:727-34.
199. Reil JC et al. Aldosterone promotes atrial fibrillation. European Heart Journal 2012;33:2098-108.
200. Rossi GP et al. Excess aldosterone Is Associated With Alterations of Myocardial Texture in Primary Aldosteronism. Hypertension 2002;40:23-7.
201. Mentz RJ et al. The past, present and future of reninGÇôangiotensin aldosterone system inhibition. International Journal of Cardiology 2013;167:1677-87.
202. Swedberg K et al. Eplerenone and Atrial Fibrillation in Mild Systolic Heart Failure: Results From the EMPHASIS-HF (Eplerenone in Mild Patients Hospitalization And SurvIval Study in Heart Failure) Study. Journal of the American College of Cardiology 2012;59:1598-603.
203. Hashim S et al. Small cytoplasmic domain peptides of natriuretic peptide receptor-C attenuate cell proliferation through Gialpha protein/MAP kinase/PI3-kinase/AKT pathways. Am J Physiol Heart Circ Physiol 2006;291:H3144-H3153.
204. Bianco A et al. Two distinct modes of guidance signalling during collective migration of border cells. Nature 2007;448:362-5.
205. Duchek P et al. Guidance of Cell Migration by the Drosophila PDGF/VEGF Receptor. Cell 2001;107:17-26.

Original Article

Loss of Epidermal Growth Factor Receptor in Vascular Smooth Muscle Cells and Cardiomyocytes Causes Arterial Hypotension and Cardiac Hypertrophy

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Abstract—The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, contributes to parainflammatory dysregulation, possibly causing cardiovascular dysfunction and remodeling. The physiological role of cardiovascular EGFR is not completely understood. To investigate the physiological importance of EGFR in vascular smooth muscle cells and cardiomyocytes, we generated a mouse model with targeted deletion of the EGFR using the SM22 (smooth muscle-specific protein 22) promoter. While the reproduction of knockout animals was not impaired, life span was significantly reduced. Systolic blood pressure was not different between the 2 genotypes—neither in tail cuff nor in intravascular measurements—whereas total peripheral vascular resistance, diastolic blood pressure, and mean blood pressure were reduced. Loss of vascular smooth muscle cell-EGFR results in a dilated vascular phenotype with minor signs of fibrosis and inflammation. Echocardiography, necropsy, and histology revealed a dramatic eccentric cardiac hypertrophy in knockout mice (2.5-fold increase in heart weight), with increased stroke volume and cardiac output as well as left ventricular wall thickness and lumen. Cardiac hypertrophy is accompanied by an increase in cardiomyocyte volume, a strong tendency to cardiac fibrosis and inflammation, as well as enhanced NADPH-oxidase 4 and hypertrophy marker expression. Thus, in cardiomyocytes, EGFR prevents excessive hypertrophic growth through its impact on reactive oxygen species balance, whereas in vascular smooth muscle cells EGFR contributes to the appropriate vascular wall architecture and vessel reactivity, thereby supporting a physiological vascular tone. **(*Hypertension*. 2013;61:000-000.)** ● **Online Data Supplement**

Key Words: blood pressure ■ growth substances ■ hypertrophy ■ receptors ■ smooth muscle

The epidermal growth factor receptor (EGFR) family consists of 4 related tyrosine kinase receptors, EGFR (ErbB1), ErbB2, ErbB3, and ErbB4.[1,2] On ligand binding, the receptors form homo- and heterodimers and activate various signaling pathways.[3] With the ability of EGFR to form functional dimers with all 4 family members, activated ErbB receptors control various signaling modules and their downstream targets, including mitogen-activated protein kinases, phosphoinositide-3 kinase, phospholipase C-γ, and cellular Src-kinase, thereby regulating cell proliferation, survival, differentiation, migration, and matrix homeostasis.[4]

In addition to activation by its classical ligands, EGFR is also subject to activation by cross-talk with nonreceptor tyrosine kinase pathways, a mechanism called transactivation.[1,5] Both mechanisms may induce pathophysiological effects that include cell proliferation and parainflammatory dysregulation of tissue homeostasis, leading, for example, to vascular dysfunction and remodeling.[6] In this regard, EGFR transactivation is supposed to be responsible for endothelin 1, angiotensin II (Ang II), or phenylephrine-mediated extracellular signal–regulated kinase1/2 phosphorylation[3] and their pathophysiological effects on vascular smooth muscle cell (VSMC) proliferation, migration, and matrix homeostasis.[7]

To elucidate the pivotal roles of the EGFR family members in development and tumorigenesis, gain- and loss-of-function mutants have been generated.[8] Their analysis revealed differential contribution of ErbB2, ErbB3, and ErbB4 to cardiac development.[9–12]

In contrast, until now only limited in-depth analysis of EGFR cardiovascular relevance has been obtained. Mice lacking the EGFR die at day 11.5 of gestation or survive until postnatal day 20, depending on the genetic background.[13] Surviving mutant mice show abnormalities in bone, heart, and epithelia of skin, hair follicles, and eyes.[13–19] The predominant heart phenotype in these mice, and in the *wa-2* mice (mice with a global reduced

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EGFR kinase activity), is a defect in valve formation.[17,20,21] As the function of the EGFR is impaired in all cell types in these models, it is difficult to draw specific conclusions regarding EGFR in different cardiovascular cell types.

The aim of the present study was to characterize the role of VSMC and myocardial EGFR (ErbB1) on cardiovascular function and tissue homeostasis. We hypothesized that EGFR contributes to the maintenance of physiological function and tissue homeostasis. To exclude interference with non-cardiovascular EGFR, we generated mice with a deletion of EGFR in VSMC and a strong reduction in cardiomyocytes ($EGFR^{\Delta/\Delta VSMC\&CM}$) and compared them with littermate control mice ($EGFR^{flox/flox}$). Here, we present the phenotype of these mice showing a reduced peripheral resistance, arterial hypotension, and cardiac hypertrophy.

Methods

See the online-only Data Supplement for details on methods.

Results

Expression of ErbB Receptors in Aortas and Hearts of $EGFR^{\Delta/\Delta VSMC\&CM}$ Mice

We generated a mouse model with a specific deletion of the EGFR in VSMC and a strong reduction in cardiomyocytes ($EGFR^{\Delta/\Delta VSMC\&CM}$, knockout [KO]) crossing $EGFR^{flox/flox}$ mice (con) with SM22-Cre mice ($SM22\text{-}Cre^{+/-}$; Figure S1A in the online-only Data Supplement).[22–26]

As demonstrated earlier,[22] EGFR expression was almost abolished in the aortas (Figure 1A), indicating a deletion of EGFR in VSMC. This result was confirmed by primary culture of VSMC demonstrating a reduction to 10±5% compared with controls.[22] In lung, liver, and kidney, EGFR expression was unchanged (Table S2). In the heart, where the SM22 promoter is active during embryogenesis,[25,26] expression of EGFR was reduced by one third (Figure 1A). To ensure that reduced cardiac EGFR expression resulted from cardiomyocytes, we analyzed isolated cardiomyocytes and observed a significant decrease in EGFR expression on mRNA level (Figure 1A) and protein level (41±13% of con; n=11; Figure 1C).[22]

Because EGF can also exert its intracellular effects by binding to a heterodimer consisting of the receptors ErbB2 and ErbB3, we analyzed mRNA expression for these receptors in hearts, aortas, and isolated cardiomyocytes. Only the expression of ErbB3 mRNA was reduced in aortas of KO animals (Table S2). Taken together, the data show a specific deletion of the EGFR in VSMC and a significant reduction in cardiomyocytes.

Population Parameters

$EGFR^{\Delta/\Delta VSMC\&CM}$ animals show a significantly reduced life span with an increased number of deaths starting from

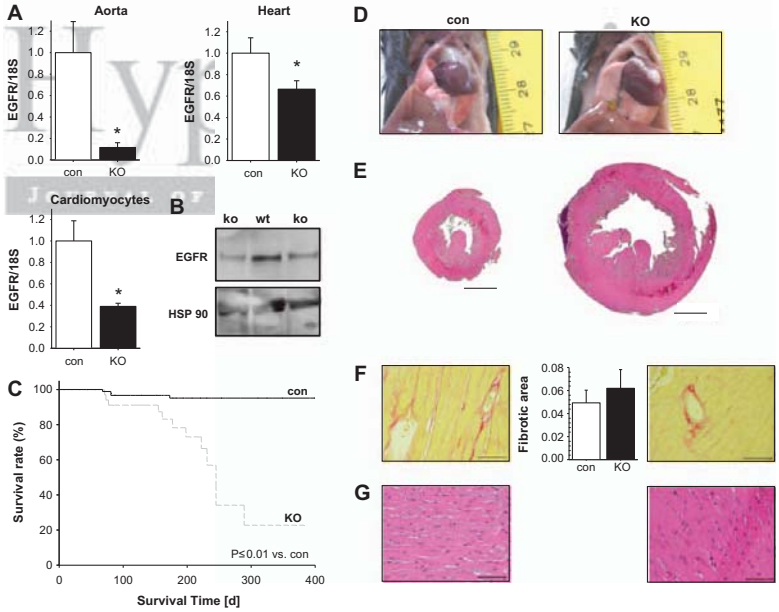

Figure 1. Epidermal growth factor receptor (EGFR) expression, heart morphology, and survival in $EGFR^{\Delta/\Delta VSMC\&CM}$ mice. **A**, In aortas of $EGFR^{\Delta/\Delta VSMC\&CM}$ animals devoid of connective tissue and endothelium, EGFR mRNA expression was reduced to 0.1 of control (n=11). In whole hearts (n=11) and in isolated cardiomyocytes (n=5), EGFR expression was reduced to ≈0.6 and ≈0.4, respectively. **P*≤0.05 vs control. **B**, The Kaplan-Meier plot shows a significantly reduced life span of $EGFR^{\Delta/\Delta VSMC\&CM}$, starting from day 100 postpartum (n=34–92 animals per genotype). **C**, Exemplary Western blot showing that EGFR is reduced in cardiomyocytes of $EGFR^{\Delta/\Delta VSMC\&CM}$ animals. Exemplary (**D**) dissection pictures from hearts of control and knockout animals showing the increase in heart size and cross-sectional dimensions of the left heart chamber (**E**). In histological semithin sections (**F**), no increase in Sirius red–stained area was obtained, whereas (**G**) hematoxylin and eosin staining revealed a slight but significant increase in cardiomyocyte diameter (×40 magnification; scale bar, 1 mm for **E** and 50 μm for **F** and **G**). n=10 to 11 per group, **P*≤0.05 vs control.

Table. Aortic and Cardiac mRNA Expression of Different Hypertrophy, Fibrosis, Inflammation, Ca^{2+} Handling, and Nutrient Supply–Related Markers

Gene	Control (n=11)	Knockout (n=11)
Aortas		
Fibrosis		
Col1a1/18S	1.0±0.3	1.4±0.4
Col3a1/18S	1.0±0.2	2.7±0.8*
FN-1/18S	1.0±0.2	1.0±0.2
PAI-1/18S	1.0±0.2	2.0±0.9
Inflammation		
TNF-α/18S	1.0±0.4	8.0±4.4
MCP-1/18S	1.0±0.3	6.3±2.9*
SPP-1/18S	1.0±0.2	0.8±0.2
Hearts		
Hypertrophy		
ANP/18S	1.0±0.5	5.4±1.9*
BNP/18S	1.0±0.2	3.6±0.8*
βMHC/αMHC	1.0±0.4	3.3±1.0*
Fibrosis		
Col 1a1/18S	1.0±0.2	2.8±1.0
Col3a1/18S	1.0±0.4	2.8±1.6
FN-1/18S	1.0±0.6	1.7±0.9
PAI-1/18S	1.0±0.2	2.3±0.7
Inflammation		
TNF-α/18S	1.0±0.7	0.5±0.3
MCP-1/18S	1.0±0.3	2.3±1.1
Calcium handling		
Serca/18S	1.0±0.2	0.9±0.1
NCX/18S	1.0±0.1	1.2±0.1
DHPR/18S	1.0±0.2	0.9±0.1
RyR/18S	1.0±0.2	1.0±0.1
Nutrient supply		
HIF1α/18S	1.0±0.2	1.0±0.1
GLUT1/18S	1.0±0.1	1.1±0.1

TNF indicates tumor necrosis factor.

*$P \leq 0.05$ vs control.

day 100 (Figure 1B). For further description of the gross population phenotype, see the online-only Data Supplement (Tables S3 and S4).

Organ Weights

Compared with controls, heart weight/tibia length of KO animals was increased 2.5-fold (Figures 1D, 1E, and 2A). In addition, lung weight was also increased significantly (Table S3).

Blood Pressure and Heart Rate

Activation of the EGFR may not only affect proliferation of VSMC but also VSMC function. Therefore, we investigated blood pressure. There was no difference in systolic blood pressure (SBP) and heart rate in conscious animals determined by the tail cuff method as indicated by preliminary measurements.[22] As tail cuff does not allow reliable assessment of diastolic blood pressure (DBP), we performed measurements in anesthetized animals using a Millar catheter. During ketamine/xylazine anesthesia, we detected a reduced DBP in KO animals, as well as mean arterial blood pressure. Systolic pressure was not different. Blood pressure amplitude was increased in KO animals (Figure 2B).

Echocardiography

Echocardiography revealed a significant increase in left ventricular wall thickness and lumen dimensions in EGFR$^{\Delta/\Delta VSMC\&CM}$ mice (Table S3). Nevertheless, ejection fraction or fractional shortening was similar in both genotypes. Besides the increase in end-diastolic and end-systolic volume, stroke volume in KO animals was higher than in control animals (Figure 2C), resulting in an increased cardiac output. Assuming a comparable right ventricular pressure, total peripheral resistance can be calculated by Ohm's law and is reduced in KO animals (con, ≈2.75±0.15 mm Hg · min · mL^{-1}; KO, ≈1.68±0.13 mm Hg · min · mL^{-1}).

Calculated systolic left ventricular wall stress (law of Laplace) was not different compared with control animals (con, 74.5±3.5 mm Hg; KO, 75.6±12.4 mm Hg; n=6–14 animals/genotype)

Estimated from pulse-wave Doppler recordings, the peak velocity and mean velocity of blood in the descending aorta were higher in KO animals compared with controls, whereas the velocity time integral was unaffected (Figure 3).

Histology of Hearts, Lungs, and Aorta

In sections, left ventricular wall thickness and left ventricular diameter were increased, confirming the echocardiography data of left ventricular hypertrophy and dilation (Figure 1D and Table S3). Cardiomyocyte diameter was increased in KO animals compared with controls (Figures 1G and 2A), albeit to a smaller degree compared with cardiac hypertrophy. Therefore, we analyzed length and area from isolated cardiomyocytes revealing a comparable relative increase in cardiomyocyte volume and heart weight/tibia length (Table S3). The increases in cardiomyocyte area and diameter result in an increase in cardiomyocyte volume—assuming a cylindrical shape of the cardiomyocyte—of ≈2.2±0.4-fold, thus explaining the increased heart weight.

No significant difference in interstitial fibrosis between controls and KO animals was observed in heart and lung sections (Figure 1F and Table S3).

Preliminary data on the histology of aortas and intramyocardial vessels indicated a difference in aortic wall/lumen area ratio.[22] In the complete series presented here, the aortic diameter determined by echocardiography (aortic sinus, n=4–11/group; Figure 3) and histology (abdominal aorta, n=10/group; Figure 4) was not different between the genotypes. Media thickness was slightly reduced in EGFR$^{\Delta/\Delta VSMC\&CM}$ animals (Figure 4A and 4B), resulting in a significant 30% decrease in the wall-to-lumen ratio of aortas from EGFR$^{\Delta/\Delta VSMC\&CM}$ animals. Consequently, aortic wall stress (law of Laplace) was increased slightly (≈1.18 of con).

Wall-to-lumen ratio of intramyocardial arteries from KO animals was also reduced compared with controls, but in

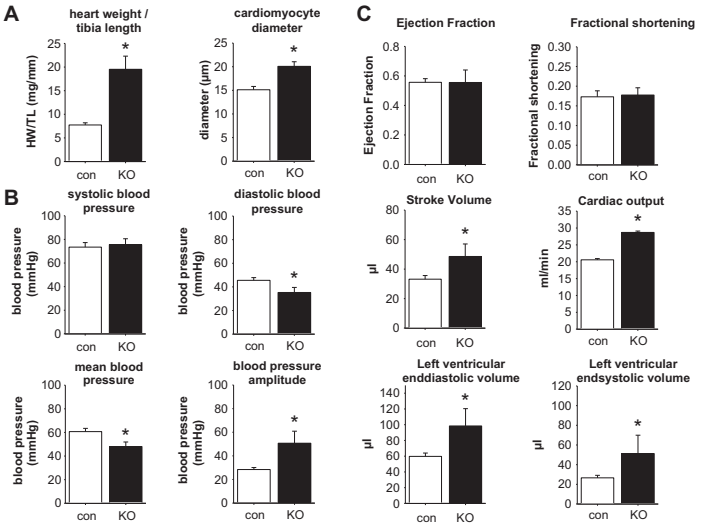

Figure 2. Blood pressure and heart function of control and epidermal growth factor receptor (EGFR)$^{\Delta/\Delta VSMC\&CM}$ animals. **A**, Heart weight/tibia length was increased in knockout animals, as well as cardiomyocyte diameter, determined in hematoxylin and eosin–stained semithin sections of the left ventricle. **B,** Systolic blood pressure was the same between both genotypes in intravascular blood pressure measurements performed with a Millar catheter. Diastolic blood pressure, as well as mean blood pressure, was significantly reduced in knockout animals, whereas the amplitude of blood pressure was increased. **C**, Echocardiography studies showed that left ventricular systolic function was not impaired in knockout animals. Stroke volume, as well as end-diastolic and end-systolic left ventricular volume, was increased in knockout animals. n=6 to 14/group, **P*≤0.05 vs control.

contrast to the aortas the luminal diameter was increased (Figure 4C). Calculated wall stress of intramyocardial arteries is significantly increased in KO animals (con, 105.3±7.0 mm Hg; KO, 172.4±20.0 mm Hg; n=9–10; *P*≤0.05).

mRNA Expression

In hearts of KO animals, mRNA expression of hypertrophy markers (atrial natriuretic peptide, B-type natriuretic peptide, β-myosin heavy chain/α-myosin heavy chain) was increased (Table). In contrast, the increase in heart size was not accompanied by significantly enhanced expression of markers of cardiac fibrosis or inflammation nor genes involved in calcium handling. However, the markers for cardiac fibrosis showed a tendency to increased expression. In addition, we detected a significant increase in cardiac NADPH-oxidase 4 (NOX4) expression in KO animals to 6.51±0.62 of control (n=5), whereas NOX2 mRNA was not significantly different from control.

Furthermore, the mRNA for genes indicating reduced nutrient supply was not increased either (Table and Table S5).

The Table shows that mRNA of aortic Col3a1 and monocyte chemoattractant protein-1 was significantly enhanced and tumor necrosis factor-α showed a strong tendency to increased expression in KO animals. There were no changes in markers for fibrosis or inflammation in the kidney (Table S6).

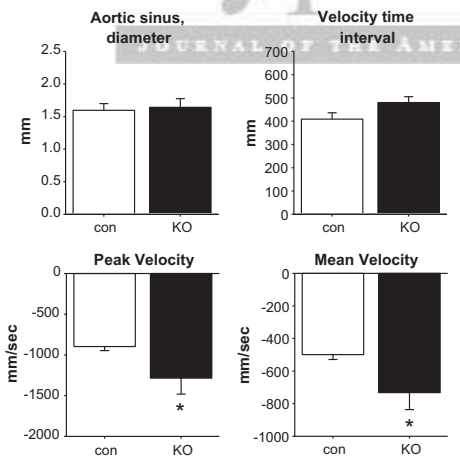

Figure 3. Estimation of intravital aortic dimension and blood flow velocity parameters by echocardiography. Aortic sinus diameter was measured during heart ventricle diastole in B-mode. There was no significant difference between the 2 genotypes. Velocity parameters were obtained from ≥5 heart cycles in the descending aortas. Although the velocity time interval was not different between the 2 genotypes, peak velocity and mean velocity of blood flow were significantly increased in *EGFR*$^{\Delta/\Delta VSMC\&CM}$ animals. n=4 to 11/group, **P*≤0.05 vs control.

Aortic Rings

Aortic rings from control animals responded with an increase in force generation during application of EGF. This response was abolished in KO animals, confirming the successful EGFR deletion (Figure 5).

Blood Pressure Increase on Acute Ang II Infusion

On infusion of Ang II, mean arterial pressure (MAP) increased initially to the same extent in wild-type (WT) and EGFR$^{\Delta/\Delta VSMC\&CM}$ mice (Figure 6), whereas the time needed to reach the maximal increase of blood pressure was significantly shorter in KO animals than in WT animals. Furthermore, the time until the blood pressure fell below the half maximal value thereafter (ΔP/2) was significantly shorter in KO animals, resulting in a reduced plateau phase of blood pressure increase.

Reactive Oxygen Species Production in Lysates of Mouse Hearts

Lucigenin assays of whole heart lysates of WT and KO animals revealed a significant increase in reactive oxygen species

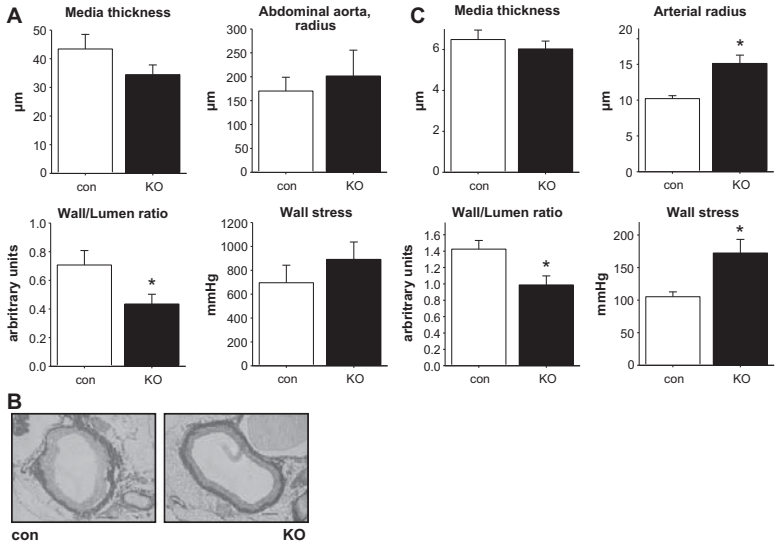

Figure 4. Morphology of aortas and intramyocardial arteries ex vivo and estimation of wall stress, respectively. Media thickness, lumen radius, wall-to-lumen ratio, and the corresponding wall stress were obtained and calculated from semithin sections of aorta (**A**) or myocardium (**C**) stained with hematoxylin and eosin, respectively. In the aortas, the wall-to-lumen ratio was reduced significantly. Media thickness was reduced, although this difference did not reach statistical significance (P=0.078 vs the respective control). The wall stress, calculated according to the law of Laplace, was the same in both genotypes. In intramyocardial arteries, the wall-to-lumen ratio was decreased and lumen radius was increased in knockout animals. This increases the wall stress in these animals. Exemplary Sirius red–stained semithin sections from con and KO animals are shown in section **B** (×40 magnification; scale bar, 100 µm). n=8 to 11/group, *P≤0.05 vs control.

(ROS) production. This increased ROS production was not inhibited by L-N^G-nitroarginine methyl ester (L-NAME) but was nearly completely abolished by diphenylene iodonium (DPI) (Figure 7). As neither NOX4 nor NOX2 mRNA expression was different in aortas of KO animals compared with WT (n=4–6 animals/group; NOX2 con, 1.0±0.2; KO, 0.6±0.2; NOX-4 con, 1.0±0.3; KO, 0.9±0.1), we did not perform lucigenin assays on isolated aortas.

Discussion

To further elucidate the role of EGFR in VSMC and cardiomyocytes, we investigated the phenotype of mice with a deletion of EGFR in VSMC and a strong reduction in cardiomyocytes. The SM22 promoter is frequently used for VSMC-specific deletions.[23] Yet, it has been shown that transient embryonic activation of SM22 in cardiomyocytes[26] leads to a partial KO of >50% of the protein of interest.[27] Here, we were able to show that EGFR mRNA and protein are reduced to ≈40% of the initial amount. As fibroblasts are the major cardiac cell type in number, a complete reduction of the EGFR cannot be expected in whole hearts. The same is true also for isolated cardiomyocytes, allowing only an enrichment of cardiomyocytes in the presence of residual fibroblasts. Thus, the model presented is suitable to gain further insight into the importance of VSMC and cardiomyocyte EGFR in vivo.

Mice with a global deletion of EGFR die in utero or within the first 20 days postpartum.[13] In EGFR$^{\Delta/\Delta VSMC\&CM}$ mice, embryonic lethality was not increased, leading to the conclusions that (1) reduction of cardiac EGFR does not impair embryonic heart development or function to a life-threatening extent and (2) EGFR deletion in VSMC does not impair placental function.

It has been suggested that EGFR is involved in the preservation of cardiac function and tissue homeostasis.[28,29] Our data support this hypothesis. The partial loss of cardiomyocyte

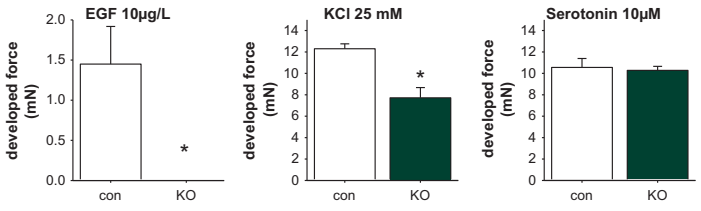

Figure 5. Functional responses of aortic rings to KCl, serotonin, and epidermal growth factor (EGF). Contractility of aortic rings on stimulation with KCl, serotonin (5-HT), or EGF, respectively, was detected using a Mulvany wire myograph. Although force development on 5-HT (10 µmol/L) stimulation was the same in both genotypes, a significant lesser force was developed by stimulation with KCl. No response could be obtained on incubation with EGF in aortic rings from knockout animals. n=5 to 10 per group, *P≤0.05 vs control.

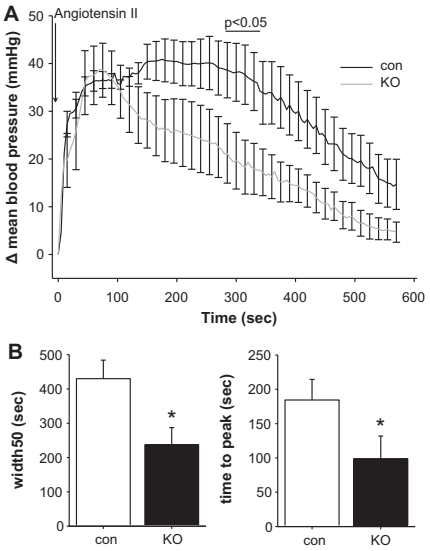

Figure 6. Change in mean arterial blood pressure induced by angiotensin II. **A,** In wild-type and knockout animals, angiotensin II induces an increase in mean arterial blood pressure to the same maximal height. **B**, But the blood pressure in knockout animals decreases and increases significantly faster than in wild-type animals, resulting in a reduced width 50 (n= 6–11 per group; *P≤0.05 vs control).

EGFR led to an eccentric hypertrophy without detectable cardiac lesions in the absence of arterial hypertension, although parameters for fibrosis were not increased. Thus, mice seem to experience a physiological heart hypertrophy. There are no signs for systolic dysfunction, as fractional shortening, stroke volume, and cardiac output were not reduced, which corresponds to physiological heart hypertrophy,[30] and the increase in cardiomyocyte volume accounts for the relative increase in heart weight. Possibly, the increase in heart size favors fatal arrhythmias, thereby increasing lethality. Future studies have to address these questions.

In *wa-2* mice on a C57BL/6 background, left ventricles are dilated while septal and chamber walls were thickened, presumably caused by aortic valve cusp thickening and aortic valve stenosis,[31] similar as for hEGFR$^{KI/KI}$ and EGFR$^{-/-}$ animals.[17] This results from accumulation of mesenchymal cells as a result of reduced endothelial/endocardial EGFR expression.[17] Heart valves originate from endocardial/endothelial cells undergoing mesenchymal transition and generating the mesenchymal cells responsible for cushion formation of heart valves.[31] To our knowledge, there is no indication for SM22 promoter activation in vivo during embryonic valve formation. Furthermore, our results do not indicate a functional relevant stenosis of the aortic valve nor developmental aortic or cardiac malformation.[19,32] Another possible explanation for the increase in heart weight would be that reduced EGFR expression leads to reduced ErbB2 activation, with subsequently reduced ErbB2/ErbB4 dimer formation, and thereby to heart hypertrophy.[33] But as ErbB2 and ErbB4 expression in the heart are unaltered, this explanation seems unlikely.

We suggest that hypertrophy results from a mild tonic dysbalance of cardiac ROS homeostasis, known to activate prohypertrophic cascades.[34] In support of this hypothesis, we detected enhanced NOX4 mRNA expression in the heart of KO mice, as well as a nonsignificant increase in protein level (preliminary data). Measurements of cardiac NOX activity by the lucigenin method[35] showed increased ROS formation in cardiac tissue of KO animals. The signal was completely abrogated by DPI but not by L-NAME, supporting the conclusion of an enhanced NOX activity. Furthermore, increased NOX4 expression under conditions of reduced

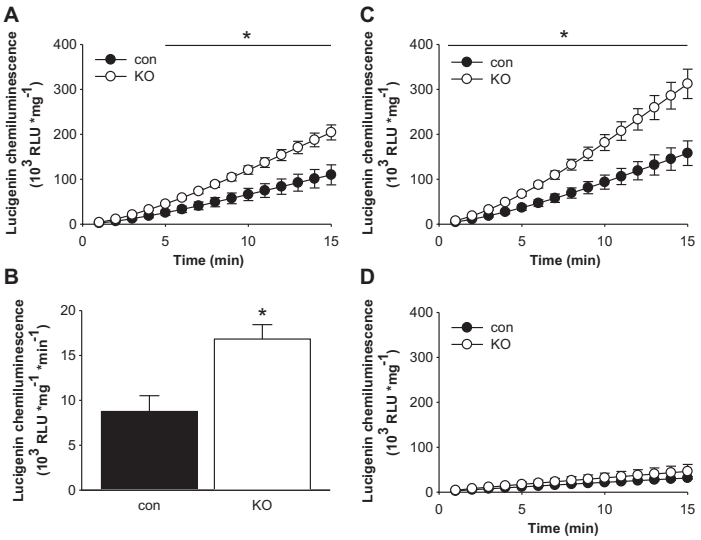

Figure 7. Change of lucigenin chemiluminescence in cardiomyocytes**. A,** Lucigenin chemiluminescence accumulates in isolated cardiomyocytes of knockout animals faster than in cardiomyocytes of control animals, indicating an enhanced ROS production in knockout cells. **B**, This increased ROS production results in an increased lucigenin chemiluminescence in cardiomyocytes of knockout animals after 15 minutes. **C**, L-NAME does not reduce the ROS production in cardiomyocyte lysates of wild-type and knockout animals. **D**, DPI abrogates the difference in ROS production between the 2 genotypes (n=3–5 per group; *P≤0.05 vs control).

EGFR activity has been reported before.[36,37] Possibly, the ROS dysbalance is not strong enough to elicit major fibrotic alterations.

In VSMC, the EGFR can be transactivated by vasoactive substances mediating physiological or pathophysiological responses.[7] In our KO model, SBP was not affected neither in conscious[22] nor in anesthetized animals, whereas DBP and MAP were reduced because of reduced peripheral vascular resistance. This does not seem to lead to reduced tissue perfusion, at least in the heart, as neither hypoxia inducible factor-1α nor glucose transporter-1 expression was herein increased. The low blood pressure levels during Millar catheter measurements are most probably because of ketamine/xylazine anesthesia, which has been reported to reduce SBP down to 46±5 mm Hg.[38] Therefore, the invasively measured blood pressure is lower compared with conscious animals independent of the genotypes. Because there is no difference in SBP between the genotypes neither in tail cuff[22] nor in Millar catheter measurements, the reduced DBP results from the loss of EGFR.

As expected from its function as a growth factor for VSMC and the reduced proliferation of VSMC$^{EGFR-/-}$,[22] media thickness was decreased in aortas of KO mice. The luminal diameter of intramyocardial vessels was increased, indicative of a reduced vascular tone when EGFR is missing. This finding corresponds with results from isolated, primary VSMC where the number of cells responding to vasoactive hormones was reduced by deletion of the EGFR.[22] On infusion of Ang II, the MAP increased to the same amount in WT and EGFR$^{\Delta/\Delta VSMC\&CM}$ mice, whereas the time needed to reach the maximal increase of blood pressure was significantly shorter in KO animals than in WT animals. Furthermore, the time, until the blood pressure fell below the half maximal value thereafter (ΔP/2) was significantly shorter in KO animals, resulting in a reduced plateau phase of blood pressure increase. Taken together, these data support our hypothesis that EGFR is also involved in blood pressure regulation of Ang II. In vivo these effects would lead to a less sustained blood pressure increase in Ang II generation and therefore a reduced range of physiological blood pressure regulation. However, under pathophysiological conditions of arterial hypertension, blood pressure increases would be smaller, protecting the arterial wall from remodeling. Taken together, these changes in VSMC function, combined with the observed structural changes, explain the reduced peripheral vascular resistance resulting in the lower DBP and MAP of KO animals, whereas the increase in stroke volume prevents a reduction in SBP.

In aortas of KO animals, markers of fibrosis and inflammation were elevated. We were able to show that in cultured VSMC from KO animals Col3a1 expression was enhanced and cells from control animals responded to EGF with reduced Col3a1 expression.[22] Thus, our data show that EGFR contributes to the physiological development of vessel structure, prevents parainflammatory alterations of tissue homeostasis, and is important for a proper vascular function.

In contrast to our results, Griol-Charhbili et al[39] did not observe significant alterations in the structure and basic function in vessels from *wa-2* mice that carry a global and spontaneous mutation of the EGFR,[21] leading to a reduction in the kinase activity but a longer plasma membrane half-life. Probably, the remaining EGFR activity was sufficient to prevent major remodeling. Furthermore, these mice[39] were kept on a mixed background (C57BL/6xC3H), and at least some EGFR effects seem to depend on the genetic background,[17] making it difficult to compare the 2 studies.

Perspective

Taken together, the data presented show that in cardiomyocytes EGFR prevents excessive hypertrophic growth through its impact on ROS balance, whereas in VSMC, EGFR contributes to the appropriate vascular wall architecture and vessel reactivity, thereby supporting a physiological vascular tone. Thus, although EGFR serves as a heterologous transducer of adverse cardiovascular stimuli, it is also required for physiological cardiovascular tissue homeostasis. EGFR is a target for cancer therapy, with specific therapeutics approved.[40] Unfortunately, cardiac toxicity is a known side effect of tyrosine kinase inhibitor treatment.[40] Therefore, it is necessary to analyze the long-term consequences of suppressed EGFR activity in VSMC and cardiomyocytes in vivo in more detail.

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Disclosures

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References

1. Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol*. 1999;11:184–189.
2. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001;2:127–137.
3. Liebmann C. EGF receptor activation by GPCRs: an universal pathway reveals different versions. *Mol Cell Endocrinol*. 2011;331:222–231.
4. Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. *Annu Rev Pharmacol Toxicol*. 2004;44:195–217.
5. Wetzker R, Böhmer FD. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol*. 2003;4:651–657.
6. Touyz RM. Mitochondrial redox control of matrix metalloproteinase signaling in resistance arteries. *Arterioscler Thromb Vasc Biol*. 2006;26:685–688.
7. Dreux AC, Lamb DJ, Modjtahedi H, Ferns GA. The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. *Atherosclerosis*. 2006;186:38–53.
8. Sibilia M, Kroismayr R, Lichtenberger BM, Natarajan A, Hecking M, Holcmann M. The epidermal growth factor receptor: from development to tumorigenesis. *Differentiation*. 2007;75:770–787.
9. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature*. 1995;378:394–398.
10. Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature*. 1997;389:725–730.
11. Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature*. 1995;378:390–394.
12. Meyer D, Birchmeier C. Multiple essential functions of neuregulin in development. *Nature*. 1995;378:386–390.
13. Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science*. 1995;269:234–238.
14. Kornblum HI, Hussain R, Wiesen J, Miettinen P, Zurcher SD, Chow K, Derynck R, Werb Z. Abnormal astrocyte development and neuronal death

in mice lacking the epidermal growth factor receptor. *J Neurosci Res*. 1998;53:697–717.
15. Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, Derynck R. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature*. 1995;376:337–341.
16. Sibilia M, Steinbach JP, Stingl L, Aguzzi A, Wagner EF. A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. *EMBO J*. 1998;17:719–731.
17. Sibilia M, Wagner B, Hoebertz A, Elliott C, Marino S, Jochum W, Wagner EF. Mice humanised for the EGF receptor display hypomorphic phenotypes in skin, bone and heart. *Development*. 2003;130:4515–4525.
18. Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science*. 1995;269:230–234.
19. Wang K, Yamamoto H, Chin JR, Werb Z, Vu TH. Epidermal growth factor receptor-deficient mice have delayed primary endochondral ossification because of defective osteoclast recruitment. *J Biol Chem*. 2004;279:53848–53856.
20. Barrick CJ, Roberts RB, Rojas M, Rajamannan NM, Suitt CB, O'Brien KD, Smyth SS, Threadgill DW. Reduced EGFR causes abnormal valvular differentiation leading to calcific aortic stenosis and left ventricular hypertrophy in C57BL/6J but not 129S1/SvImJ mice. *Am J Physiol Heart Circ Physiol*. 2009;297:H65–H75.
21. Luetteke NC, Phillips HK, Qiu TH, Copeland NG, Earp HS, Jenkins NA, Lee DC. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev*. 1994;8:399–413.
22. Schreier B, Döhler M, Rabe S, Schneider B, Schwerdt G, Ruhs S, Sibilia M, Gotthardt M, Gekle M, Grossmann C. Consequences of epidermal growth factor receptor (ErbB1) loss for vascular smooth muscle cells from mice with targeted deletion of ErbB1. *Arterioscler Thromb Vasc Biol*. 2011;31:1643–1652.
23. Holtwick R, Gotthardt M, Skryabin B, Steinmetz M, Potthast R, Zetsche B, Hammer RE, Herz J, Kuhn M. Smooth muscle-selective deletion of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. *Proc Natl Acad Sci USA*. 2002;99:7142–7147.
24. Natarajan A, Wagner B, Sibilia M. The EGF receptor is required for efficient liver regeneration. *Proc Natl Acad Sci USA*. 2007;104:17081–17086.
25. Li L, Miano JM, Mercer B, Olson EN. Expression of the SM22α promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J Cell Biol*. 1996;132:849–859.
26. Miano JM, Ramanan N, Georger MA, de Mesy Bentley KL, Emerson RL, Balza RO Jr, Xiao Q, Weiler H, Ginty DD, Misra RP. Restricted inactivation of serum response factor to the cardiovascular system. *Proc Natl Acad Sci USA*. 2004;101:17132–17137.
27. Malhowski AJ, Hira H, Bashiruddin S, Warburton R, Goto J, Robert B, Kwiatkowski DJ, Finlay GA. Smooth muscle protein-22-mediated deletion of Tsc1 results in cardiac hypertrophy that is mTORC1-mediated and reversed by rapamycin. *Hum Mol Genet*. 2011;20:1290–1305.
28. Esposito G, Perrino C, Cannavo A, Schiattarella GG, Borgia F, Sannino A, Pironti G, Gargiulo G, Di Serafino L, Franzone A, Scudiero L, Grieco P, Indolfi C, Chiariello M. EGFR trans-activation by urotensin II receptor is mediated by β-arrestin recruitment and confers cardioprotection in pressure overload-induced cardiac hypertrophy. *Basic Res Cardiol*. 2011;106:577–589.
29. Williams-Pritchard G, Knight M, Hoe LS, Headrick JP, Peart JN. Essential role of EGFR in cardioprotection and signaling responses to A1 adenosine receptors and ischemic preconditioning. *Am J Physiol Heart Circ Physiol*. 2011;300:H2161–H2168.
30. Weeks KL, McMullen JR. The athlete's heart vs. the failing heart: can signaling explain the two distinct outcomes? *Physiology (Bethesda)*. 2011;26:97–105.
31. Nakajima Y, Mironov V, Yamagishi T, Nakamura H, Markwald RR. Expression of smooth muscle α-actin in mesenchymal cells during formation of avian endocardial cushion tissue: a role for transforming growth factor β3. *Dev Dyn*. 1997;209:296–309.
32. Terzi F, Burtin M, Hekmati M, Federici P, Grimber G, Briand P, Friedlander G. Targeted expression of a dominant-negative EGF-R in the kidney reduces tubulo-interstitial lesions after renal injury. *J Clin Invest*. 2000;106:225–234.
33. Rajagopalan V, Zucker IH, Jones JA, Carlson M, Ma YJ. Cardiac ErbB-1/ErbB-2 mutant expression in young adult mice leads to cardiac dysfunction. *Am J Physiol Heart Circ Physiol*. 2008;295:H543–H554.
34. Tsutsui H, Kinugawa S, Matsushima S. Oxidative stress and heart failure. *Am J Physiol Heart Circ Physiol*. 2011;301:H2181–H2190.
35. Rueckschloss U, Villmow M, Klöckner U. NADPH oxidase-derived superoxide impairs calcium transients and contraction in aged murine ventricular myocytes. *Exp Gerontol*. 2010;45:788–796.
36. Orcutt KP, Parsons AD, Sibenaller ZA, Scarbrough PM, Zhu Y, Sobhakumari A, Wilke WW, Kalen AL, Goswami P, Miller FJ Jr, Spitz DR, Simons AL. Erlotinib-mediated inhibition of EGFR signaling induces metabolic oxidative stress through NOX4. *Cancer Res*. 2011;71:3932–3940.
37. Sancho P, Bertran E, Caja L, Carmona-Cuenca I, Murillo MM, Fabregat I. The inhibition of the epidermal growth factor (EGF) pathway enhances TGF-β-induced apoptosis in rat hepatoma cells through inducing oxidative stress coincident with a change in the expression pattern of the NADPH oxidases (NOX) isoforms. *Biochim Biophys Acta*. 2009;1793:253–263.
38. Janssen BJ, De Celle T, Debets JJ, Brouns AE, Callahan MF, Smith TL. Effects of anesthetics on systemic hemodynamics in mice. *Am J Physiol Heart Circ Physiol*. 2004;287:H1618–H1624.
39. Griol-Charhbili V, Fassot C, Messaoudi S, Perret C, Agrapart V, Jaisser F. Epidermal growth factor receptor mediates the vascular dysfunction but not the remodeling induced by aldosterone/salt. *Hypertension*. 2011;57:238–244.
40. Orphanos GS, Ioannidis GN, Ardavanis AG. Cardiotoxicity induced by tyrosine kinase inhibitors. *Acta Oncol*. 2009;48:964–970.

Novelty and Significance

What Is New?

- Physiological role of epidermal growth factor receptor in the cardiovascular system.
- Genetic model for the cardiovascular epidermal growth factor receptor in adult animals.

What Is Relevant?

- Hearts of knockouts show an increase in heart weight, stroke volume, cardiac output, and hypertrophy markers, but not for markers of fibrosis or inflammation.
- Total peripheral vascular resistance, diastolic blood pressure, and mean blood pressure were reduced.
- In aortas, markers for fibrosis and inflammation were increased.
- Knockouts show a dilated vascular phenotype.

Summary

- Although epidermal growth factor receptor serves as a heterologous transducer of adverse cardiovascular stimuli, it is also required for healthy basal tissue homeostasis.

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Original Paper

Consequences of postnatal vascular smooth muscle EGF-receptor deletion on acute angiotensin II action

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Abstract

Epidermal growth factor (EGF) receptor (EGFR) is activated by its canonical ligands and trans-activated by various vasoactive substances, e.g. angiotensin II (Ang II). Vascular EGFR has been proposed to be involved in vascular tissue homoeostasis and remodelling. Thus, most studies focused on its role during long-term vascular changes whereas the relevance for acute regulation of vascular function *in vivo* and *ex vivo* is insufficiently understood. To investigate the postnatal role of VSMC (vascular smooth muscle cells) EGFR *in vivo* and *ex vivo*, we generated a mouse model with cell-specific and inducible deletion of VSMC–EGFR and studied the effect on basal blood pressure, acute pressure response to, among others, Ang II *in vivo* as well as *ex vivo*, cardiovascular tissue homoeostasis and vessel morphometry in male mice. In knockout (KO) animals, systolic, diastolic and mean blood pressures were reduced compared with wild-type (WT). Furthermore, Ang II-induced pressure load was lower in KO animals, as was Ang II-induced force development and extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation in aortic rings from KO animals. By contrast, we observed no difference in force development during application of serotonin, KCl, endothelin-1 or endothelin-1-induced pressure load in KO animals. In addition, nitric oxide (NO)-mediated vasodilation was not affected. Heart weight (HW) increase and up-regulation of aortic and cardiac expression of Ccl2 (chemoattractant protein-2) and Serpine1 (plasminogen activator inhibitor 1) during the transition from 4- to 10-months age were prevented by VSMC–EGFR KO. We conclude that VSMC–EGFR is involved in basal blood pressure homoeostasis, acute pressure response to Ang II and thereby contributes to maturation-related remodelling.

Key words: angiotensin II, endothelin-1, epidermal growth factor receptor, hypotension, mouse model, vascular smooth muscle cells.

INTRODUCTION

The epidermal growth factor (EGF) receptor (EGFR), member of the ErbB-receptor tyrosine kinase family [1,2], can be activated by EGF or heparin-bound EGF (HB-EGF) [3] in an autocrine or paracrine fashion [1,4]. Upon ligand binding, EGFR forms homo- or heterodimers with all four family members and activates various signalling pathways which affect, among others, cell differentiation, migration and matrix homoeostasis [5,6].

The EGFR can also be trans-activated by vasoactive substances, like angiotensin II (Ang II) [6–9], α_1- and β-adrenergic agonists [10,11], thrombin [12], endothelin-1 [13], purinergic receptor ligands [14] or aldosterone [15,16], after binding to their distinct receptor. Thereby, EGFR is supposed to mediate part of their pathophysiological effects, including atherosclerosis [17] or heart hypertrophy [8,16]. Thus, EGFR has the potential to serve as a pathologically-relevant heterologous signal transducer for non-EGFR ligands in the vasculature, thereby supporting

Abbreviations: Ang II, angiotensin II; AT1/2-receptor, angiotensin II type 1/2 receptor; ATRAP, angiotensin II type I receptor-associated protein; AUC360, area under the curve for 360 s after angiotensin II application; BW, body weight; Ccl-2, chemoattractant protein-2; CM, cardiomyocyte; Col1/3a1, collagen, type I/III, α 1; EGF, epidermal growth factor; EGFR, EGF-receptor; $EGFR^{\Delta/\Delta VSMC\ \&\ CM}$, mice with a constitutive deletion of the EGFR in VSMC and CM; ERK1/2, extracellular signal-regulated kinase 1 and 2; HW, heart weight; $iEGFR^{\Delta/\Delta VSMC}$, mice with a inducible deletion of the EGFR in VSMC; KO, knockout; LW, lung weight; qRT-PCR, quantitative reverse transcription PCR; ROS, reactive oxygen species; Serpine-1, plasminogen activator inhibitor 1; SMMHC, smooth muscle myosin heavy chain; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; TL, tibia length; VSMC, vascular smooth muscle cells; WT, wild-type.

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para-inflammatory dysregulation and finally vascular dysfunction and fibrosis [18].

The intracellular signalling pathways affected by transactivation and potentially involved in long-term phenotypic changes of vascular smooth muscle cells (VSMC) have been investigated in detail. It has been demonstrated that EGFR transactivation leads to an increase in, e.g., extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation [19,20], involves reactive oxygen species (ROS)-dependent activation of c-Src [21], activation of phosphoinositide 3-kinase (PI3K), intracellular Ca^{2+}-signalling, endoplasmic reticulum (ER) stress [22], ADAM (A disintegrin and metalloproteinase) proteins or NADPH oxidases [23]. The impact of transactivation includes VSMC proliferation, migration, alterations in ROS defence, cell survival and matrix homoeostasis [19]. Additionally, it has been suggested that VSMC–EGFR is involved in the development of hypertension induced by Ang II [8], endothelin-1 [24] or α_1-adrenergic substances [11]. However, there are also studies using pharmacological approaches that challenge this view [9,25], assigning EGFR a role in remodelling or hypertrophy but not in hypertension. It is conceivable that the role of vascular EGFR is context-dependent, including, under particular condition, blood pressure regulation.

Less effort has been made to elucidate the importance of EGFR for the acute vascular contractile response to vasoactive substances. Yet, it was shown that intracellular Ca^{2+}-handling and ERK1/2 activation in VSMC depend, at least partially, on EGFR [19,26]. Therefore, we tested the hypothesis that VSMC–EGFR is also involved in the acute action of vasoactive substances, *in vivo* and *ex vivo*. Because there is evidence that age-associated vascular remodelling processes are modulated by VSMC–EGFR [27–29] we investigated the effect of VSMC–EGFR deletion in 4- and 10-month-old male animals with a special focus on Ang II.

Many attempts to delete or overexpress the members of the EGFR family or their ligands have been made, revealing the importance of EGFR for perinatal and postnatal development [30,31]. Lately, we established a constitutive, conditional knockout (KO) mouse model with deletion of the EGFR in VSMC and cardiomyocytes (CMs) [6]. Loss of EGFR in this model affected total peripheral vascular resistance and mean blood pressure due to a dilated vascular phenotype with minor fibrosis and inflammation. In addition, the animals showed cardiac hypertrophy with increased cardiac output but no major signs of fibrosis or inflammation [6]. Furthermore, we obtained first indication of a reduced vascular responsiveness to Ang II. Unfortunately it could not be excluded that the difference in blood pressure increase is due to alterations in vessel development, morphology or heart function. Therefore, we report herein the generation of a second mouse model with inducible conditional deletion of the EGFR in VSMC without alterations in heart morphology.

MATERIALS AND METHODS

All mouse experiments described in the present manuscript were approved by the local government (Landesverwaltungsamt Sachsen-Anhalt, Germany). All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines.

Animals

Mice were kept at constant temperature of $22 \pm 2\,^{\circ}C$ under a 12/12-h light–dark cycle with *ad libitum* access to water and standard chow in the facilities of the University of Halle-Wittenberg.

We generated an inducible KO mouse model for the EGFR in VSMC via the Cre/LoxP system by mating $EGFR^{flox/flox}$ mice [32] (kindly provided by Professor M. Sibilia, Vienna) with smooth muscle myosin heavy chain (SMMHC)–$Cre^{+/-}$ mice [33] (kindly provided by Dr S. Offermanns, Nauheim). In the $iEGFR^{\Delta/\Delta VSMC}$ (mice with a inducible deletion of the EGFR in VSMC) littermates Cre recombinase is expressed under control of the SMMHC promoter and can be activated in VSMC by intraperitoneal injection of tamoxifen (1 mg/50 μl Miglyol 812/mouse/day for five consecutive days) [34]. As control group mice (WT, $CRE^{+/-}$) without LoxP-sites but carrying the Cre-recombinase under control of the SMMHC promotor were employed. In initial experiments, we analysed the time point of maximal EGFR reduction in mouse aorta. For the $iEGFR^{\Delta/\Delta VSMC}$ mice, control animals were also treated with tamoxifen. Therefore, we performed the experiments 56 days after tamoxifen application in mice, 4 months of age and approximately 236 days (10 months), according to McCurley et al. [28]. This protocol also allows long-term comparison of EGFR–KO-induced effects. $EGFR^{\Delta/\Delta VSMC\&CM}$ (mice with a constitutive deletion of the EGFR in VSMC and CM) mice were analysed at an age of approximately 4 months, too. The mouse models were kept on a C57/Bl6J background. Genotyping was performed on tail biopsies by PCR, as previously described [6,33].

Aortas were dissected, carefully freed from adjacent tissue and EGFR expression was analysed by quantitative reverse transcription (qRT)-PCR (Primers: Supplementary Table S1). According to qPCR analysis of whole aortic tissue, the EGFR expression was reduced down to $26 \pm 18\,\%$ of WT animals ($N = 6$ animals/group, $P < 0.05$). A further reduction was not expected, because EGFR is also expressed in other cell types of the vessel wall, like endothelial cells and fibroblasts. Heart, lung, skeletal muscle and liver EGFR expression were not affected (Supplementary Figure S1).

Measurement of blood pressure

Blood pressure measurements via tail-cuff were performed as described before [6]. Additionally intravasal blood pressure measurements (diastolic, systolic, mean) were performed in anaesthetized [80 mg/kg BW (body weight) ketamine and 120 mg/kg BW xylazine, Sigma–Aldrich] mice. The right jugular vein was cannulated for infusion of 2 % BSA (Sigma–Aldrich) in Ringer lactate solution (for 1 litre: NaCl 5.9 g, KCl 0.3 g, $CaCl_2$ 0.22 g, Na-Lactate 2.8 g) at 4 μl/g BW/min. A Millar catheter (size 1 F, Millar Instruments) was inserted into the right carotid artery. After a 10-min stabilization period, systolic, diastolic and mean blood pressures were obtained (PowerLab data acquisition systems) and pulse pressure was calculated with the LabChart7

software (ADI instruments). To analyse the reactivity of blood pressure upon Ang II, a bolus of 1 μg of Ang II/kg BW was given followed by an infusion of 100 μl of Ringer lactate solution, as described above, within a minute (infusion rate: 6 ml/h). The change in blood pressure was analysed for a subsequent 30-min time period.

Harvesting of organs

Mice were killed by cervical dislocation. Immediately after death, livers, kidneys, lungs, hearts and aortas were excised, carefully freed from adjacent tissue and partially weighed. Furthermore, tibia length (TL) was measured for normalization of organ weights. Part of the tissues were immediately snap frozen in liquid nitrogen for later RNA extraction whereas parts were put in 3% paraformaldehyde solution for fixation. Tissues were dehydrated by bathing in increasing concentrations of methanol or isopropanol respectively. After embedding in paraffin, 3 μm sections were cut.

Microscopic analysis of aorta, heart and lung

Morphometric analysis of aorta, heart and kidneys was performed as described before [6]. For wall-to-lumen ratio media thickness was measured at 10 different locations within the vessel wall and was divided by the internal circumference of the aorta according to Liang et al. [35].

Gene expression

Gene expression analysis was carried out as described before [6,19]. The primers used are given in Supplementary Table S1. qRT-PCR efficiency was >90%. The relative expression of the two genes of interest was calculated according to the $2^{-\Delta\Delta Ct}$ method, using the 18S signal for normalization. Each sample was analysed as triplicate. All values are expressed as mean difference between WT and KO or between 4- and 10-month-old animals $\pm$ S.E.M.

Immunoblotting

Immunoblotting was performed as described before [19]. All antibodies were purchased from Cell Signaling Technologies. pERK 1/2 was measured in aortic rings from $EGFR^{\Delta/\Delta VSMC\&CM}$ mice 24 h after dissection. After this time-period the aortic rings were stimulated for 10 min with 100 nM Ang II or serum-free cell culture medium.

Aortic ring force measurement

Before starting the genotype comparison experiments, we determined whether the aeration with carbogen (5% CO_2, 95% O_2), frequently used for CO_2/HCO_3^--buffered solutions, affects aortic ring reactivity as compared with aeration with 5% CO_2 in compressed air (20% O_2). Because we observed differences in force generation (e.g. for 25 mmol/l KCl 5.2 ± 0.6 mN at 20% O_2 and 8.1 ± 1.1 mN at 95% O_2, $n = 4$; for 10 μg/l EGF -0.2 ± 0.1 mN at 20% O_2 and 1.3 ± 0.6 mN at 95% O_2, $n = 11$) we performed all subsequent experiments at near physiological 20% O_2. We did not investigate the underlying mechanism for the observed O_2-dependent difference further, but assume that an enhanced load of ROS are involved.

Aortic rings were equilibrated in modified aerated Krebs–Ringer solution at 37 °C for 30 min. At the beginning and at the end of the equilibration, the physiological salt solution was changed once, followed by the application of a strain to the vessel wall resulting in a force of 12 mN [36]. This strain resulted in a similar change in vessel circumference (dL) and similar effective pressure values in both genotypes [36] (Figure 2) and was applied for 10 min prior to the first substance application. Wall stress and effective pressure were calculated as described by Mulvany and Halpern [36]. After each measurement, the chambers were flushed five times with Krebs–Ringer solution, achieving an approximately 100000-fold dilution of the substance, before a new reagent was tested. This did not apply for the relaxants carbamoylcholine chloride (carbachol) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP). These substances were given at the point of stable force development of the previously administered vasoconstrictor (10 μmol/l serotonin).

Materials

Unless otherwise stated, all materials were purchased from Sigma–Aldrich.

Statistics

The data are presented as mean $\pm$ S.E.M. ANOVA, followed by post-hoc testing or Student's *t*test were used as applicable according to pre-test data analysis by SigmaPlot 12.5. A $P < 0.05$ was considered significant.

RESULTS

Impact of VSMC–EGFR deletion on vessel morphology and basal blood pressure

In the conditional, constitutive model ($EGFR^{\Delta/\Delta VSMC\&CM}$) wall-to-lumen ratio of aortas and intramyocardial arteries was slightly reduced in KO animals [6]. In the conditional, inducible model ($iEGFR^{\Delta/\Delta VSMC}$) no significant differences in wall-to-lumen ratio (4 months: WT: 0.033 ± 0.002, KO: 0.036 ± 0.005, n = 8–9 animals/group) or aortic wall thickness (4 months: WT: 31.4 ± 2.9 μm, KO: 30.8 ± 4.2 μm, n = 8–9 animals/group) could be observed in HE-stained ultrathin sections. Intramyocardial arteries of KO animals also showed no difference in wall thickness compared with WT mice (4 months: WT: 5.8 ± 0.5 μm and KO: 5.6 ± 0.5 μm, $n = 4$–5 animals/group) whereas wall-to-lumen ratio was reduced in KO animals compared with age-matched WT animals (4 months: WT: 0.068 ± 0.005 and KO: 0.052 ± 0.006, n = 4–5 animals/group; $P < 0.05$, for representative Sirius Red stained ultrathin sections see Supplementary Figures S2A and S2B). In contrast with intramyocardial arteries, no significant difference in intrarenal-resistance wall thickness (4 months: WT: 9.5 ± 1.0 μm and KO: 9.7 ± 0.4 μm, $n = 4$–5 animals/group) or wall-to-lumen ratio (4 months: WT: 0.104 ± 0.015 and KO: 0.090 ± 0.011, n = 4–5 animals/group) could be observed. These data indicate a slightly dilated vascular phenotype in KO animals, mainly in the heart, as observed in the $EGFR^{\Delta/\Delta VSMC\&CM}$ model, too. Normalized BW (4 months: BW/TL; WT: 1.5 ± 0.1 g/mm, KO: 1.5 ± 0.1 g/mm,

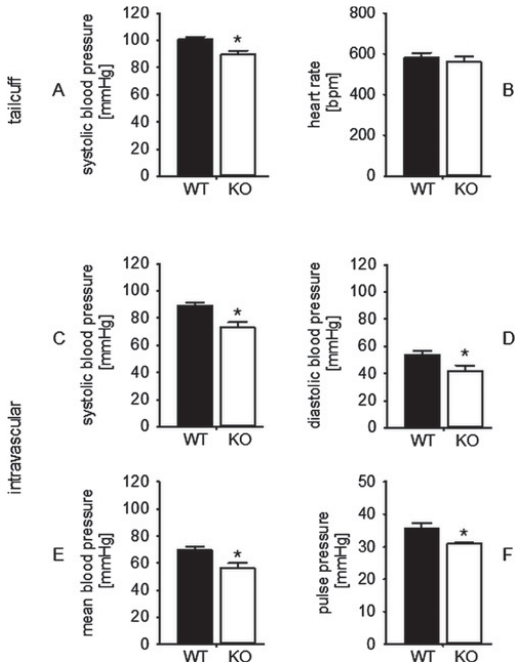

Figure 1 **Influence of the deletion of VSMC–EGFR on blood pressure (A, C–F) and heart rate (B) in conscious (A and B, $N = 14$ animals/group) and anaesthetized (C–F, $N = 5$–7 animals/group) male $iEGFR^{\Delta/\Delta VSMC}$ mice at an age of approximately 4 months**
*$P < 0.05$ compared with respective WT animals.

$n = 10$ animals/group), heart weight (HW; 4 months: HW/TL; WT: 7.2 ± 0.3 mg/mm, KO: 7.4 ± 0.5 g/mm, $n = 10$ animals/group) and lung weight (LW; 4 months: LW/TL; WT: 8.5 ± 0.5 g/mm, KO: 8.5 ± 0.5 mg/mm, $n = 10$ animals/group) were the same in animals of both genotypes. Thus, with the $iEGFR^{\Delta/\Delta VSMC}$ mouse, a model exists that allows (a) inducible postnatal deletion of EGFR in VSMC without (b) affecting cardiomyocyte (CM) EGFR. The $iEGFR^{\Delta/\Delta VSMC}$ mouse model is suitable to analyse the physiological role of the EGFR in fully developed vessels without confounding effects due to altered cardiac EGFR expression. These data also show that the cardiac phenotype observed in mice with a constitutive conditional deletion of the EGFR in CMs and VSMC [6] results from EGFR deletion in CMs.

To evaluate the impact of postnatal VSMC–EGFR deletion ($iEGFR^{\Delta/\Delta VSMC}$ model) on blood pressure we initially performed tail-cuff blood pressure measurements in conscious animals and subsequently intravascular blood pressure measurements in anaesthetized mice using a Millar catheter. Tail-cuff measurements in KO animals compared with age-matched WT animals revealed a significantly reduced systolic blood pressure by ~10 mmHg (Figure 1A). As a reduced blood pressure could induce counter-regulatory mechanisms, we analysed heart rate as one read-out parameter for the baroreceptor-reflex and volume intake. No difference in heart rate of conscious animals could be observed (Figure 1B), making an increase in vascular sympathetic tone as counter-regulatory mechanism unlikely. In contrast, although food intake was the same between the two genotypes (4.13 ± 0.10 compared with 4.10 ± 0.06 g/day), water intake was increased in KO animals (WT: 3.7 ± 0.1 ml and KO: 4.3 ± 0.2 ml; $P < 0.05$; $N = 9$–11 animals/group). Previously, we have already shown that aldosterone plasma levels are not increased in animals with VSMC–EGFR deletion [16]. These data indicate that there is a certain behavioural adaptation that, however, does not compensate blood pressure decrease completely.

Intravascular measurements confirmed the reduced systolic blood pressure in KO animals (Figure 1C). In addition, these measurements revealed a reduced diastolic (Figure 1D), mean (Figure 1E) and pulse pressure (Figure 1F) in KO animals compared with WT animals, thereby affirming that VSMC–EGFR is involved in basal blood pressure homoeostasis.

Influence of VSMC–EGFR on vasoconstriction

To evaluate the influence of VSMC–EGFR on acute vasoconstriction, we performed wire myography with aortic rings. To assure successful preparation and aortic tissue functionality, only vessels with a reaction upon KCl administration were used for analysis. To compare the impact of a deletion of the EGFR in

VSMC in adulthood to the effect of a missing EGFR during embryogenesis and adulthood, the experiments were also performed in aortic rings from $EGFR^{\Delta/\Delta VSMC\&CM}$ mice.

In an initial series of experiments, we determined force, stress and effective pressure development during distension in aortae from KO and WT animals according to Mulvany and Halpern [36]. As shown in Figure 2, within the functional range, i.e. at an effective pressure of ∼100 mmHg, no major differences between the genotypes were observed. Force-strain and stress-strain curves diverged slightly at higher strain (Figures 2D and 2E), due to the by-trend greater vessel circumference in KO animals, as reported before [6] (WT $= 0.63 \pm 0.08$ mm, KO $= 0.83 \pm 0.06$ mm for abdominal aortae at 1 mN force; $N = 10$; $P = 0.075$). The effective pressure relationships compared with strain as well as compared with stress were not significantly different between the genotypes (Figures 2F and 2G), indicating that the aortic walls of both genotypes are exposed to similar physical conditions *in vivo*. These data correspond to the lack of effect of EGFR inhibition on vessel distensibility [25].

KCl (25 mM in modified Krebs–Ringer solution, osmolarity corrected) induced similar force development in KO and control animals in either mouse model (Figures 3A and 3B; additional dose-response curve in Supplementary Figure S3). Serotonin [10 μM, vasoconstrictor acting mostly via serotonin type 2A receptor (5-HT2A receptor)] also induced a similar force development in both genotypes (Figures 3C and 3D). Carbachol (300 nM, a vasodilator acting via the endothelium, Figures 3E and 3F) and SNAP (300 nM, a vasodilator acting as NO-donor directly in VSMC, Figures 3G and 3H) were applied to the aortic rings after pre-constriction with serotonin. No difference between the genotypes could be observed, indicating that endothelium-dependent and -independent vasorelaxation does not depend on VSMC–EGFR. This was confirmed by additional dose-response experiments (Supplementary Figure S3).

Endothelin-1 (1 μM, vasoconstrictor acting via ETa/b-receptors), induced similar force development in wild-type (WT) and KO animals (Figures 3I and 3K). Further dose-response experiments confirmed these findings (Figure 4D). Thus, VSMC–EGFR, known to be trans-activated by endothelin-1 [19,24], is not required for the acute vasoconstrictor response to endothelin-1.

By contrast, Ang II [100 nM, acting vasoconstrictive via angiotensin II type 1 receptors (AT1-receptors)], induced a significant smaller force development in KO as compared with WT animals (Figures 3L and 3M). Further dose-response experiments confirmed these findings (Figures 4A and 4C). As shown in Figure 4(B), the Ang II-induced force was significantly smaller in KO animals, also in the presence of the AT2-receptor inhibitor (PD-123319), excluding the involvement of this receptor type. Thus, the acute vasoconstrictor response, via the AT1-receptor, to Ang II depends on the VSMC–EGFR.

Analysis of the mRNA expression of the endothelin-1 receptors Ednra and Ednrb and the AT1a- and AT1b-receptor revealed no reduction in aortae from KO animals as compared with aortae from WT animals (Figure 4G). However, Ang II-induced ERK1/2-phosphorylation was reduced in KO animals (Figure 4H), again confirming the role of EGFR as a signal transducer for Ang II. Supplementary Figures S4 ($EGFR^{\Delta/\Delta VSMC\&CM}$) and S5 ($iEGFR^{\Delta/\Delta VSMC}$) demonstrate that EGFR expression is reduced in aortas from VSMC–EGFR KO mice.

Because the basal components of the pharmaco-mechanical and electro-mechanical coupling seem to be intact (there were no differences in force development in response to KCl, serotonin or endothelin-1 in aortae from KO animals) and because we did not detect differences in mRNA-receptor expression, we investigated whether differences in functional homologous desensitization can explain the observed reduction in responsiveness to Ang II. For this purpose, aortic rings were exposed to 100 nM Ang II for 5 min followed by 10 min of complete washout to allow relaxation. This procedure was repeated six times and the changes in force development compared with application number 1 were analysed. For comparison, similar experiments were performed with 1 μM endothelin-1. Figure 4(E) shows that Ang II-induced homologous desensitization is accelerated in aortae from KO animals. For endothelin-1, no difference between the genotypes was observed (Figure 4F). To investigate if the alteration in reduced Ang II response is due to an alteration in the expression of proteins contributing to AT1-receptor desensitization, we analysed the mRNA content of β-arrestin 1 and 2 as well as ATRAP (AT1-receptor-associated protein) in aortas from $iEGFR^{\Delta/\Delta VSMC}$ mice. No difference between the two genotypes could be observed (Supplementary Figure S6).

Influence of EGFR on acute blood pressure effects of angiotensin II

To confirm that the alteration in force generation by aortic rings is of *in vivo* relevance, we infused Ang II into the jugular vein for 1 min and analysed blood pressure changes via Millar catheter. Blood pressure increase upon vehicle infusion was less than 15 mmHg in both genotypes and returned to baseline within ∼2 min (Supplementary Figures S7A–S7F). Regarding Ang II, deletion of VSMC–EGFR leads to a reduced pressure load, indicated by the areas under the curves of mean, diastolic and systolic blood pressure for the first 360 s after the start of Ang II infusion (Figures 5A–5H). The impact of Ang II on pulse pressure was similar in animals of both genotypes of the $iEGFR^{\Delta/\Delta VSMC}$ model (Figures 5D and 5H), indicating no major difference in cardiac stroke volume [37]. Analysis of Ang II-induced blood pressure load in the constitutive conditional model [6] revealed a similar setting, with reduced blood pressure load in KO animals [4 months: AUC360 (area under the curve for 360 s after angiotensin II application; mean blood pressure) Ang II: WT 15543 ± 1620, KO 9034 ± 2906 mmHg × s, $P = 0.07$ compared with WT, $n =$ 3–5 animals/group).

Additionally, we analysed the impact of endothelin-1 on the blood pressure in VSMC–EGFR KO animals in the same setting as for Ang II and observed no difference between the two genotypes [4 months: ΔSBP (difference of systolic blood pressure between time point 0 and time point x) WT 60.6 ± 5.5 mmHg, KO 63.1 ± 4.1 mmHg, ΔDBP (difference of diastolic blood pressure between time point 0 and time point x) WT 32.8 ± 2.8 mmHg, KO 28.6 ± 0.6 mmHg, ΔMBP (difference of mean blood pressure between time point 0 and time point x) WT 42.4 ± 4.1 mmHg, KO 39.3 ± 1.2 mmHg, $N = 4$–7 animals/group; AUC360 was not different either). These data confirm *in vivo* the results from

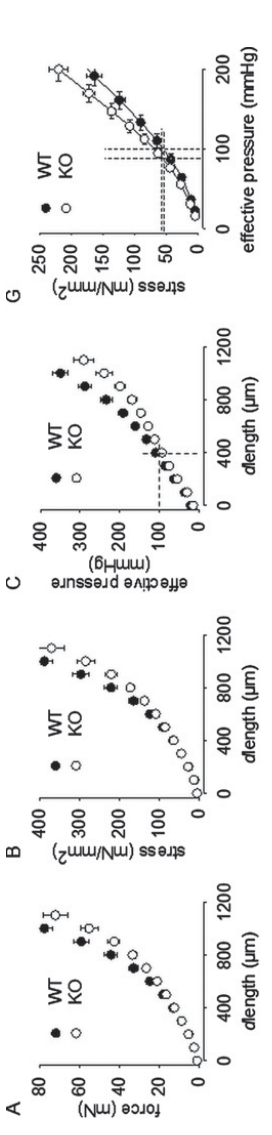

Figure 2 **In an initial series of experiments, we analysed force, stress and effective pressure development during distension in aortae from KO and WT animals according to Mulvany and Halpern [36]**

(**A**) Force development upon increase in aortic ring diameter (length). (**B**) Development of wall stress, calculated according to the law of Laplace [$K = P \times r/(2 \times d)$] upon increase in aortic diameter, (**C**) resulting effective pressure compared with aortic diameter, (**D**) force development compared with relative changes in aortic diameter (strain), (**E**) change in wall stress, calculated according to the law of Laplace, upon relative changes in aortic diameter, (**F**) change in effective pressure upon relative changes in aortic diameter. (**G**) Stress changes upon effective pressure changes. These data show that, within the functional range, i.e. at an effective pressure of ~100 mmHg, no major differences between the genotypes were observed. Similar results were obtained in *iEGFR*$^{\Delta/\Delta VSMC}$ mice at an age of approximately 4 month. $N = 6$–8 animals/group.

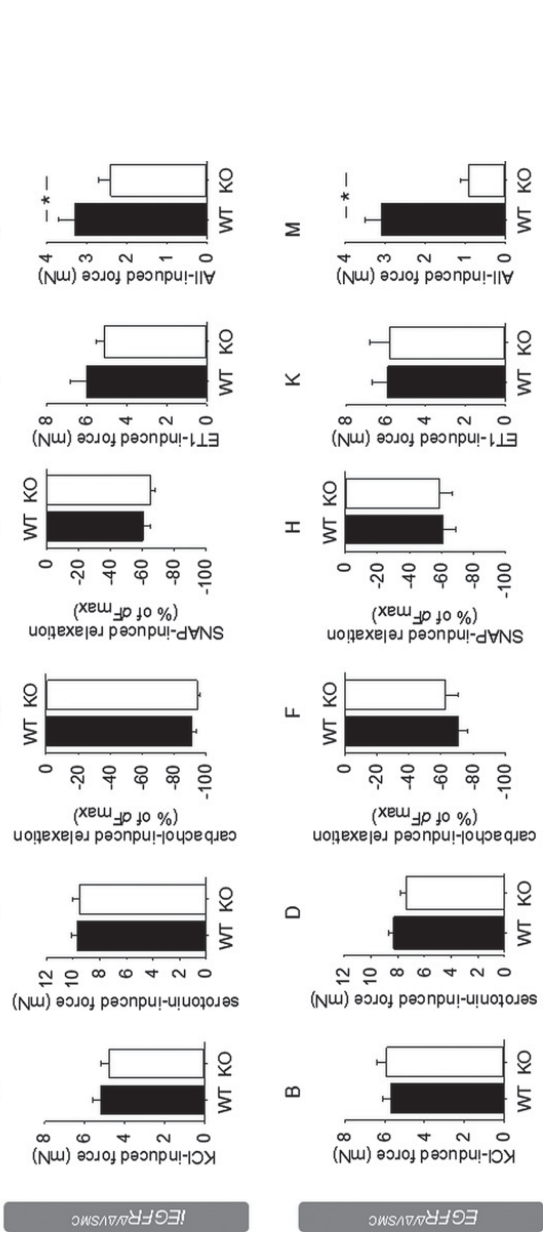

Figure 3 **Impact of VSMC–EGFR on vasoconstriction or vasodilation upon KCl (25 mM; A and B), serotonin (10 μmol/l; C and D), carbachol (300 nM; E and F), SNAP (300nM; G and H), endothelin-1 (1 μM; I and K) and Ang II (100 nM; L and M) application in *iEGFR*$^{\Delta/\Delta VSMC}$ (upper row) and *EGFR*$^{\Delta/\Delta VSMCeCre}$ (bottom row) mice at an age of approximately 4 month**

$N = 6$–10 animals/group, $^{*}P < 0.05$ compared with respective WT animals.

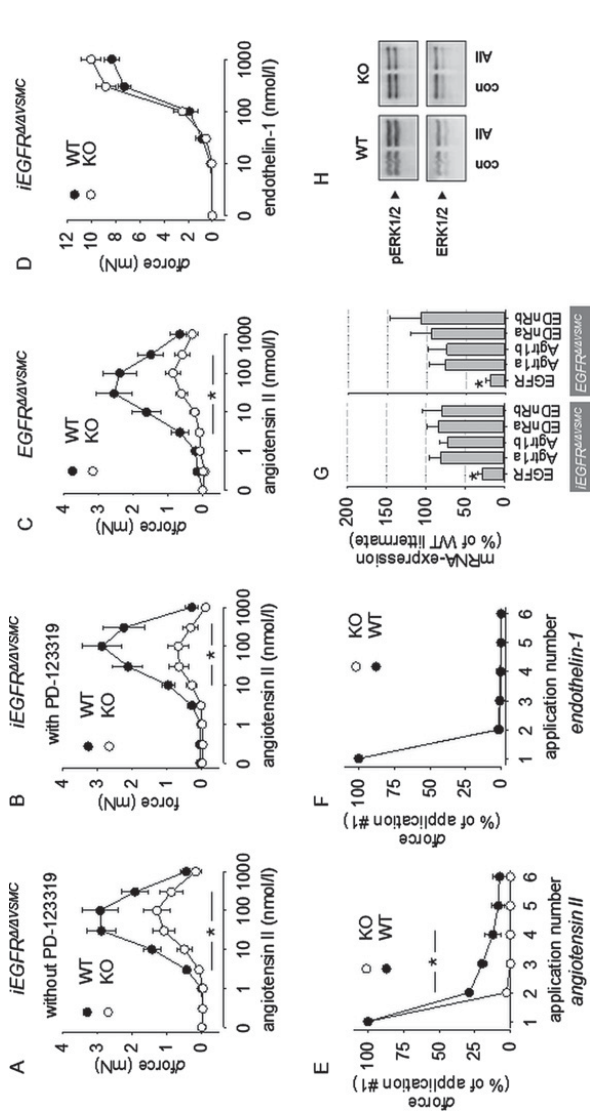

Figure 4 **Cumulative dose-response curves for Ang II (A) and endothelin-1 (D) in *iEGFR*$^{\Delta/\Delta VSMC}$ mice**

PD-123319 (2 μmol/l), an AT2-receptor antagonist, had no significant effect on the dose-response curves for Ang II in *iEGFR*$^{\Delta/\Delta VSMC}$ WT or KO animals (**B**). Ang II (**C**) also exerted a significantly smaller effect on vessels from *EGFR*$^{\Delta/\Delta VSMC\&CM}$ mice, comparable to *iEGFR*$^{\Delta/\Delta VSMC}$. To test if desensitization of the receptors was involved in the reduced Ang II response, 100 nM Ang II or 1 μM endothelin-1 were applied six times to aortic rings with interim complete washout for relaxation. Although no difference in desensitization between the two genotypes could be observed for endothelin-1 (**E**), desensitization by Ang II was more pronounced in KO animals (**F**). (**G**) Expression of receptors for endothelin-1 and Ang II was analysed via qRT-PCR. $N = 6$–8 animals/group, $^*P < 0.05$ compared with respective WT animals. (**H**) Western blot analysis of Ang II-induced ERK1/2 phosphorylation in aortic rings from inducible KO and respective WT animals after 10 min stimulation.

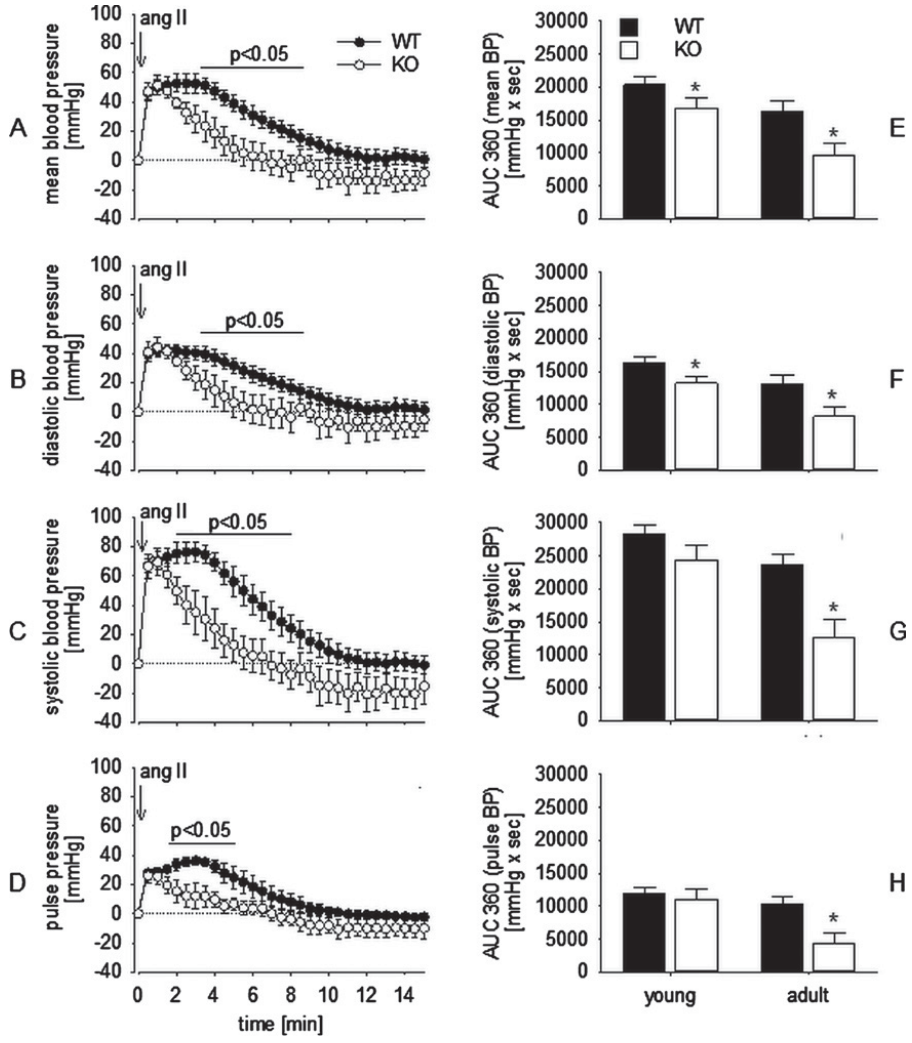

Figure 5 **Increase in mean (A), diastolic (B), systolic (C) and pulse pressure (D) is reduced in *iEGFR*$^{\Delta/\Delta VSMC}$ mice upon Ang II infusion (Ang II), resulting in a reduced AUC (= pressure load) in the first 360 s after start of infusion for mean (E), diastolic (F), systolic (G) and pulse pressure (H)**
$N = 5$–7 animals/group, $^{*}P < 0.05$ compared with respective WT animals.

our Mulvany and Halpern [36] experiments and show that the VSMC–EGFR is necessary for acute vasoconstriction induced by Ang II but not by endothelin-1.

Impact of prolonged EGFR inactivation on vascular homoeostasis

As the deletion of the EGFR in VSMC reduces systemic blood pressure and pressure response to Ang II, we hypothesized that prolonged inactivation of the EGFR might reduce maturation-associated alterations of the vascular system. It has been shown that pathological relevant alterations are apparent in mice starting at an age of 9 months [28]. Therefore, we compared mice aged 4 and 10 months and investigated the effect of VSMC–EGFR deletion on Ang II-induced blood pressure changes, marker gene expression and cardiovascular morphology.

As shown in Figures 5(E)–5(G), deletion of VSMC–EGFR reduced Ang II-induced blood pressure load in 4- and 10-month-old animals. By contrast with 4-month-old animals, the impact

of Ang II on pulse pressure of 10-month-old animals depends on VSMC–EGFR (Figure 5H), possibly due to the reduced HW/TL in KO animals compared with 10-month-old WT animals (Figure 6A).

It has been suggested that EGFR contributes to a para-inflammatory phenotype [18] and is involved in pathophysiological dysregulation of matrix homoeostasis [5,6,38]. Thus, we analysed the expression of marker genes for fibrosis {Col1a1 (collagen, type I, α 1) [Ref.Seq.: NM_007742], Col3a1 [Ref.Seq.: NM_009930]}, inflammation {Serpine1 (plasminogen activator inhibitor 1) [Ref.Seq.: NM_008871], Ccl2 (chemoattractant protein-2) [Ref.Seq.: NM_011333]} or heart hypertrophy {Nppb (B-type natriuretic peptide) [Ref.Seq.: NM_001287348.1], Myh6 (α-myosin heavy chain) [Ref.Seq.: NM_001164171]}. In 4-month-old animals, no difference between WT and KO was observed (Supplementary Table S2). However, we observed differences in age-related changes of gene expression in KO as compared with WT animals. In WT animals, aortic Ccl2 mRNA expression was approximately 4.5-fold higher in 10-month-old compared with 4-month-old animals (Figure 6B). This age-related increase was absent from KO animals, indicating that the age-related increase in Ccl2 in vessel walls is mediated by the VSMC–EGFR. These age-related changes were, in tendency, also detectable in heart sections of 10-month-old animals, with an increased CM diameter and percentage of fibrotic area in WT compared with KO animals at an age of 10 months (Supplementary Figure S8). For Serpine1, Col1a1 and Col3a1 no age-related differences in aortic gene expression were observed (Figure 6B; Supplementary Figure S9). Furthermore, in WT, but not in KO animals, cardiac Serpine1 mRNA expression was 3-fold higher in 10-month-old compared with 4-month-old animals (Figure 6C), demonstrating that the age-related increase in cardiac Serpine1 is mediated by VSMC–EGFR.

We further analysed if the increase in age also induces changes in resistance artery morphology. Intramyocardial arteries of KO animals showed no difference in wall thickness (10 months: WT 5.8 ± 0.5 μm and KO 5.9 ± 0.4 μm, $n = 6$–7 animals/group) or wall-to-lumen ratio (10 months: WT 0.055 ± 0.001 and KO 0.058 ± 0.004, $n = 6$–7 animals/group; $P < 0.05$, Supplementary Figures S2C and S2D) between age-matched WT and KO animals. The same was true for intrarenal resistance arteries (wall thickness 10 months: WT 11.3 ± 0.8 μm and KO 10.5 ± 0.9 μm; wall-to-lumen ratio 10 months: WT 0.108 ± 0.010 and KO 0.100 ± 0.004; $n = 6$–7 animals/group). In both genotypes, age-related BW/TL increase is the same, as well as the increase in systolic blood pressure measured by tail-cuff, whereas the age-related HW/TL increase observed in WT animals was completely abolished in KO animals (Figure 6A). These data are another example for the important vascular contribution to cardiac hypertrophy induced by ligands acting via G-protein-coupled receptors [39].

DISCUSSION

The evidence for a role of EGFR in vascular reactivity and control of vascular tone relied mainly on pharmacological approaches or studies in cultured cells and thereby could not identify the cellular origin of EGFR involved or exclude off-target effects. Recently, we reported on a mouse model with deletion of the EGFR in VSMC and a strong reduction in CMs [6], where the induction of gene deletion takes place during embryonic development [40] and therefore differences observed after birth might be due to developmental effects. Additionally, the contemporaneous deletion of the EGFR in VSMC and CMs makes it more difficult to distinguish between effects elicited by the VSMC–EGFR and effects by CM–EGFR. Thus, the inducible model presented now, allows the investigation of the role of VSMC–EGFR for vascular function in normally developed animals without confounding effects from other cell types.

iEGFR$^{\Delta/\Delta VSMC}$ mice show no alteration in morphology of the aorta and of intrarenal arteries but a reduced wall-to-lumen ratio of intramyocardial arterial vessels in 4-month-old animals with virtually preserved wall thickness. These data suggest that VSMC–EGFR deletion results in a slightly dilated vascular phenotype [6] in smaller arteries of some but not all tissues, which vanishes with age. The mechanism underlying these mild morphological alterations as well as the apparent tissue heterogeneity will be the scope of further studies.

As mentioned before, *EGFR*$^{\Delta/\Delta VSMC\&CM}$ mice show an increase in heart size with preserved systolic function [6]. In *iEGFR*$^{\Delta/\Delta VSMC}$ mice, no signs of heart hypertrophy could be observed, leading to the conclusion that the deletion of EGFR in CMs caused heart hypertrophy in the previously described *EGFR*$^{\Delta/\Delta VSMC\&CM}$ mice. Further investigations, beyond the scope of the present study, are required to determine the mechanistic role of CM–EGFR in the prevention of heart hypertrophy during development as well as postnatally [16].

Functionally, inducible conditional VSMC–EGFR–KO led to a reduction in basal blood pressure without any evidence for an altered stroke volume, supporting the hypothesis that VSMC–EGFR is necessary for the maintenance of a physiological vascular tone and therefore blood pressure homoeostasis. The slightly larger difference of systolic blood pressure in anaesthetized compared with conscious animals could be the result of a sympathetic counter-regulation. As the heart rate does not differ between the genotypes under both conditions, activation of the baroreceptor-reflex is unlikely. Furthermore, we did not detect increased aldosterone levels in in VSMC–EGFR–KO animals [16]. Nevertheless, telemetric in-depth analysis will be performed in future studies in order to obtain more detailed information on (a) blood pressure and (b) baroreceptor-reflex control. According to our data, KO animals try to counteract the reduced blood pressure by increased volume intake. In 10-month-old animals, the difference in basal blood pressure between WT and KO animals is reduced and only present in tendency, whereas the Ang II sensitivity was reduced even further. We hypothesize that successful physiological regulatory adaptations of the cardiovascular system led to the equalization of basal blood pressure at higher age. Although the underlying mechanisms have not yet been studied in detail, it is conceivable that differences in the vascular sympathetic tone, leading to an increased vasotonus, play an important role, besides altered volume homoeostasis. Alternatively, vessel sensitivity for vasoconstrictors other than Ang II, may be compensatory

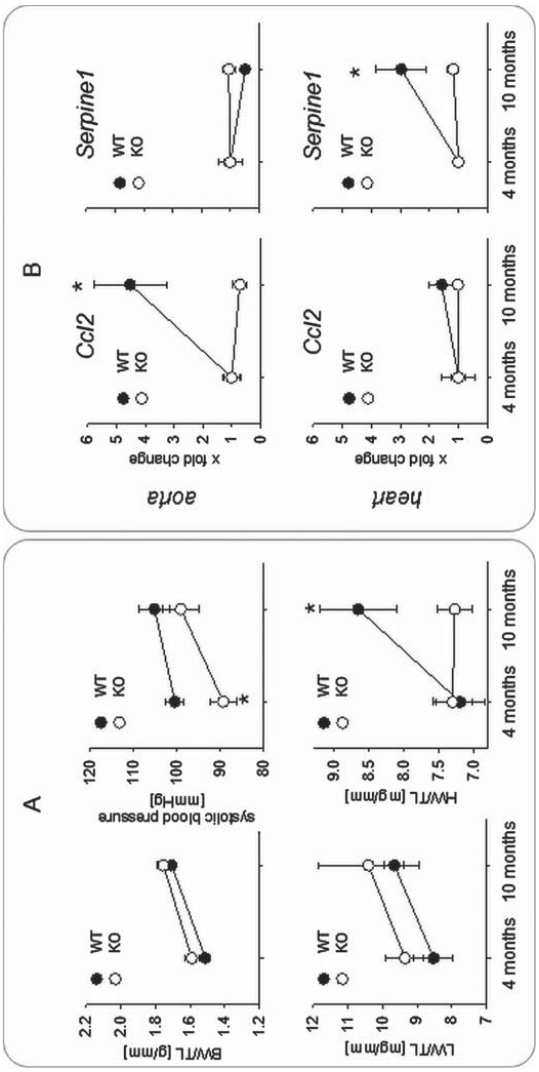

Figure 6 **(A) Deletion of VSMC–EGFR does not influence normalized BW (upper panel), HW (middle) and LW (lower) in 4-month-old *iEGFR*$^{\Delta/\Delta VSMC}$ mice**

Upon aging BW/TL increases in both genotypes to the same extent, whereas HW/TL is only increased in 10-month-old WT animals. $N = 6–9$ animals/group. $^{*}P < 0.05$ compared with respective WT animals. (**B**) Aortic mRNA expression. Ccl-2 expression increases with age up to 4-fold in the aortas of WT *iEGFR*$^{\Delta/\Delta VSMC}$ animals. This effect is completely abolished in KO animals. The expression of Serpine-1 increases in hearts of WT animals in contrast with KO. $N = 6–13$ animals/group. $^{*}P < 0.05$ compared with respective WT animals.

enhanced. According to our data, an increase in left ventricular stroke volume is an unlikely cause for the equalization of basal blood pressure at higher age.

As we did not observe alterations in heart rate or heart size in VSMC–EGFR–KO animals, we conclude that a decrease in total peripheral resistance causes the reduced mean arterial blood pressure, as it was the case in the constitutive model [6]. In contrast with constitutive VSMC–EGFR–KO animals [6] systolic blood pressure is reduced in inducible VSMC–EGFR–KO animals too. This difference results most probably from the cardiac hypertrophy in the constitutive model that partially compensates the reduced peripheral vascular resistance. In addition, CM-specific expression of a dominant-negative EGFR has been reported to increase systolic blood pressure [7]. We conclude that VSMC–EGFR has minor effects on basal short-time vascular tissue homoeostasis (Supplementary Table S2) but is relevant for physiological vessel function.

Isometric force measurements upon depolarization with KCl and in response to serotonin, carbachol or SNAP revealed no difference in reactivity of aortae from KO animals as compared with the respective WT in either model under the experimental conditions of the present study, showing (a) that the preparations were intact (including the endothelium), (b) that absence of VSMC–EGFR does not impair reactivity in terms of relaxation, (c) that the basal components for pharmaco-mechanical and electromechanical coupling are not affected and (d) that the extend of force generation is not restricted in the absence of VSMC–EGFR. This allowed the direct comparison of vasoactive agents in KO and WT animals, in order to determine the importance of EGFR for their action. The vasoconstrictive effects of endothelin-1 was not affected by EGFR deletion, strongly suggesting that this vasoactive agent, although known to be capable of EGFR transactivation [13,19], does not require VSMC–EGFR for the acute vascular action, at least in the aorta.

In contrast with the above described vasoactive substances, appropriate Ang II-induced force generation relies on VSMC–EGFR and there is no compensatory mechanism when EGFR is deleted constitutively. These data are in good agreement with the reduced Ang II-induced blood pressure load in mice with VSMC–EGFR deletion as compared with WT animals [6]. The long-known bell-shaped dose-response curve for Ang II, which most probably results from receptor type 1b expression and/or endothelial effects at higher concentrations [41–43], was observed for WT and KO animals, with a strong reduction in dF_{max} (Ang II efficacy). According to our results, this difference does not result from a decrease in AT1a/b-receptor expression or an unequal impact of Ang II type 2 receptors, but can be explained, at least in part, by an accelerated homologous desensitization. Thus, VSMC–EGFR affects the vasoconstrictor action of Ang II not just by supporting MAPK (mitogen-activated protein kinase) activation, but by modulating cellular AT1-receptor dynamics, possibly due to their interaction in Cav1 (caveolin 1)-enriched lipid rafts which seem to modulate AT1R trafficking [44]. We made first attempts to gain insight into possible underlying mechanisms, but so far can only conclude from our preliminary data that the observed difference does presumably not result from an alteration in β-arrestin or ATRAP expression, two known modulators of Ang II receptor sensitivity. Thus, further studies are needed to evaluate the Ang II-receptor-induced signal transduction and its modulation, like G-protein receptor-coupled kinases or regulators of G-protein-coupled signalling, in EGFR KO animals. At the moment, we hypothesize that the differences are most probably due to functional alterations of protein–protein interactions, as the time course of desensitization argues against a transcriptional or a protein degradation mechanism. The differential importance of EGFR for Ang II and endothelin-1 shown here is in accordance with its differential role in protein synthesis reported some time ago [45]. At the moment, we cannot exclude that the importance of VSMC–EGFR differs between large and small vessels and will address this question in future studies.

The reduced blood pressure load in KO animals, upon Ang II infusion, corresponds to the reduced Ang II-induced maximum force development in aortic rings, thereby confirming that Ang II mediates part of its direct, acute vascular effects *in vivo* via the VSMC–EGFR [8]. It has been suggested that VSMC–EGFR contributes to hypertension induced by prolonged application of Ang II [8], but to our knowledge this is the first study to show the importance of VSMC–EGFR for short-time regulation of blood pressure by Ang II *in vivo*. Our study defines the role of VSMC–EGFR in acute vascular Ang II-action *in vivo* and extends the observation of previous studies, where global inactivation of EGFR was applied in order to unveil its contribution to the chronic cardiovascular effects of Ang II [6,8,46]. The data obtained strongly suggest that VSMC–EGFR is required for a full physiological effect of Ang II on the acute vascular tone, i.e. the induction of a physiological pressure load (AUC360). Thus, VSMC–EGFR not only contributes to the blood pressure load under resting conditions but also under conditions of an activated renin–angiotensin system. In future studies, it will be important to investigate the impact of VSMC–EGFR on chronic blood pressure regulation and vascular tissue homoeostasis, especially in response to Ang II challenge.

A central finding of our study is the diverging importance of VSMC–EGFR with respect to the regulation of vascular tone for different stimuli reported to be capable of EGFR-transactivation. Thus, the fact of a molecular interaction of a given vasoactive substance with EGFR does not *a priori* imply that VSMC–EGFR is required for an appropriate acute vasoreaction, because different categories of EGFR-dependence seem to exist. As reports regarding the contribution of the EGFR to Ang II-induced chronic blood pressure increase and vascular remodelling are conflicting [6,8,9,46], we aim to evaluate the impact of VSMC–EGFR in this respect using the KO model.

EGFR is supposed to be involved in the regulation of transcriptional programmes leading to proliferation, fibrosis and para-inflammation [5,6,38]. In our study, we can confirm the increase in Ccl2 mRNA expression with age in WT animals [47]. As Ccl2 acts as a chemoattractant for monocytes, this probably contributes to the development of an age-related para-inflammatory phenotype. In KO animals, the Ccl2 increase was abolished. In addition, deletion of VSMC–EGFR prevented the age-related increase in heart size and the concomitant increase in Serpine1. The cardiac phenotype of VSMC–EGFR deletion results most probably from the reduced pressure load under basal

conditions as well as induced by Ang II. Future studies have to evaluate the role of the VSMC–EGFR for the maturation-associated alterations of the vessel wall, especially the impact on age-related para-inflammatory vessel remodelling, in more depth.

In conclusion, we provide *in vivo* evidence that VSMC–EGFR can be involved in basal blood pressure homoeostasis and has an agonist-dependent major impact on vascular function without affecting postnatal vascular morphology. VSMC–EGFR contributes to the Ang II-induced blood pressure load in 4-month-old and 10-month-old animals and thereby to the life-long pressure burden that determines pathologically-relevant cardiovascular remodelling, including heart hypertrophy. Thus, an increase in VSMC–EGFR expression, as e.g. during aging [27] or inappropriate activation, may represent a significant cardiovascular risk factor that increases blood pressure at rest and enhances vasoconstriction in response to Ang II. Our data underscore the importance of the vasculature for cardiac hypertrophy, induced by vasoactive substances like Ang II and emphasize the role of vascular EGFR in this process.

CLINICAL PERSPECTIVES

- VSMC–EGFR is involved in basal blood pressure homoeostasis and has an agonist-dependent major impact on vascular function regulation without affecting postnatal vascular morphology.
- VSMC–EGFR contributes to the Ang II-induced blood pressure load in animals 4 and 10 months of age and thereby to the life-long pressure burden that determines pathologically-relevant cardiovascular remodelling, including heart hypertrophy.
- Thus, an increase in VSMC–EGFR expression, as e.g. during aging or inappropriate activation, represents a significant cardiovascular risk factor that increases blood pressure at rest and enhances vasoconstriction in response to Ang II. Our data underscore the importance of the vasculature for cardiac hypertrophy, induced by vasoactive substances like Ang II and emphasize the role of vascular EGFR in this process.

AUTHOR CONTRIBUTION STATEMENT

Barbara Schreier and Michael Gekle conceived and designed the study. Barbara Schreier co-ordinated the study. Barbara Schreier, Mirja Hünerberg, Sindy Rabe, Daniel Bethmann, Sigrid Mildenberger and Christian Heise performed the experiments. Barbara Schreier, Michael Gekle, Sigrid Mildenberger, Christian Heise, Mirja Hünerberg and Daniel Bethmann analysed the data. Maria Sibilia and Stefan Offermanns contributed reagents/materials/analysis tools. Barbara Schreier and Michael Gekle wrote the paper. Mirja Hünerberg contributed to the writing of the paper. All authors contributed to drafting of the paper and revising it critically for intellectual content and the authors approved the final version to be published. Michael Gekle was responsible for funding.

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REFERENCES

1 Hackel, P.O., Zwick, E., Prenzel, N. and Ullrich, A. (1999) Epidermal growth factor receptors: critical mediators of multiple receptor pathways. Curr. Opin. Cell Biol. **11**, 184–189 CrossRef PubMed

2 Yarden, Y. and Sliwkowski, M.X. (2001) Untangling the ErbB signalling network. Nat. Rev. Mol. Cell. Biol. **2**, 127–137 CrossRef PubMed

3 Harris, R.C., Chung, E. and Coffey, R.J. (2003) EGF receptor ligands. Exp. Cell Res. **284**, 2–13 CrossRef PubMed

4 Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature **402**, 884–888 PubMed

5 Schlessinger, J. (2002) Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell **110**, 669–672 CrossRef PubMed

6 Schreier, B., Rabe, S., Schneider, B., Bretschneider, M., Rupp, S., Ruhs, S., Neumann, J., Rueckschloss, U., Sibilia, M., Gotthardt, M. et al. (2013) Loss of epidermal growth factor receptor in vascular smooth muscle cells and cardiomyocytes causes arterial hypotension and cardiac hypertrophy. Hypertension **61**, 333–340 CrossRef PubMed

7 Messaoudi, S., Di Zhang, A., Griol-Charhbili, V., Escoubet, B., Sadoshima, J., Farman, N. and Jaisser, F. (2012) The epidermal growth factor receptor is involved in angiotensin II but not aldosterone/salt-induced cardiac remodelling. PLoS One **7**, e30156 CrossRef PubMed

8 Kagiyama, S., Eguchi, S., Frank, G.D., Inagami, T., Zhang, Y.C. and Phillips, M.I. (2002) Angiotensin II-induced cardiac hypertrophy and hypertension are attenuated by epidermal growth factor receptor antisense. Circulation **106**, 909–912 CrossRef PubMed

9 Takayanagi, T., Kawai, T., Forrester, S.J., Obama, T., Tsuji, T., Fukuda, Y., Elliott, K.J., Tilley, D.G., Davisson, R.L., Park, J.Y. et al. (2015) Role of epidermal growth factor receptor and endoplasmic reticulum stress in vascular remodeling induced by angiotensin II. Hypertension **65**, 1349–1355 CrossRef PubMed

10 Noma, T., Lemaire, A., Naga Prasad, S.V., Barki-Harrington, L., Tilley, D.G., Chen, J., Le Corvoisier, P., Violin, J.D., Wei, H., Lefkowitz, R.J. et al. (2007) Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. J. Clin. Invest. **117**, 2445–2458 CrossRef PubMed

11 Ulu, N., Gurdal, H., Landheer, S.W., Duin, M., Guc, M.O., Buikema, H. and Henning, R.H. (2010) alpha1-Adrenoceptor-mediated contraction of rat aorta is partly mediated via transactivation of the epidermal growth factor receptor. Br. J. Pharmacol. **161**, 1301–1310 CrossRef PubMed

12 Hsieh, H.L., Tung, W.H., Wu, C.Y., Wang, H.H., Lin, C.C., Wang, T.S. and Yang, C.M. (2009) Thrombin induces EGF receptor expression and cell proliferation via a PKC(delta)/c-Src-dependent pathway in vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. **29**, 1594–1601 CrossRef PubMed

13 Chansel, D., Ciroldi, M., Vandermeersch, S., Jackson, L.F., Gomez, A.M., Henrion, D., Lee, D.C., Coffman, T.M., Richard, S., Dussaule, J.C. et al. (2006) Heparin binding EGF is necessary for vasospastic response to endothelin. FASEB J. **20**, 1936–1938 CrossRef PubMed

14 Morris, J.B., Pham, T.M., Kenney, B., Sheppard, K.E. and Woodcock, E.A. (2004) UTP transactivates epidermal growth factor receptors and promotes cardiomyocyte hypertrophy despite inhibiting transcription of the hypertrophic marker gene, atrial natriuretic peptide. J. Biol. Chem. **279**, 8740–8746 CrossRef PubMed

15 Kagiyama, S., Matsumura, K., Fukuhara, M., Sakagami, K., Fujii, K. and Iida, M. (2007) Aldosterone-and-salt-induced cardiac fibrosis is independent from angiotensin II type 1a receptor signaling in mice. Hypertens. Res. **30**, 979–989 CrossRef PubMed

16 Schreier, B., Rabe, S., Winter, S., Ruhs, S., Mildenberger, S., Schneider, B., Sibilia, M., Gotthardt, M., Kempe, S., Mader, K. et al. (2014) Moderate inappropriately high aldosterone/NaCl constellation in mice: cardiovascular effects and the role of cardiovascular epidermal growth factor receptor. Sci. Rep. **4**, 7430 CrossRef PubMed

17 Jagadeesha, D.K., Takapoo, M., Banfi, B., Bhalla, R.C. and Miller, F.J. (2012) Nox1 transactivation of epidermal growth factor receptor promotes N-cadherin shedding and smooth muscle cell migration. Cardiovasc. Res. **93**, 406–413 CrossRef PubMed

18 Pullar, C.E. and Isseroff, R.R. (2006) The beta 2-adrenergic receptor activates pro-migratory and pro-proliferative pathways in dermal fibroblasts via divergent mechanisms. J. Cell Sci. **119**, 592–602 CrossRef PubMed

19 Schreier, B., Dohler, M., Rabe, S., Schneider, B., Schwerdt, G., Ruhs, S., Sibilia, M., Gotthardt, M., Gekle, M. and Grossmann, C. (2011) Consequences of epidermal growth factor receptor (ErbB1) loss for vascular smooth muscle cells from mice with targeted deletion of ErbB1. Arterioscler. Thromb. Vasc. Biol. **31**, 1643–1653 CrossRef PubMed

20 Inagami, T. and Eguchi, S. (2000) Angiotensin II-mediated vascular smooth muscle cell growth signaling. Braz. J. Med. Biol. Res. **33**, 619–624 CrossRef PubMed

21 Yang, C.M., Lin, M.I., Hsieh, H.L., Sun, C.C., Ma, Y.H. and Hsiao, L.D. (2005) Bradykinin-induced p42/p44 MAPK phosphorylation and cell proliferation via Src, EGF receptors, and PI3-K/Akt in vascular smooth muscle cells. J. Cell. Physiol. **203**, 538–546 CrossRef PubMed

22 Fleisch, J.H. (1981) Age-related decrease in beta adrenoceptor activity of the cardiovascular system. Trends Pharmacol. Sci. **2**, 337–339 CrossRef

23 Miller, Jr, F.J., Chu, X., Stanic, B., Tian, X., Sharma, R.V., Davisson, R.L. and Lamb, F.S. (2010) A differential role for endocytosis in receptor-mediated activation of Nox1. Antioxid. Redox Signal. **12**, 583–593 CrossRef PubMed

24 Flamant, M., Tharaux, P.L., Placier, S., Henrion, D., Coffman, T., Chatziantoniou, C. and Dussaule, J.C. (2003) Epidermal growth factor receptor trans-activation mediates the tonic and fibrogenic effects of endothelin in the aortic wall of transgenic mice. FASEB J. **17**, 327–329 PubMed

25 Chan, S.L., Umesalma, S. and Baumbach, G.L. (2015) Epidermal growth factor receptor is critical for angiotensin II-mediated hypertrophy in cerebral arterioles. Hypertension **65**, 806–812 CrossRef PubMed

26 Iwasaki, H., Eguchi, S., Marumo, F. and Hirata, Y. (1998) Endothelin-1 stimulates DNA synthesis of vascular smooth-muscle cells through transactivation of epidermal growth factor receptor. J. Cardiovasc. Pharmacol. **31** (Suppl 1), S182–S184 CrossRef PubMed

27 Krug, A.W., Allenhofer, L., Monticone, R., Monticone, R., Spinetti, G., Gekle, M., Wang, M. and Lakatta, E.G. (2010) Elevated mineralocorticoid receptor activity in aged rat vascular smooth muscle cells promotes a proinflammatory phenotype via extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase and epidermal growth factor receptor-dependent pathways. Hypertension **55**, 1476–1483 CrossRef PubMed

28 McCurley, A., Pires, P.W., Bender, S.B., Aronovitz, M., Zhao, M.J., Metzger, D., Chambon, P., Hill, M.A., Dorrance, A.M., Mendelsohn, M.E. et al. (2012) Direct regulation of blood pressure by smooth muscle cell mineralocorticoid receptors. Nat. Med. **18**, 1429–1433 CrossRef PubMed

29 Wang, M., Monticone, R.E. and Lakatta, E.G. (2014) Proinflammation of aging central arteries: a mini-review. Gerontology **60**, 519–529 CrossRef PubMed

30 Sibilia, M. and Wagner, E.F. (1995) Strain-dependent epithelial defects in mice lacking the EGF receptor. Science **269**, 234–238 CrossRef PubMed

31 Sibilia, M., Kroismayr, R., Lichtenberger, B.M., Natarajan, A., Hecking, M. and Holcmann, M. (2007) The epidermal growth factor receptor: from development to tumorigenesis. Differentiation **75**, 770–787 CrossRef PubMed

32 Natarajan, A., Wagner, B. and Sibilia, M. (2007) The EGF receptor is required for efficient liver regeneration. Proc. Natl. Acad. Sci. U.S.A. **104**, 17081–17086 CrossRef PubMed

33 Wirth, A., Benyo, Z., Lukasova, M., Leutgeb, B., Wettschureck, N., Gorbey, S., Orsy, P., Horvath, B., Maser-Gluth, C., Greiner, E. et al. (2008) G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. Nat. Med. **14**, 64–68 CrossRef PubMed

34 Groneberg, D., Koenig, P., Wirth, A., Offermanns, S., Koesling, D. and Friebe, A. (2010) Smooth muscle-specific deletion of nitric oxide-sensitive guanylyl cyclase is sufficient to induce hypertension in mice. Circulation **121**, 401–409 CrossRef PubMed

35 Liang, M., Ekblad, E., Lydrup, M.L. and Nilsson, B.O. (2003) Combined lack of estrogen receptors alpha and beta affects vascular iNOS protein expression. Cell Tissue Res. **313**, 63–70 CrossRef PubMed

36 Mulvany, M.J. and Halpern, W. (1977) Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ. Res. **41**, 19–26 CrossRef PubMed

37 Freeman, G.L., Little, W.C. and O'Rourke, R.A. (1986) The effect of vasoactive agents on the left ventricular end-systolic pressure-volume relation in closed-chest dogs. Circulation **74**, 1107–1113 CrossRef PubMed

38 Lemmon, M.A. (2009) Ligand-induced ErbB receptor dimerization. Exp. Cell Res. **315**, 638–648 CrossRef PubMed

39 Keys, J.R., Greene, E.A., Koch, W.J. and Eckhart, A.D. (2002) Gq-Coupled receptor agonists mediate cardiac hypertrophy via the vasculature. Hypertension **40**, 660–666 CrossRef PubMed

40 Miano, J.M., Ramanan, N., Georger, M.A., Mesy Bentley, K.L., Emerson, R.L., Balza, R.O., Xiao, Q., Weiler, H., Ginty, D.D. and Misra, R.P. (2004) Restricted inactivation of serum response factor to the cardiovascular system. Proc. Natl. Acad. Sci. U.S.A. **101**, 17132–17137 CrossRef PubMed

41 Dumont, E.C., Lambert, C. and Lamontagne, D. (1996) Modification of aortic contractility in the cardiomyopathic hamster. Br. J. Pharmacol. **118**, 1141–1146 CrossRef PubMed

42 Babaei, H., Ebrahimi, F., Shahbazi, M.J., Azarmi, Y. and Gharehbagheri, A. (2011) Vasorelaxant effect of a newly synthesized dihydropyridine ethyl ester (DHPEE) on rat thoracic aorta: dual mechanism of action. Adv. Pharm. Bull. **1**, 10–17 PubMed

43 Kuroda, S., Konishi, H., Okishio, M. and Fujisawa, Y. (1994) Novel subtype of human angiotensin II type 1 receptor: analysis of signal transduction mechanism in transfected Chinese hamster ovary cells. Biochem. Biophys. Res. Commun. **199**, 475–481 CrossRef PubMed

44 Czikora, I., Feher, A., Lucas, R., Fulton, D.J.R. and Bagi, Z. (2015) Caveolin-1 prevents sustained angiotensin II-induced resistance artery constriction and obesity-induced high blood pressure. Am. J. Physiol. Heart Circ. Physiol. **308**, H376–H385 CrossRef PubMed

45 Beaucage, P. and Moreau, P. (2004) EGF receptor transactivation in angiotensin II and endothelin control of vascular protein synthesis *in vivo*. J. Cardiovasc. Pharmacol. **44** (Suppl 1), S20–S23 CrossRef PubMed

46 Bokemeyer, D., Schmitz, U. and Kramer, H.J. (2000) Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor. Kidney Int. **58**, 549–558 CrossRef PubMed

47 Spinetti, G., Wang, M., Monticone, R., Zhang, J., Zhao, D. and Lakatta, E.G. (2004) Rat aortic MCP-1 and its receptor CCR2 increase with age and alter vascular smooth muscle cell function. Arterioscler. Thromb. Vasc. Biol. **24**, 1397–1402 CrossRef PubMed

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Moderate inappropriately high aldosterone/NaCl constellation in mice: cardiovascular effects and the role of cardiovascular epidermal growth factor receptor

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Non-physiological activation of the mineralocorticoid receptor (MR), e.g. by aldosterone under conditions of high salt intake, contributes to the pathogenesis of cardiovascular diseases, although beneficial effects of aldosterone also have been described. The epidermal growth factor receptor (EGFR) contributes to cardiovascular alterations and mediates part of the MR effects. Recently, we showed that EGFR is required for physiological homeostasis and function of heart and arteries in adult animals. We hypothesize that moderate high aldosterone/NaCl, at normal blood pressure, affects the cardiovascular system depending on cardiovascular EGFR. Therefore we performed an experimental series in male and female animals each, using a recently established mouse model with EGFR knockout in vascular smooth muscle cells and cardiomyocytes and determined the effects of a mild-high aldosterone-to-NaCl constellation on a.o. marker gene expression, heart size, systolic blood pressure, impulse conduction and heart rate. Our data show that (i) cardiac tissue of male but not of female mice is sensitive to mild aldosterone/NaCl treatment, (ii) EGFR knockout induces stronger cardiac disturbances in male as compared to female animals and (iii) mild aldosterone/NaCl treatment requires the EGFR in order to disturb cardiac tissue homeostasis whereas beneficial effects of aldosterone seem to be independent of EGFR.

The mineralocorticoid receptor (MR) with its endogenous ligand aldosterone is one of the main effectors in the renin-angiotensin-aldosterone-system and has a pivotal role in water-electrolyte homeostasis and regulation of blood pressure. Recently the MR has gained further interest after the importance of the MR for pathological changes in the cardiovascular system and the kidneys became apparent. In two pivotal clinical studies the beneficial effect of MR antagonists like spironolactone and eplerenone for patients with cardiovascular disease was proven; however, without understanding the underlying mechanisms[1,2]. Since then, MR activation has been shown to be involved in different pathophysiological effects in the reno-cardiovascular system including endothelial dysfunction, inflammation, hypertrophy and fibrosis in both clinical studies and animal experiments[1,3–5]. The trigger that causes the MR to turn from a receptor regulating water-electrolyte homeostasis and not causing any harm into a receptor mediating pathological effects in the cardiovascular system is still an enigma. One way to achieve this is by having inappropriately high aldosterone levels in relation to a normal salt status in an individual. Although such a scenario is likely in case of hyperaldosteronism caused by adrenal adenoma or hyperplasia, this does not seem to apply for the majority of patients, where aldosterone levels were unremarkable. In animal studies, it is striking that aldosterone application only leads to pathological changes in the presence of additional permissive factors like salt, aging or oxidative stress, in other words a parainflammatory micro-milieu[6]. Among the plethora of molecules involved in aldosterone effects[7] the epidermal growth factor receptor (EGFR) has been attributed major importance especially for the pathological effects of activated MR in the cardiovascular system[8,9].

Beside its affore-mentioned detrimental effects aldosterone has also been reported to exert beneficial effects in the cardiovascular system[10], e.g. in a mouse model of type 2 diabetes cardiac hyperaldosteronism prevented inflammation, oxidative stress and capillary rarefaction in the heart. The mechanisms mediating these beneficial effects are not completely understood.

EGFR is a receptor tyrosine kinase, that can be activated by its ligands, e.g. EGF, TGF-α, HB-EGF, which derive from neighbouring or the same cell by shedding[11,12]. But it can also be transactivated by binding of vasoactive substances to their distinct receptor, like angiotensin II[13,14], α1- and β-adrenergic agonists[15,16], and aldosterone[17–19] and thereby support their pathophysiological effects in the cardiovascular system[13,20]. Thus, EGFR - via transactivation - has the potential to mediate signalling of non-EGFR ligands and thereby serve as a heterologous transducer of cellular signalling. Pathophysiological effects of EGFR include parainflammatory dysregulation of tissue homeostasis leading for example to vascular dysfunction and fibrosis as well as electrical remodelling by altered ion channel activity or expression[21]. Many attempts to delete or overexpress the EGFR family members or their ligands have been made[22], revealing the importance of EGFR for perinatal and postnatal development. Mice lacking the EGFR die at day 11.5 of gestation or survive until the postnatal day 20, depending on the genetic background[23].

In order to investigate the physiological importance of EGFR in the cardiovascular system further we recently generated a mouse model with simultaneous deletion of the EGFR in cardiomyocytes (CM) and vascular smooth muscle cells (VSMC)[24]. Under baseline conditions analysis without gender differentiation revealed cardiac hypertrophy, probably as a result of enhanced growth-promoting ROS formation due to greater NOX4 activity. Cardiac output was increased but no signs of fibrosis or inflammation could be observed. Furthermore, a reduced total peripheral vascular resistance and mean blood pressure due to dilated vascular phenotype with minor fibrosis and inflammation in knockout animals was detected. Thus, EGFR besides being a possible heterologous transducer of adverse cardiovascular stimuli it is also required for healthy basal cardiovascular tissue homeostasis[24].

In the present study we extend our investigations regarding the cardiovascular importance of EGFR in two dimensions. First, we tested whether the loss of EGFR in CM and VSMCs[24] leads to gender-related effects, comparing male and female knockout mice with their wildtype littermates. Second, we tested whether pathological cardiovascular effects of MR-activation by aldosterone depend on EGFR in CM and VSMC, again using the gender-specific approach. Because a growing body of evidence indicates that it is not the aldosterone concentration per se that induces pathological, blood pressure-independent, alterations but an inappropriately high aldos-

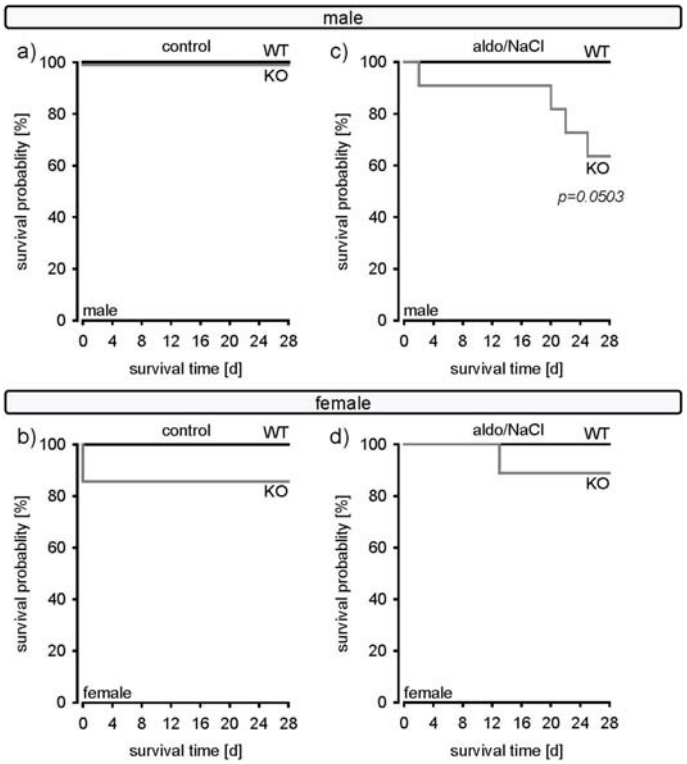

Figure 1 | **Kaplan-Meier-Plots on survival of wildtype and knockout animals depending on gender and treatment.** Mantel-Cox-Test for survival rate was performed revealing that male EGFR-knockout animals had a higher probability to die during the 28 days of aldosterone/NaCl treatment than male animals under control conditions or female EGFR-knockout animals upon aldosterone/NaCl treatment. a) survival of male wildtype and knockout animals during 28 days of control treatment (N (WT) =9, N(KO) =8), b) survival of female wildtype and knockout animals during 28 days of control treatment (N (WT) =9, N(KO) =7), c) survival of male wildtype and knockout animals during 28 days of aldosterone/NaCl treatment (N (WT) =10, N(KO) =9) and d) survival of female wildtype and knockout animals during 28 days of aldosterone/NaCl treatment (N (WT) =9, N(KO) =11).

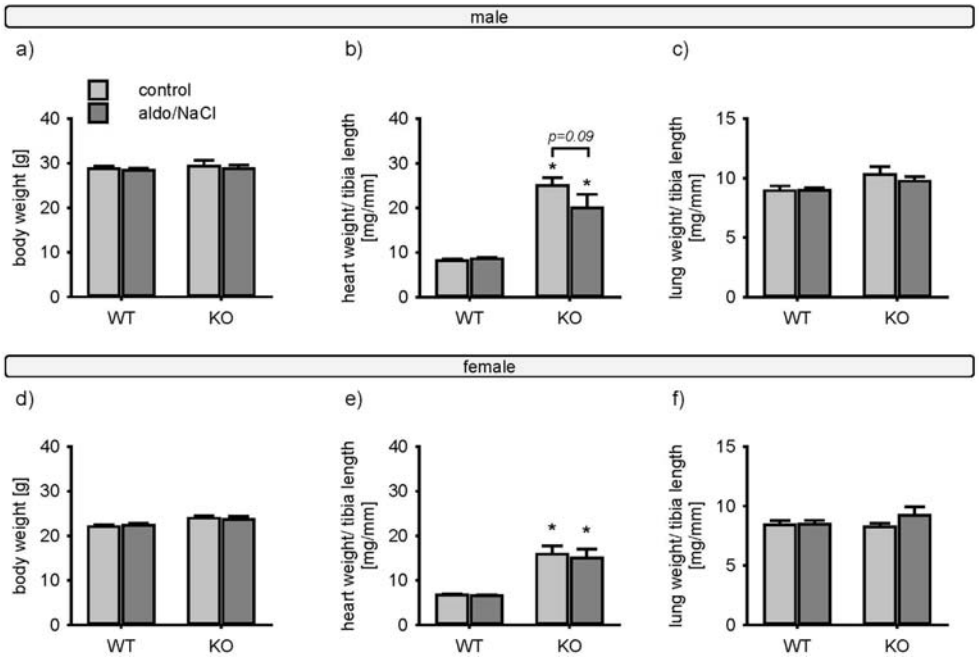

Figure 2 | **Effect of genotype and treatment on organ weights in male and female animals.** Wildtype and knockout animals were either untreated or treated with aldosterone/NaCl for 28 days. Afterwards the animals were sacrificed and the body weight (a, d), heart weight/tibia length (b, e) and lung weight/tibia length (c, f) were determined with respect to gender. Data are given as mean ± SEM, * $p<0.05$ compared to respective wildtype, # $p<0.05$ compared to respective control, N=9–12 animals/group.

terone concentration in relation to the NaCl status or intake - even at aldosterone concentrations within the "normal" range[25,26] -, we establish an experimental setup in which the intake of NaCl is increased and the aldosterone levels are inadequately high but in the physiological range. Furthermore, in our model no iatrogenic reduction of renal function was induced and blood pressure remained in the physiological range. This constellation represents most probably the situation of a large proportion of patients benefiting from MR blocking therapy. Using this model we show that (i) male are more susceptible to inappropriate high aldosterone, (ii) loss of EGFR leads to larger detrimental cardiac effects in male animals and (iii) mild aldosterone/NaCl treatment requires the EGFR in order to disturb cardiac tissue homeostasis whereas beneficial effects of aldosterone seem to be independent of EGFR.

Results

Plasma aldosterone concentrations. Our aim was to reproduce a situation with inappropriately high plasma aldosterone relative to salt intake with absolute aldosterone plasma concentrations in the normal range, because it is known that this is most probably of high clinical prevalence. In order to obtain this condition animals received 1% NaCl in the drinking water - increasing daily NaCl intake from ~10 mg to ~60 mg per animal - and low dose aldosterone infusion from subcutaneous pellets. Analysis of plasma aldosterone concentrations in preparation of this study showed that the hormone levels were not different between the groups (WT: 341.6 ± 54.4 pM, KO: 342.1 ± 79.1 pM, aldosterone/NaCl (WT and KO): 262.0 ± 52.6 pM, N=7–11 animals/group). Thus, in our experimental system aldosterone levels are indeed inappropriately high although still in a physiological range.

Survival. We have demonstrated before that mortality of the KO animals is higher as compared to WT animals aged > 6 month[24]. Yet, over the relative short 28d period of aldo/NaCl treatment in the present study, there was no significant difference in mortality for the two genotypes under control conditions (Fig. 1A and B). Aldosterone/NaCl-treatment enhanced mortality in male knockout animals (Fig. 1C) but not in the other groups (Fig. 1D). Treatment resulted in an increase in knockout male mortality by 45.5% over 28d, whereas mortality in knockout female animals was virtually not affected.

As aldosterone can induce the expression of EGFR we analyzed EGFR mRNA expression via realtime qRT PCR. Inadequately high aldosterone/salt treatment indeed increased the expression of EGFR in the heart of male wildtype (196% of control, 95%CI: 140–252%, n=9–11 animals/group) but not of female wildtype or the knockout animals. The expression of the other ErbB-family members, namely ErbB2-4, was not affected.

Organ weight. To rule out growth defects caused by the knockout of EGFR and as heart failure can lead to lung congestion organ weights were evaluated. There was no difference in body (Fig 2A and 2D) and lung weight (Fig 2C and 2F) for any of the four groups of both genders. As predicted from a previous study, heart weight was enhanced in knockout animals of the control groups (Fig 2B and 2E). In extension to previous studies, the data presented here show that the degree of cardiac hypertrophy is significantly higher in male knockout animals (306 ± 22% of WT for male KO animals; 218 ± 23% of WT for female KO animals, N=8–10). In order to gain more insight into the time course of hypertrophy development we investigated heart size of newborn animals of either gender (N=5 for each group). Heart weight per body weight was 5.73 ± 0.18 mg/g

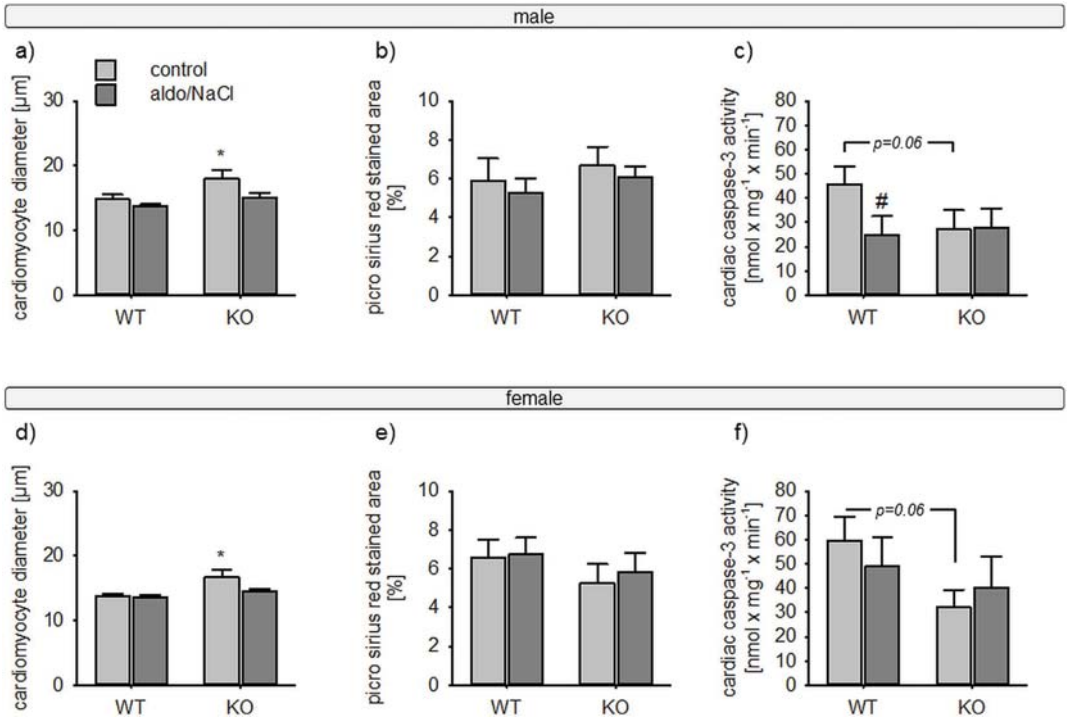

Figure 3 | **Effect of genotype and treatment on cardiomyocyte diameter, caspase-3 activity and fibrosis depending on gender.** Wildtype and knockout animals were either untreated or treated with aldosterone/NaCl for 28 days. Afterwards the animals were sacrificed and the hearts were either partially embedded with paraffin or snap frozen for further analysis. Cardiomyocyte diameter (a, d) was determined in haematoxylin/eosin stained slices. At least 100 cardiomyocytes were analysed per mouse. Cardiac fibrosis was determined in picro sirius red stained, paraffin embedded heart sections as % red stained area per field in male (b) and female (e) animals. Cardiac caspase-3 activity was determined from snap frozen cardiac homogenates in male (c) and female (f) animals. Data are given as mean ± SEM, * $p<0.05$ compared to respective wildtype, # $p<0.05$ compared to respective control. N=9–12 animals/group.

for male and 5.64 ± 0.16 mg/g for female wildtype animals. The values for knockout animals were 7.98 ± 0.26 mg/g (male) and 8.04 ± 0.52 mg/g (female). Thus, there is a similar (~35%) cardiac hypertrophy at birth in male and female knockout animals. After birth hypertrophy aggravates, yet to a greater extend in male animals as compared to females.

Treatment with aldosterone/NaCl did not affect heart weight with statistical significance in any of the groups, however hypertrophy in male KO animals was reduced by ~25% with $p=0.09$ (Fig. 2B).

The slight increase in lung weight in male knockout animals results most probably from the strong cardiac hypertrophy with enhanced enddiastolic volume[24].

Tissue analysis. To analyze gross morphological changes underlying the observed heart hypertrophy cardiomyocyte diameter, fibrosis (SR staining) and apoptosis (caspase-3 activity) were determined. Cardiomyocyte diameter was enhanced in knockout animals of both gender (Fig. 3 A and 3D). Aldosterone/NaCl treatment exerted not significant effects in wildtype animals but abolished cell hypertrophy in EGFR knockout, irrespective of gender, indicating a beneficial effect of the treatment.

The fractions of fibrotic tissue were identical in between the different groups of both genders (Fig. 3B and 3E), which is not surprising, because histomorphologically detectable changes during mild pathological stimulation occur only after prolonged exposure.

In contrast, cardiac caspase-3 activity (Fig. 3C and 3F) was reduced in knockout animals of both genders as compared to sex-matched wildtype animals. Furthermore, aldosterone/NaCl reduced caspase-3-activity in male wildtype but not in female wildtype animals as shown in figure 3, again indicating a certain protective effect of treatment. As aldosterone/NaCl-treatment did not enhance the effect of EGFR-KO we conclude that the effect of aldo/NaCl requires the EGFR.

Blood pressure, heart rate, electrocardiography. Aldosterone influences blood pressure via the regulation of salt/water homeostasis, therefore systolic blood pressure via tail-cuff was determined. As expected in a situation of moderate high aldosterone/NaCl with intact renal function, neither systolic blood pressure nor heart rate were altered significantly (male: Fig. 4A and 4B, female: Fig. 4C and 4D) in any of the groups. These data confirm that we are investigating blood pressure independent effects.

Electrocardiography was performed to analyze the electrical conduction in the heart. Electrical conduction (P-duration, PR-, QRS and QTc-interval) did not differ between the female groups (Fig. 5E–H), by contrast to the male groups (Fig. 5A–D), where EGFR-KO caused prolonged P-duration and QRS- as well as QTc-intervals (and a PR-prolongation in tendency) that were reversed by aldosterone/NaCl-treatment. The observed prolongation mirrors at least in part the cardiac hypertrophy.

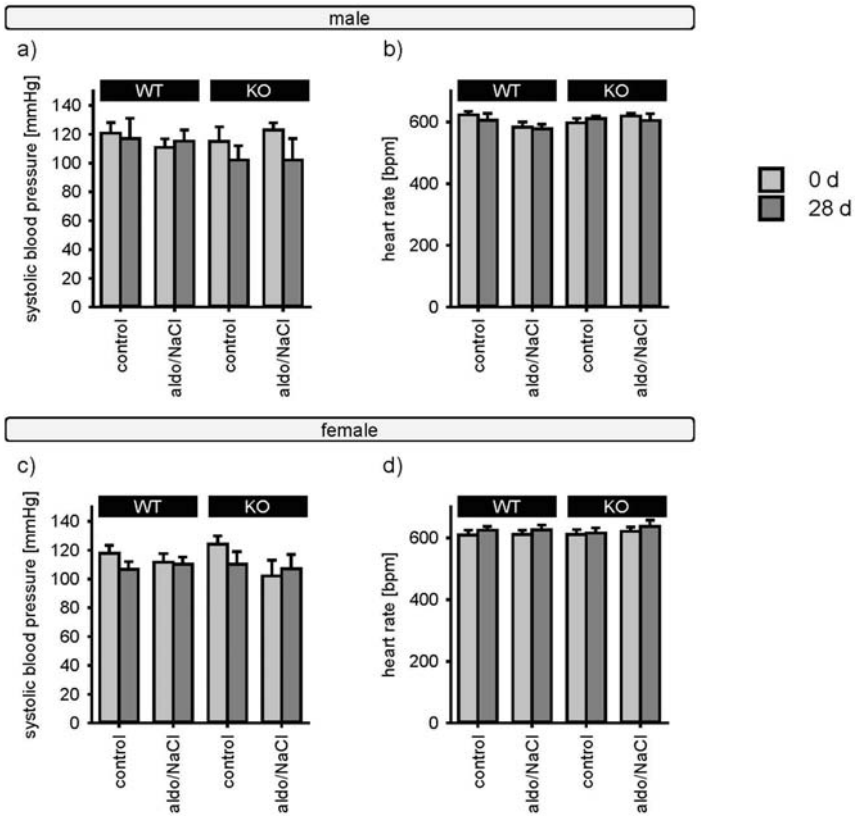

Figure 4 | **Effect of genotype and treatment on systolic blood pressure and heart rate depending on gender.** Systolic blood pressure (a, c) and heart rate (b,d) was measured in 5–7month-old animals for 5 consecutive days (0 d) using external tail pulse detection. After this time period aldosterone releasing pellets were implanted subcutaneously in the back of the animals. After a three day recovery period blood pressure and heart rate were measured twice weekly for additional 28 days. Data are given as mean ± SEM, * $p<0.05$ compared to respective wildtype, # $p<0.05$ compared to respective control. N=9–12 animals/group.

Of note, aldosterone/NaCl-treatment induced a PR-interval prolongation in male wildtype but not in male knockout or female animals (Fig. 5A and 5E). PR-interval prolongation corresponds to enhanced PQ-intervals in patients with hyperaldosteronism[27] and in mice with cardiac MR overexpression[4], substantiating the validity of our model.

The parameters for heart rate variability were not different in the female groups (Fig. 6B). In contrast, all three parameters were significantly enhanced by aldosterone/NaCl in male EGFR knockout animals (Fig. 6A, C and D), leading to an increased heart rate variability.

mRNA expression. In the heart of male knockout animals (Fig. 7) an increase in the expression of marker genes for hypertrophy (Nppa and Nppb), fibrosis (Col1a1, Fn1 and Serpine1) and ROS balance (Nos1 and Nox4) was observed under control conditions compared to wildtype. In contrast, expression of Ccl2, Tnf or Tgfb1 was not altered, arguing against a proinflammatory phenotype.

Aldosterone/NaCl treatment increased the expression of marker genes for fibrosis (Col1a1, Col3a1 and Serpine1) and ROS balance (Nos1) in hearts of wildtype males (Fig. 7).

In the heart of male knockout animals (Fig. 7) aldosterone/NaCl treatment prevented the increase in Nppb expression, indicating again an EGFR-independent protective effect. On the other side, the expression of none of the genes affected in male wildtype animals was altered in male knockouts upon aldosterone/NaCl treatment, suggesting that EGFR is required for at least some of the detrimental effects of aldosterone/NaCl.

In the aorta of male animals (Supplementary figure S1), with the exception of Ccl2, no changes in mRNA expression were observed in wildtype or knockout animals with or without treatment.

In the hearts (Fig. 8) of female knockout animals the expression of Nppa, Nppb and Nos1 was enhanced compared to wildtype females. The expression of all other mRNAs investigated was not altered.

Aldosterone/NaCl-treatment exerted no significant effect on mRNA expression in hearts of female wildtype and female knockout animals (Fig. 8).

In the aortae (Supplementary figure S2) of female animals no major change in mRNA expression was observed for any of the groups.

Discussion

In the present study we investigate the cardiovascular impact of the clinical highly relevant constellation of normal plasma aldosterone levels despite enhanced NaCl intake, independent of blood pressure. In this constellation, achieved by feeding a 1% NaCl drinking solu-

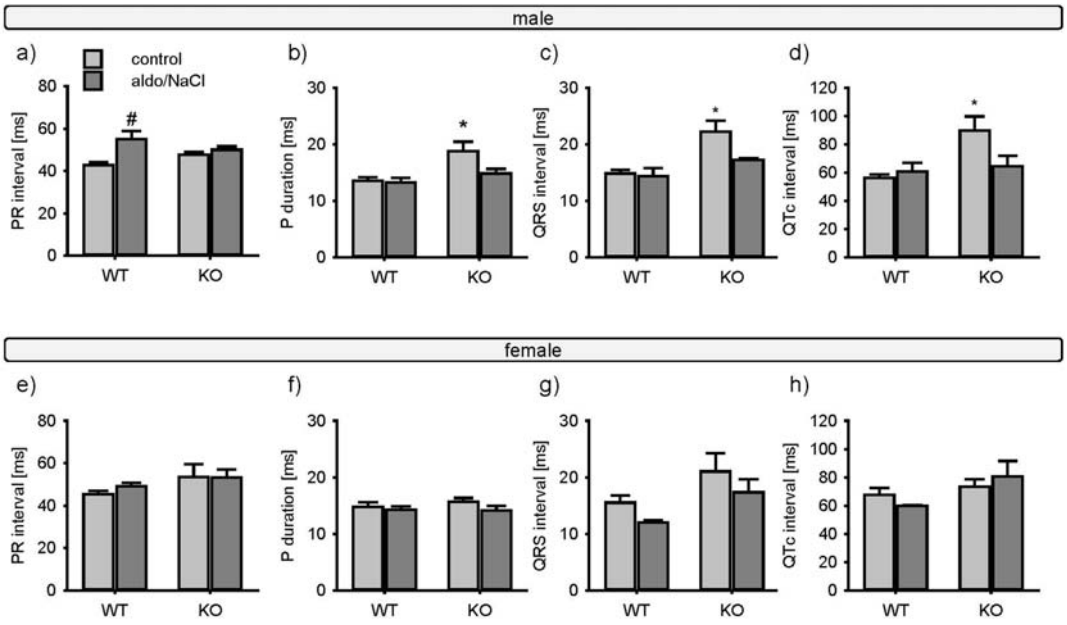

Figure 5 | **Effect of genotype and treatment on electrocardiography parameters distinguished by gender.** After 28days with or without aldo/NaCl treatment animals were anaesthetized with isoflurane and electrocardiography recordings were obtained for at least ten minutes employing an Einthoven I limb lead recording. PR interval (a, e) was measured from the beginning of the P wave to the start of the chamber complex, P duration (b, f) was defined as the time from start to end of the P wave, QRS interval (c, g) was measured from start to end of the chamber excitation and the QTc interval (d,h; time from start of the chamber excitation to the end of the chamber repolarization, heartrate corrected according to Bazette). Data are given as mean ± SEM, * $p<0.05$ compared to respective wildtype, # $p<0.05$ compared to respective control. N=3-5 animals/group.

tion (leading to an additional NaCl intake of >50 mg per day, which corresponds to a total NaCl intake of ~600% of control animals) and clamping plasma aldosterone to physiological values by continuous infusion with subcutaneous pellets, aldosterone is inappropriately high, because normally enhanced NaCl intake suppresses aldosterone secretion. The model refrains from partial kidney removal and is not accompanied by blood pressure elevations within the observation period of 28 days as expected, because it had been demonstrated that in mice[28,29] and rats[30] without nephrectomy even the treatment with higher salt concentrations does not result in an increase in blood pressure[31]. Thus, the model allows the investigations of effects elicited by inappropriately high aldosterone-to-NaCl concentrations, known to lead to inappropriate MR activation[32]. Furthermore, the dimension of increase in NaCl intake in the treated groups represents a known risk factor for cardiovascular morbidity and mortality in humans[33].

Effects of Aldosterone/NaCl in wildtype animals. According to our data a moderate inappropriate high aldosterone/NaCl constellation induces a mild profibrotic cardiac phenotype with slowed atrioventricular conduction in hearts of male animals. In extension to previous studies, that reported a higher susceptibility of male animals under conditions of extensively increased aldosterone/NaCl in combination with reduced kidney function[34–36], we now show that female animals are apparently not affected at all by mildly elevated aldosterone/NaCl, at least during the experimental period used in this study. Thus, we report, to our knowledge for the first time, that in the absence of changes in blood pressure and at preserved kidney function male but not female animals are affected by aldosterone/NaCl. Under our experimental conditions and with respect to the parameters investigated, the effects were restricted to the heart without significant alterations in the aorta. Of course we cannot exclude that treatment over a period exceeding 28 days would also affect the vasculature.

The prolonged PR interval is in line with the observations that (i) in uninephrectomized rats receiving aldosterone intraatrial and atrioventricular conduction are slower compared to untreated animals[37], (ii) mice with cardiac MR overexpression[4] also show extended PR intervals and (iii) human patients with hyperaldosteronism have longer PQ-intervals as compared to patients with essential hypertension[27]. It was hypothesized, that hyperaldosteronism might lead to site-specific conduction delay due to atrial anisotropy and thereby stabilize or promote atrial arrhythmias. There is additional evidence from humans, that the mineralocorticoid receptor might be involved in the onset of atrial fibrillation[38,39]. The pro-arrhythmogenic effect of aldosterone, suggested by several studies, most probably results from the cellular consequences of cardiac mineralocorticoid receptor activation, like disturbed control of cellular calcium homeostasis, action potential lengthening, alteration of calcium and potassium conductance and sarcoplasmic reticulum diastolic leaks[40]. In order to refine the analysis of electrical heart activity telemetrical studies will be performed in the future.

Effect of EGFR KO. In the last years our group, among others, demonstrated that the activated mineralocorticoid receptor is able to increase EGFR expression and can transactivate the EGFR[8,17,18]. Therefore we aimed to analyse the contribution of the EGFR to the aldo/NaCl induced cardiovascular alterations.

The ErbB-receptor family and its role in the heart gained interest as the anti-cancer drug herceptin (ErbB2-receptor antagonist) induced a dilative cardiomyopathy in a subset of patients[14]. Cardiac phenotypes in transgenic mice lacking HB-EGF[41,42], or with

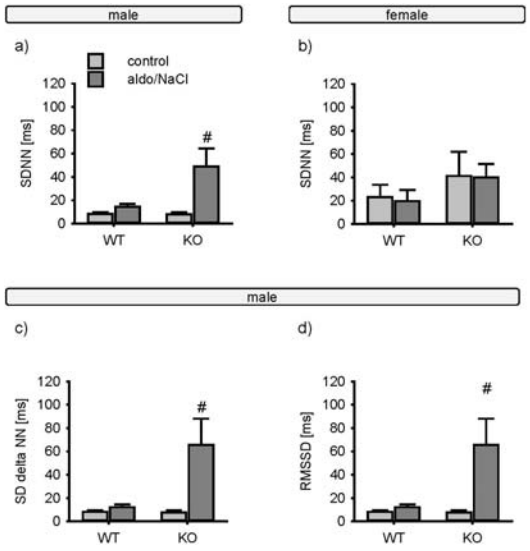

Figure 6 | Effect of genotype and treatment on heart rate variability distinguished by gender. After 28 days with or without aldo/NaCl treatment animals were anaesthetized with isoflurane and electrocardiography recordings were obtained for at least ten minutes employing an Einthoven I limb lead recording, from these recordings heart rate variability was determined. SDNN (a, b), as standard deviation of RR interval of normal-to-normal intervals was analysed first. As no difference could be obtained in female animals, SD delta NN (standard deviation of averages of normal R-R intervals, c) and RMSSD (Square root of the mean of the sum of the squares of differences between adjacent NN intervals, d) was only analysed in male animals. Data are given as mean ± SEM, * $p<0.05$ compared to respective wildtype, # $p<0.05$ compared to respective control. N=3–5 animals/group.

a mutant EGFR[43], developing cardiomyopathy, cardiac hypertrophy and premature death have been reported, indicating that beside ErbB2, EGFR is also important. Recently, we introduced the genetic mouse model with EGFR deletion in vascular smooth muscle cells and cardiomyocytes which now allows investigating the role of EGFR under pathophysiological conditions more specifically than using pharmacological inhibition[24]. In light of the above mentioned gender differences in wildtype animals we first had to perform a gender analysis with respect to VSMC- and CM-EGFR knockout. Our data show, to our knowledge for the first time, that the importance of cardiac EGFR is more pronounced in male as compared to female animals. Adult female animals are much less affected by EGFR-KO with respect to cardiac hypertrophy, cardiac and aortic parainflammation as well as electrical remodelling, indicating that the EGFR is of greater importance for tissue homeostasis in adult males. By contrast, cardiac hypertrophy in newborn animals was significantly smaller as compared to adult animals and there was no gender difference. These data indicate that more than one mechanism contributes to cardiac pathophysiology. Prenatally, there is a gender-independent mechanism, indicating that not the sex of the cardiomyocyte per se is responsible for part of the hypertrophy. Postnatal, hypertrophy aggravates, yet now with a significant gender difference, indicating that sex hormones may play a role, with androgens acting prohypertrophic. According to our data, it is conceivable that EGFR deletion leads to cardiac hypertrophy driven by at least two mechanisms, one gender-independent the other gender-dependent. The underlying molecular mechanisms of these effects will be the subject of future studies.

Recently we showed, that cardiac EGFR deletion leads to an increase of NADPH-oxidase 4 (NOX4) expression and activity[24] with enhanced ROS formation that presumably exerts the hypertrophic growth in adult animals. By contrast to adult animals, in the hearts of newborn animals we were not able to detect an enhanced NOX4-expression (84 ± 10% of wildtype, N=5). Thus, prenatal gender-independent hypertrophy does not result from altered NADPH-oxidase expression. One mechanism for the postnatal gender-dependent hypertrophy, might be the gender-dependent increase in NOX4 expression (303 ± 57% of wildtype in male; 174 ± 33% of wildtype in female; figures 7 and 8), that can be explained by the stimulatory effect of androgens on NOX4 expression[44]. Additionally, the protective cardiovascular effects of oestrogens leading to greater non-specific resilience, e.g. to ROS, may also contribute. Furthermore, it is also conceivable that cardiac EGFR modulates cardiac androgen and oestrogen signalling – the receptors of both being able to interact with EGFR activity and/or expression – differently, resulting in sex hormone dependent cardiac phenotype after EGFR deletion. EGFR knockout could lead to a disinhibition especially of androgen-driven effects, by e.g. enhanced androgen receptor expression or insulin-like growth factor-1 signalling[45,46].

From the clinical point of view our data suggest the need of more detailed investigations of side-effects during EGFR-targeted pharmacotherapy. In line with our findings, Barrick et al (2008)[47] demonstrated that long-term treatment with EGFR-inhibitor (e.g. AG1478 or EKB-569) leads to a significant decrease in cardiovascular function. Taken, together with our data, these side-effects resulted, at least in part, from inhibition of cardiomyoctye EGFR.

The enhanced atrial and ventricular conduction times in male knockout mice are most probably not entirely attributable to enhanced heart size, because female knockout animals also presented with substantial cardiac hypertrophy, albeit not as extreme as male animals. The interaction of EGFR with cardiac ion channels (e.g. Kir3.1, Kv4.3) has been shown and therefore a lack of EGFR activity is expected to alter cardiac action potentials[48,49]. The enhanced conduction time combined with the increased heart size and the prolonged QTc-interval represent a high risk constellation for cardiac arrhythmias due to reentries, that may explain the previously reported increase in mortality in animals aged >6 month. In line with this conclusion are previous reports showing a contribution of EGFR to cardiac arrhythmias due to the modulation of Na^+ and Ca^{2+}-channel activity[50]. In future telemetry studies we will analyse also heart arrhythmia.

Effects of Aldosterone/NaCl in knockout animals. In male knockout animals aldosterone/NaCl treatment induced (i) a partial beneficial effect with respect to cardiac hypertrophy and altered intraatrial and intraventricular conduction induced by EGFR deletion, (ii) an increase in heart rate variability but (iii) no further increase of the profibrotic/parainflammatory phenotype compared to controls. According to these findings we propose that the detrimental effects aldosterone/NaCl exerts in the heart dependent, at least in part, on EGFR. On the contrary, the beneficial cardiac effects appear independent of EGFR. Recently, Akt-mediated cardioprotective effects of mineralocorticoid receptor activation have been described[10]. If these effects were EGFR-independent, they could explain also the beneficial effect observed in our study. Of course, this hypothesis needs further investigation in the future.

The development of heart fibrosis and hypertrophy induced by aldosterone has been described to depend on a high salt diet[51,52], but the mechanisms involved are still under discussion[53]. Our data add another piece to the mechanistic puzzle of pathological MR action, namely the EGFR. At present we cannot quantify the relative importance of enhanced activation of preexisting EGFR and enhanced expression of EGFR as the underlying molecular mechanisms. The fact that cardiac EGFR-expression was enhanced in WT animals by

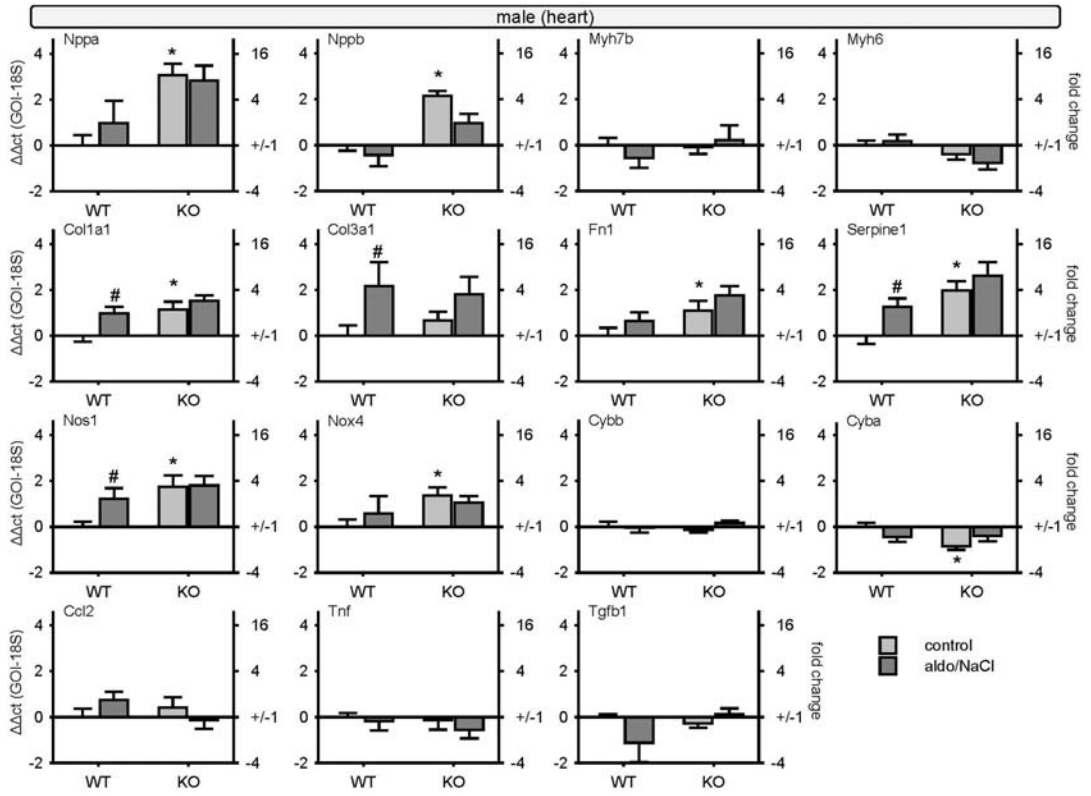

Figure 7 | **Alteration of marker gene expression in hearts of male animals regarding genotype and treatment.** After 28 days with or without aldo/NaCl treatment animals were sacrificed, the hearts dissected and snap frozen in liquid nitrogen. Gene expression was analysed according to the -Δ/Δct-method normalizing the individual mRNA amount to 18S and the mean mRNA amount in the untreated wildtype. In each graph the Δ/Δct amount is given on the left y-axis while the corresponding fold change in expression is given on the right y-axis. Data are given as mean ± SEM, * $p<0.05$ compared to respective wildtype, # $p<0.05$ compared to respective control. N=6–11 animals/group.

aldosterone/NaCl suggests that this mechanism plays at least a partial role for the detrimental effects.

In conclusion, our data demonstrate that EGFR and moderately high aldosterone/NaCl seem to elicit Ying and Yang-like effects: While EGFR has a beneficial effect on physiological tissue homeostasis in hearts and aortas of male and female animals; it mediates at least some of the detrimental effects of high aldosterone-to-NaCl intake in male animals. On the other hand, high aldosterone-to-NaCl besides is detrimental effect also elicits beneficial effects in the heart, but presumably not mediated by the EGFR.

Methods

Ethics statement. All mouse experiments described in this manuscript were approved by the local government (Landesverwaltungsamt Sachsen-Anhalt, Germany, permit number: 203.k-42502-2-1039 MLU G) and were performed according to the guidelines of the American Physiological Society.

Generation of $EGFR^{\Delta/\Delta\ VSMC}$ mice. Generation of the mice and baseline phenotype were described before[24,54]. Mice were kept in the facilities of the University of Halle-Wittenberg at a 12 h/12 h light/dark cycle with a room temperature of 20 ± 1°C. $EGFR^{\Delta/\Delta VSMC\&CM}$ mice were generated by mating C57BL/6 mice containing floxed EGFR alleles (EGFRflox/flox) to SM22-Cre mice. Genotyping was performed on tail biopsies by PCR as described before[24]. All animals were homozygous for the EGFR flox allele (EGFRflox/flox) and either heterozygous for the SM22-Cre allele (SM22-CRE+/-; $EGFR^{\Delta/\Delta VSMC\&CM}$ = KO) or negative for the SM22-Cre allele (SM22-Cre-/-, WT).

Measurement of blood pressure. 5-7month-old animals were trained 14 d before starting the blood pressure measurement for 5 consecutive days (0 d) using external tail pulse detection (ADI instruments, Spechbach, Germany) in awake animals. After this time period aldosterone releasing pellets (Innovative Research of America, Sarasota, USA) were implanted subcutaneously in the back of the animals under isoflurane anaesthesia (2%, 1 L/min O2). After a three day recovery period blood pressure and heart rate were measured twice weekly for additional 28 days. Release rate of aldosterone pellets was chosen to increase endogenous aldosterone levels to physiological plasma concentrations of mice even under high salt diet (see below).

Plasma aldosterone levels. The daily aldosterone synthesis rate of mice can be estimated from the known plasma half-life (t0,5 = 10–20 minutes), the approximate distribution volume (VD = 3–6 ml per animal) and the plasma concentration (CP = 100–200 ng/l) according to the formula synthesis rate = VD × CP × (ln2/t0,5) to range between 20 to 120 ng per day. In order to achieve a similar aldosterone application rate we used the above mentioned pellets that are supposed to release ~220 ng/d as determined by pre-tests in vitro. Our results show that by these means the animals maintained physiological plasma aldosterone concentrations despite consuming water containing 1% NaCl, known to reduce plasma aldosterone to ~1/3 of controls[55].

Blood was collected after 28 d of treatment via retroorbital puncture and anticoagulated with Na-citrate. After centrifugation (1.500 g, 10 min) plasma was removed from the reaction tube and transferred into a new 1.5 ml reaction tube. Immediately thereafter the plasma was snap frozen in liquid nitrogen. For plasma aldosterone estimation plasma was thawed and an ELISA was performed according to the manufacturer's protocol (IBL, Hamburg).

Electrocardiography. 5–7 month old animals were anaesthetised with isoflurane 2% (1 L/min O2) and electrocardiography recordings were obtained for at least ten minutes employing AD Instruments LabChart equipment and an Einthoven I limb

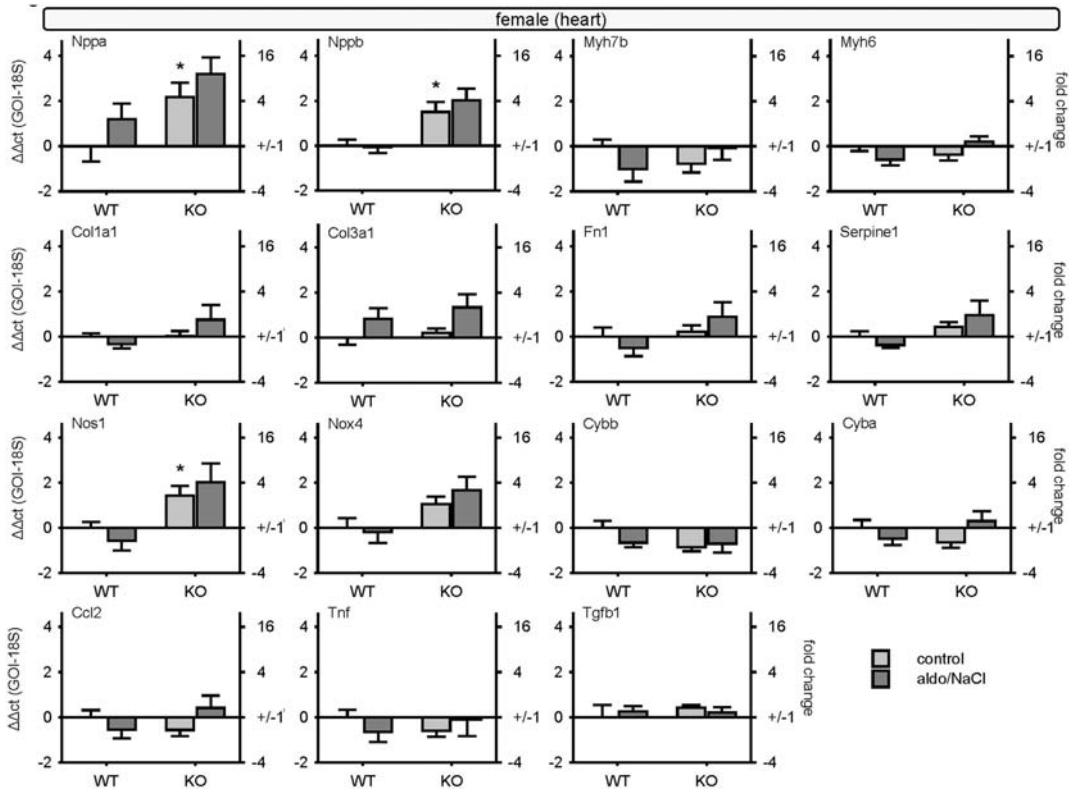

Figure 8 | **Alteration of marker gene expression in hearts of female animals regarding genotype and treatment.** After 28 days with or without aldo/NaCl treatment animals were sacrificed, the hearts dissected and snap frozen in liquid nitrogen. Gene expression was analysed according to the -Δ/Δct- method normalizing the individual mRNA amount to 18S and the mean mRNA amount in the untreated wildtype. In each graph the Δ/Δct amount is given on the left y-axis while the corresponding fold change in expression is given on the right y-axis. Data are given as mean ± SEM, * $p<0.05$ compared to respective wildtype, # $p<0.05$ compared to respective control. N=6–12 animals/group.

lead recording. The following parameters were analysed for standard ECG evaluation: PR interval (measured from the beginning of the P wave to the start of the chamber complex, a measure for the conduction time), P duration (time from start to end of the P wave, a measure for the dispersion of the excitation in the atria), QRS interval (time from start to end of the chamber excitation, a measure for the dispersion of the excitation in the chambers) and QTc interval (time from start of the chamber excitation to the end of the chamber repolarization, heart rate corrected according to Bazette, QTc = QT interval/√RR-interval). Heart rate variability was analysed in the above mentioned ECG tracings via the time-domain analysis according to Thireau et al (2008)[56] with ΔNN=6 ms. The following parameters were analysed: SDNN (standard deviation of RR interval of normal-to-normal intervals, a representation of overall heart rate variability), SD delta NN (standard deviation of averages of normal R-R intervals), RMSSD (Square root of the mean of the sum of the squares of differences between adjacent NN intervals).

Harvesting of organs and cardiomyocytes. Immediately after death, livers, kidneys, lungs, hearts and aortas were excised, carefully freed from adjacent tissue, and partially weighed. Subsequently, the left ventricle was separated and also weighed. Furthermore, tibia length was measured for normalization of organ weights. Part of the kidneys and left ventricles were immediately snap frozen in liquid nitrogen for later RNA extraction while parts were stored in 3% paraformaldehyde solution for fixation. Paraformaldehyde fixated tissues were dehydrated by bathing in increasing concentrations of methanol or isopropanol, respectively. After embedding in paraffin, 3 µm sections were cut.

Microscopic analysis of hearts. The degree of interstitial fibrosis in hearts was determined by evaluation of Sirius Red stained area from at least 10 microscopic fields per animal, utilizing a point counting technique[57]. To analyse cardiomyocyte diameter haematoxylin/eosin stained crosssections of the left chamber were used. At least 100 cardiomyocytes were analysed per mouse.

Morphometric analysis was performed with a Keyence Biozero Fluorescence Microscope with 20× magnification. Images were analysed with the BZ image analysis software and ImageJ.

Caspase-3-activity. Caspase-3 activity was measured as described previously[58]. Snap frozen hearts were incubated with 100 µl cell lysis buffer (10 mmol/l TRIS, 100 mmol/l NaCl, 1 mmol/l EDTA, 0.01% Triton X-100, pH 7.5) for 10 min on ice and centrifuged at 16000 g for 10 min at 4°C. 60 µl of the supernatant was incubated with 65 µl reaction buffer (20 mmol/l PIPES, 4 mmol/l EDTA, 0.2% CHAPS, 10 mmol/l DTT, pH 7.4) containing 42 µmol/l DEVD-AFC (end-concentration) at 37°C, and fluorescence of the cleaved product, 7-amino-4-trifluoromethylcoumarin (AFC), was measured at 400 nm excitation and 505 nm emission wavelength using a multiwell-multilabel counter (Infinite, Tecan, Berlin). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. Protein content was determined with bicinchoninic acid assay (Interchim, Montluçon, France) using bovine serum albumin as standard.

Gene expression. Gene expression analysis was carried out as described before[24,54]. RNA was isolated using the RNeasy Mini Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Subsequently residual remaining DNA was digested with DNase I. Reverse transcription was performed with 1 µg RNA and random primers using qScript from Quanta Biosciences according to the manufacturers' protocol. Additionally each sample was analysed without reverse transcription. The signals obtained without RT were neglectable (<1%). Finally, realtime amplification was performed with the Stratagene Mx3005P using the Platinum SYBR Green kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Primer sequences, names of the corresponding proteins, ref-sequences, as well as annealing temperature and fragment length are given in supplementary table S1. qPCR efficiency was >90%. All primers for genes of interest are intron-spanning and have been validated by RT-PCR and gel electrophoresis.

Furthermore, the primers were validated by melting curve analysis. The relative expression of the two genes of interest was calculated using the 18S signal for normalization. Each sample was analysed as triplicate. All values are expressed as mean ± standard error of mean normalized to the wildtype control group.

Materials. Unless otherwise stated all materials were purchased from Sigma, Munich, Germany.

Statistical analysis. The data are presented as mean ± standard error of mean (SEM). Anova, followed by post hoc testing and Mantel-Cox (for survival rates) were applied. A p-value <0.05 was considered significant.

1. Pitt, B. *et al.* The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N. Engl. J. Med.* **341**, 709–717 (1999).
2. Pitt, B. *et al.* The EPHESUS trial: eplerenone in patients with heart failure due to systolic dysfunction complicating acute myocardial infarction. Eplerenone Post-AMI Heart Failure Efficacy and Survival Study. *Cardiovasc. Drugs Ther.* **15**, 79–87 (2001).
3. Nakano, S., Kobayashi, N., Yoshida, K., Ohno, T. & Matsuoka, H. Cardioprotective mechanisms of spironolactone associated with the angiotensin-converting enzyme/epidermal growth factor receptor/extracellular signal-regulated kinases, NAD(P)H oxidase/lectin-like oxidized low-density lipoprotein receptor-1, and Rho-kinase pathways in aldosterone/salt-induced hypertensive rats. *Hypertens. Res.* **28**, 925–936 (2005).
4. Ouvrard-Pascaud, A. *et al.* Conditional mineralocorticoid receptor expression in the heart leads to life-threatening arrhythmias. *Circulation.* **111**, 3025–3033 (2005).
5. Pitt, B. Effect of aldosterone blockade in patients with systolic left ventricular dysfunction: implications of the RALES and EPHESUS studies. *Mol. Cell Endocrinol.* **217**, 53–58 (2004).
6. Gekle, M. & Grossmann, C. Actions of aldosterone in the cardiovascular system: the good, the bad, and the ugly? *Pflugers Arch.* **458**, 231–246 (2009).
7. Grossmann, C. & Gekle, M. New aspects of rapid aldosterone signaling. *Mol. Cell Endocrinol.* **308**, 53–62 (2009).
8. Grossmann, C. & Gekle, M. Interaction between mineralocorticoid receptor and epidermal growth factor receptor signaling. *Mol. Cell Endocrinol.* **350**, 235–241 (2012).
9. Yavas, G., Elsurer, R., Yavas, C., Elsurer, C. & Ata, O. Does spironolactone ameliorate trastuzumab-induced cardiac toxicity? *Med. Hypotheses.* **81**, 231–234 (2013).
10. Fazal, L. *et al.* Akt-mediated cardioprotective effects of aldosterone in type 2 diabetic mice. *FASEB J.* **28**, 2430–2440 (2014).
11. Hackel, P. O., Zwick, E., Prenzel, N. & Ullrich, A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr. Opin. Cell Biol.* **11**, 184–189 (1999).
12. Prenzel, N. *et al.* EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature.* **402**, 884–888 (1999).
13. Kagiyama, S. *et al.* Angiotensin II-induced cardiac hypertrophy and hypertension are attenuated by epidermal growth factor receptor antisense. *Circulation.* **106**, 909–912 (2002).
14. Smith, N. J., Chan, H. W., Osborne, J. E., Thomas, W. G. & Hannan, R. D. Hijacking epidermal growth factor receptors by angiotensin II: new possibilities for understanding and treating cardiac hypertrophy. *Cell Mol. Life Sci.* **61**, 2695–2703 (2004).
15. Noma, T. *et al.* Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J. Clin. Invest.* **117**, 2445–2458 (2007).
16. Ulu, N. *et al.* alpha1-Adrenoceptor-mediated contraction of rat aorta is partly mediated via transactivation of the epidermal growth factor receptor. *Br. J. Pharmacol.* **161**, 1301–1310 (2010).
17. Grossmann, C. *et al.* Human mineralocorticoid receptor expression renders cells responsive for nongenotropic aldosterone actions. *Mol. Endocrinol.* **19**, 1697–1710 (2005).
18. Grossmann, C. *et al.* Aldosterone-induced EGFR expression: interaction between the human mineralocorticoid receptor and the human EGFR promoter. *Am. J. Physiol Endocrinol. Metab.* **292**, E1790–E1800 (2007).
19. Kagiyama, S. *et al.* Aldosterone-and-salt-induced cardiac fibrosis is independent from angiotensin II type 1a receptor signaling in mice. *Hypertens. Res.* **30**, 979–989 (2007).
20. Asakura, M. *et al.* Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat. Med.* **8**, 35–40 (2002).
21. Pullar, C. E. & Isseroff, R. R. The beta 2-adrenergic receptor activates pro-migratory and pro-proliferative pathways in dermal fibroblasts via divergent mechanisms. *J. Cell Sci.* **119**, 592–602 (2006).
22. Sibilia, M. *et al.* The epidermal growth factor receptor: from development to tumorigenesis. *Differentiation.* **75**, 770–787 (2007).
23. Sibilia, M. & Wagner, E. F. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science.* **269**, 234–238 (1995).
24. Schreier, B. *et al.* Loss of epidermal growth factor receptor in vascular smooth muscle cells and cardiomyocytes causes arterial hypotension and cardiac hypertrophy. *Hypertension.* **61**, 333–340 (2013).
25. Rossi, G. P. *et al.* Primary aldosteronism: cardiovascular, renal and metabolic implications. *Trends Endocrin. Met.* **19**, 88–90 (2008).
26. Catena, C., Colussi, G. & Sechi, L. A. Aldosterone, organ damage, and dietary salt. *Clin. Exp. Pharmacol. Physiol.* **40**, 922–928 (2013).
27. Rossi, G. P. *et al.* Excess aldosterone is associated with alterations of myocardial texture in primary aldosteronism. *Hypertension.* **40**, 23–27 (2002).
28. Pruthi, D. *et al.* Aldosterone promotes vascular remodeling by direct effects on smooth muscle cell mineralocorticoid receptors. *Arterioscler. Thromb. Vasc. Biol.* **34**, 355–364 (2014).
29. McCurley, A. *et al.* Direct regulation of blood pressure by smooth muscle cell mineralocorticoid receptors. *Nat. Med.* **18**, 1429–1433 (2012).
30. Kittikulsuth, W., Looney, S. W. & Pollock, D. M. Endothelin ET(B) receptors contribute to sex differences in blood pressure elevation in angiotensin II hypertensive rats on a high-salt diet. *Clin. Exp. Pharmacol. Physiol.* **40**, 362–370 (2013).
31. Kim, S. M. *et al.* Salt sensitivity of blood pressure in NKCC1-deficient mice. *Am. J. Physiol. Renal Physiol.* **295**, F1230–F1238 (2008).
32. Shibata, H. & Itoh, H. Mineralocorticoid receptor-associated hypertension and its organ damage: clinical relevance for resistant hypertension. *Am. J. Hypertens.* **25**, 514–523 (2012).
33. Alderman, M. H. & Cohen, H. W. Dietary sodium intake and cardiovascular mortality: controversy resolved? *Am. J. Hypertens.* **25**, 727–734 (2012).
34. Crofton, J. T. & Share, L. Gonadal hormones modulate deoxycorticosterone-salt hypertension in male and female rats. *Hypertension.* **29**, 494–499 (1997).
35. Bayorh, M. A., Socci, R. R., Eatman, D., Wang, M. & Thierry-Palmer, M. The role of gender in salt-induced hypertension. *Clin. Exp. Hypertens.* **23**, 241–255 (2001).
36. Ouchi, Y., Share, L., Crofton, J. T., Iitake, K. & Brooks, D. P. Sex difference in the development of deoxycorticosterone-salt hypertension in the rat. *Hypertension.* **9**, 172–177 (1987).
37. Reil, J. C. *et al.* Aldosterone promotes atrial fibrillation. *Eur. Heart J.* **33**, 2098–2108 (2012).
38. Swedberg, K. *et al.* Eplerenone and atrial fibrillation in mild systolic heart failure: results from the EMPHASIS-HF (Eplerenone in mild patients hospitalization and survival study in heart failure) Study. *J. Am. Coll. Cardiol.* **59**, 1598–1603 (2012).
39. Mentz, R. J. *et al.* The past, present and future of renin-angiotensin aldosterone system inhibition. *Int. J. Cardiol.* **167**, 1677–1687 (2013).
40. Gravez, B., Tarjus, A. & Jaisser, F. Mineralocorticoid receptor and cardiac arrhythmia. *Clin. Exp. Pharmacol. Physiol.* **40**, 910–915 (2013).
41. Iwamoto, R. *et al.* Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 3221–3226 (2003).
42. Iwamoto, R. & Mekada, E. ErbB and HB-EGF signaling in heart development and function. *Cell Struct. Funct.* **31**, 1–14 (2006).
43. Chen, B. *et al.* Mice mutant for egfr and shp2 have defective cardiac semilunar valvulogenesis. *Nat. Genet.* **24**, 296–299 (2000).
44. Chignalia, A. Z. *et al.* Testosterone induces vascular smooth muscle cell migration by NADPH oxidase and c-Src-dependent pathways. *Hypertension.* **59**, 1263–1271 (2012).
45. Nahrendorf, M. *et al.* Effect of testosterone on post-myocardial infarction remodeling and function. *Cardiovasc. Res.* **57**, 370–378 (2003).
46. Phillip, M., Maor, G., Assa, S., Silbergeld, A. & Segev, Y. Testosterone stimulates growth of tibial epiphyseal growth plate and insulin-like growth factor-1 receptor abundance in hypophysectomized and castrated rats. *Endocrine.* **16**, 1–6 (2001).
47. Barrick, C. J., Yu, M., Chao, H. H. & Threadgill, D. W. Chronic pharmacologic inhibition of egfr leads to cardiac dysfunction in C57BL/6J mice. *Toxicol. Appl. Pharmacol.* **228**, 315–325 (2008).
48. Kobrinsky, E., Mirshahi, T., Zhang, H., Jin, T. & Logothetis, D. E. Receptor-mediated hydrolysis of plasma membrane messenger pip2 leads to K+-current desensitization. *Nat. Cell Biol.* **2**, 507–514 (2000).
49. Zhang, Y. H. *et al.* Modulation of human cardiac transient outward potassium current by egfr tyrosine kinase and src-family kinases. *Cardiovasc. Res.* **93**, 424–433 (2012).
50. Feng, M. *et al.* Activation of epidermal growth factor receptor mediates reperfusion arrhythmias in anaesthetized rats. *Cardiovasc. Res.* **93**, 60–68 (2012).
51. Robert, V. *et al.* Biological determinants of aldosterone-induced cardiac fibrosis in rats. *Hypertension.* **26**, 971–978 (1995).
52. Robert, V. *et al.* Angiotensin AT(1) receptor subtype as a cardiac target of aldosterone - Role in aldosterone-salt-induced fibrosis. *Hypertension.* **33**, 981–986 (1999).
53. Robert, V. *et al.* Increased cardiac type-i and type-iii collagen messenger-rnas in aldosterone-salt hypertension. *Hypertension.* **24**, 30–36 (1994).
54. Schreier, B. *et al.* Consequences of epidermal growth factor receptor (erbB1) loss for vascular smooth muscle cells from mice with targeted deletion of erbB1. *Arterioscler. Thromb. Vasc. Biol.* **31**, 1643–1653 (2011).
55. Huang, D. Y. B. K. Resistance of mice lacking the serum- and glucocorticoid-inducible kinase SGK1 against salt-sensitive hypertension induced by a high-fat diet. *Am. J. Physiol. - Renal.* **291**, F1264–F1273 (2006).
56. Thireau, J., Zhang, B. L., Poisson, D. & Babuty, D. Heart rate variability in mice: a theoretical and practical guide. *Exp. Physiol.* **93**, 83–94 (2008).

57. Rangan, G. K. & Tesch, G. H. Quantification of renal pathology by image analysis. *Nephrology.* **12**, 553–558 (2007).
58. Schwerdt, G. *et al.* Long-term effects of ochratoxin A on fibrosis and cell death in human proximal tubule or fibroblast cells in primary culture. *Toxicology.* **232**, 57–67 (2007).

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Author contributions

B.S. performed and designed most of the experiments, analysed and interpreted the data, designed the artwork, performed the statistical analysis and is the main author of the manuscript. Si. Ra. performed most of the experiments, S.W. performed the genotyping on the tail biopsies, St. Ru. took part in the tailcuff measurements and assisted in the organ harvesting, S.M. took part in the tailcuff measurements, Be. Sch. cut and stained the paraffin embedded organs, M.S. generated and provided the EGFR flox/flox mouse, Michael Gotthardt generated and provided the SM22-Cre mouse, S.K. and K.M. performed the experiments on the aldosterone release, C.G. took part in the experiment designing and the fund raising. M.G. wrote the manuscript, discussed the data and gave substantial input to the interpretation of the data, designed part of the experiments and is responsible for funding. All authors reviewed the manuscript.

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Consequences of Epidermal Growth Factor Receptor (ErbB1) Loss for Vascular Smooth Muscle Cells From Mice With Targeted Deletion of ErbB1

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Objective—Pathophysiological effects of the epidermal growth factor receptor (EGFR or ErbB1) include vascular remodeling. EGFR transactivation is proposed to contribute significantly to heterologous signaling and remodeling in vascular smooth muscle cells (VSMC).

Methods and Results—We investigated the importance of EGFR in primary VSMC from aorta of mice with targeted deletion of the EGFR ($EGFR^{\Delta/\Delta\ VSMC}\rightarrow VSMC^{EGFR-/-}$ and $EGFR^{\Delta/+\ VSMC}\rightarrow VSMC^{EGFR+/-}$) and the respective littermate controls ($EGFR^{+/+\ VSMC}\rightarrow VSMC^{EGFR+/+}$) with respect to survival, pentose phosphate pathway activity, matrix homeostasis, extracellular signal–regulated kinase 1/2 (ERK1/2) phosphorylation, and Ca^{2+} homeostasis. In $VSMC^{EGFR-/-}$, epidermal growth factor–induced signaling was abolished; $VSMC^{EGFR+/-}$ showed an intermediate phenotype. EGFR deletion enhanced spontaneous cell death, reduced pentose phosphate pathway activity, disturbed cellular matrix homeostasis (collagen III and fibronectin), and abolished epidermal growth factor sensitivity. In $VSMC^{EGFR-/-}$ endothelin-1- or α_1-adrenoceptor-induced ERK1/2 phosphorylation and the fraction of Ca^{2+} responders were significantly reduced, whereas responsive cells showed a significantly stronger Ca^{2+} signal. Oxidative stress (H_2O_2) induced ERK1/2 activation in $VSMC^{EGFR+/+}$ and $VSMC^{EGFR+/-}$ but not in $VSMC^{EGFR-/-}$. The Ca^{2+} signal was enhanced in $VSMC^{EGFR-/-}$, similar to purinergic stimulation by ATP.

Conclusion—In conclusion, EGFR was found to be important for basal VSMC homeostasis and ERK1/2 activation by the tested G-protein–coupled receptors or radical stress. Ca^{2+} signaling was modulated by EGFR differentially with respect to the fraction of responders and magnitude of the signal. Thus, EGFR seems to be Janus-faced for VSMC biology. **(*Arterioscler Thromb Vasc Biol*. 2011;31:1643-1652.)**

Key Words: growth factors ■ receptors ■ signal transduction ■ vascular biology ■ vascular muscle

The epidermal growth factor receptor (EGFR) family consists of 4 related tyrosine kinase receptors: EGFR (ErbB1), HER2 (ErbB2, with no ligand described), HER3 (ErbB3, without kinase function), and HER4 (ErB4).[1,2] There are more than 10 ligands for these receptors with different specificities.[3] On ligand binding, the receptors undergo dimerization, leading to Tyr phosphorylation of the cytosolic domain and subsequent activation of various signaling pathways, including mitogen-activated protein kinases (extracellular signal–regulated kinase 1/2 [ERK1/2], p38, c-Jun N-terminal kinase), protein kinase B, c-Src kinase, and phospholipase C γ, which affect extra- and intranuclear events.[4] Activation includes formation of homo- and heterodimers, with the EGFR possessing the ability to interact with all 3 family members. Activated ErbB receptors regulate various aspects of cell fate, such as proliferation, survival, differentiation, migration, and matrix homeostasis.[5]

EGFR (ErbB1) dimers can be activated by epidermal growth factor (EGF), transforming growth factor-α, amphiregulin, heparin-binding (HB)-EGF, β-cellulin, and epiregulin. ErbB1/ErbB4-dimers can be activated by neuregulins.[6] The ligands may derive from neighboring cells (paracrine) or from the same cell (autocrine), as in the case of shedding of membrane-bound pro-HB-EGF.[1] As pro-HB-EGF shedding can also be induced by activation of other receptors (eg, G-protein–coupled receptors), EGFR is subject to activation by other hormones, a mechanism called transactivation.[7] In addition, transactivation can also be achieved by intracellular pathways, involving c-Src-mediated EGFR phosphorylation.[8] Thus, EGFR, via transactivation, has the potential to mediate signaling of non-EGFR ligands and thereby serve as a heterologous transducer of cellular signaling. This mechanism has been proposed for the ERK1/2-activating action of

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a variety of hormones also in vascular smooth muscle cells (VSMC).[6,9]

Pathophysiological effects of EGFR include cell transformation and tumorigenesis, as well as parainflammatory dysregulation of tissue homeostasis, leading, for example, to vascular dysfunction and fibrosis.[6] There are several reports suggesting that EGFR transactivation is responsible for G-protein–coupled receptor–mediated ERK1/2 phosphorylation (eg, induced by angiotensin II [ang II],[10,11] α_1- and β-adrenergic agonists,[12,13] thrombin,[14] endothelin-1 [ET-1],[15–17] and purinergic receptor ligands[18]) and the resulting pathophysiological effects on VSMC and vascular function, including atherosclerosis.[6] However, other hormones also, such as steroids[19] or prostaglandins[20] as well as reactive oxygen species[21] and cardiotonic steroids,[22] (mis)use the EGFR as signal transducer.

EGFR supports fibrosis in cardiovascular and renal tissue.[15,23,24] The importance of EGFR in mediating pathophysiological effects of heterologous signaling systems is supported by its role in endothelin-induced fibrogenic effects.[15] Endothelin-induced phosphorylation of the mitogen-activated protein kinase and endothelin-induced increase in collagen I gene activity were completely prevented by an inhibitor of the EGFR kinase. Ang II also "uses" the EGFR to elicit certain effect in cardiovascular cells. For example, Bokemeyer et al reported that ang II–induced growth of VSMC requires activation of EGFR,[25] and Mazak et al showed that ang II– or aldosterone-induced stimulation of ERK1/2 in VSMC depends on EGFR.[19] In cardiomyocytes, ang II receptor type 1A promotes hypertrophy via transactivation of the EGFR,[10,26,27] thus proving that EGFR serves as a heterologous transducer for pathological vascular signals such as ET-1, ang II, and catecholamines, all of which have been linked to vascular remodeling.[1]

EGFR affects not only protein kinases and transcription factors but also influences cellular Ca^{2+} signaling via phospholipase Cγ.[28,29] Phospholipase Cγ can induce inositoltrisphosphate-mediated Ca^{2+} release and also Ca^{2+} influx across the cell membrane.[30] This effect is elicited by, eg, cAMP, arachidonic acid metabolites, or internal store depletion.[31,32] Additionally, EGFR kinase activity may also contribute to or support the Ca^{2+} response to other mediators after transactivation. For ang II and ET-1, a small contribution of EGFR to Ca^{2+} signaling in VSMC has been suggested on the basis of pharmacological data.[17,33] Thus, EGFR is not only relevant for mitogen-activated protein kinase but also for Ca^{2+} signaling.

Most of the studies that have been performed relied on pharmacological or immunologic tools to unveil the contribution of EGFR to the aforementioned deleterious effects on vascular tissue homeostasis. Our work, presented here, aims at testing the hypothesis of EGFR as a heterologous signal transducer in VSMC using the genetic model of targeted EGFR deletion. We generated mice with deletion of EGFR in VSMC ($EGFR^{\Delta/\Delta\ VSMC}$) and compared the cells in primary culture with cells derived from wild-type littermates ($EGFR^{+/+\ VSMC}$), with respect to ERK1/2 phosphorylation, Ca^{2+} homeostasis, and cell survival.

Methods

Generation of $EGFR^{\Delta/\Delta\ VSMC}$ Mice

C57BL/6 mice containing floxed *EGFR* alleles ($EGFR^{f/f}$) after removal of the *neo* cassette were obtained from Dr M. Sibilia (University of Vienna) and used for further breeding.[34] *EGFR* was inactivated tissue specifically in smooth muscle cells by using *SM22-cre* transgenic mice, in which the cyclization recombination recombinase is under the control of the smooth muscle cell–specific SM22 promoter.[35] Genotyping of the mice was performed on tail biopsies by polymerase chain reaction (PCR) against the floxed EGFR allele as well as cyclization recombination. Mice were kept in the facilities of the University of Halle-Wittenberg in accordance with institutional policies and federal guidelines.

Cell Culture

Primary culture of VSMC from 4- to 5-month-old mice ($EGFR^{\Delta/\Delta\ VSMC} \rightarrow VSMC^{EGFR-/-}$, $EGFR^{\Delta/+\ VSMC} \rightarrow VSMC^{EGFR-/+}$, $EGFR^{+/+\ VSMC} \rightarrow VSMC^{EGFR+/+}$) was performed as described by Ray et al.[36] Cells from passages 2 to 6 were cultured in DMEM with 10% fetal bovine serum. Cell number and cell size were determined with a CASY cell counter system (CASY, Reutlingen, Germany).

Western Blot

Cells were lysed with Cell Signaling Technology lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris [Base], pH 7.4, 1% Triton X-100, protease inhibitor cocktail I from Calbiochem, 1 mmol/L EDTA, and 184 mg/L sodium orthovanadate), and proteins were separated by 12% SDS-PAGE for ERK/phospho-ERK. Proteins were transferred to nitrocellulose and incubated overnight with anti-phospho-ERK or anti-ERK (1:1000, Cell Signaling Technology) respectively with anti-EGFR or anti-heat shock protein 90 (1:1000, Cell Signaling Technology) according to the manufacturer's protocol. Protein bands were detected by horseradish peroxidase–conjugated secondary antibody (anti-rabbit IgG, Biotrend, Cologne, Germany).

Determination of Cytosolic Ca^{2+}

Cytosolic free calcium was determined using the Ca^{2+} sensitive dye fura-2 (Molecular Probes, Leiden, the Netherlands) as described previously[37] with an inverted Zeiss 100 TV microscope (magnification ×400, oil immersion, Zeiss, Oberkochen, Germany) and an automatic filter change device (Hamamatsu, Herrsching, Germany). Cells were cultivated on glass coverslips, and serum was removed 24 hours before the measurements. Subsequently, cells were incubated for 15 minutes with 5 μmol/L fura-2-AM. Finally, coverslips were transferred to the stage of the microscope. The fluorescence signal was monitored at 510 nm, with excitation wavelength alternating between 334 and 380 nm, using a 100-W xenon lamp (fura-2 ratio). The sampling rate was 1 ratio every 2 s. At the end of each experiment thapsigargin was applied as a positive control and only cells responding to thapsigargin were included for further analysis. We did not observe significant differences with respect to the thapsigargin response for the 3 genotypes. Stimulus-induced changes in Ca^{2+} were accepted only if the induced change in the fura-2 ratio exceeded signal standard deviation of 10 preceding measurements by more than 4-fold. Thus, a cell was categorized as a responder if the Ca^{2+} signal increased by more than 4 times the SD of the baseline signal before the addition of the stimulus. The percentage of responders was determined from the sum of all measurements under a certain experimental condition (number responding/number measured). The response behavior of the cells during the different measurements for a given condition was very similar, with a variance of 10% or less.

Real-Time Quantitative Polymerase Chain Reaction

RNA was isolated using the RNeasy Mini Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Subsequently, remaining DNA was digested with DNase I. Reverse transcription was performed with 1 μg of RNA and random primers using qScript from Invitrogen according to the manufacturer's instructions. As a control, each sample was also analyzed without reverse transcription. The signals obtained without reverse transcription were negligible (<1%). Finally, real-time amplification was performed with the Stratagene Mx 3005P using the Platinum SYBR Green kit (Invitrogen) according to the manufacturer's instructions. Quantitative PCR (qPCR) efficiency was >90%. The relative expression of mRNA was calculated according to the $2^{\Delta\Delta Ct}$ method, using the 18S signal for normalization. Each sample was analyzed in triplicate, as follows: EGFR sense primer, GACCTTCACATCCTGCCAGTEGFR; EGFR antisense primer, GCATGGAGGTCAGTCCAGTT; 18S sense primer, GTTGGTGGAGCCGATTTGTCTGG; 18S antisense primer, AGGGCAGGGACTTAATCAACGC. Annealing temperatures were 63°C and 64°C for EGFR and 18s, respectively.

Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) activity in media and in cell lysates was measured using a standard protocol[38] adapted to a lower scale (200 μL) in a multiwell-multilabel reader (Infinite, Tecan, Berlin).

Caspase-3 Activity

Caspase-3 activity was measured as described previously.[38] Briefly, cells were washed once with PBS buffer (4°C) and incubated with 100 μL of cell lysis buffer (10 mmol/L TRIS, 100 mmol/L NaCl, 1 mmol/L EDTA, 0.01% Triton X-100, pH 7.5) for 10 minutes on ice, harvested, and centrifuged at 16 000g for 10 minutes at 4°C. Sixty microliters of the supernatant was incubated with 65 μL of reaction buffer (20 mmol/L piperazine-N,N′-bis(ethanesulfonic acid), 4 mmol/L EDTA, 0.2% CHAPS, 10 mmol/L dithiothreitol, pH 7.4) containing 42 μmol/L DEVD-7-amino-4-trifluoromethylcoumarin (AFC) (end concentration) at 37°C, and fluorescence of the cleaved product, AFC, was measured at 400 nm excitation and 505 nm emission wavelength using a multiwell-multilabel counter (Infinite). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. Protein content was determined with bicinchoninic acid assay (Interchim, Montluçon, France) using bovine serum albumin as a standard.

Glucose-6-Phosphate Dehydrogenase Activity

Glucose-6-phosphate dehydrogenase (G6PD) activity was measured as described by Leopold and Loscalzo.[39] Briefly, cells were washed with phosphate-buffered saline (0.9%), scraped from the plate, lysed, and centrifuged at 2000g at 4°C for 10 minutes. Enzyme activity was determined using a plate-reader spectrophotometer (Sunrise, Tecan, Crailsheim, Germany) by measuring the rate of increase of absorbance at 340 nm due to the conversion of $NADP^+$ to NADPH by either G6PD or 6-phosphogluconate dehydrogenase (6-PGDH). To determine total dehydrogenase activity, we added 40 μL of supernatant to a well that contained 160 μL of assay buffer and substrates for both enzymes. In a second well, substrates for the enzyme 6-PGDH were added to determine the activity of this enzyme. G6PD activity was then determined by subtracting 6-PGDH activity from total dehydrogenase activity. Substrate concentrations were glucose-6-phosphate (200 μmol/L), 6-phosphogluconate (200 μmol/L), and $NADP^+$ (100 μmol/L). Protein levels were determined for each sample, and activity results were standardized to protein concentration.

Collagen-3 and Fibronectin ELISA

Extracellular collagen III and fibronectin were determined by ELISA as described previously.[40,41] Media and collagen standards (Sigma-Aldrich, Deisenhofen, Germany) were incubated for 24 hours in 96-well Nunc-Immuno Maxisorp plates (Nalge Nunc International, Naperville, IL) followed by washing and blocking with 2% bovine serum albumin. Subsequently, the wells were incubated with rabbit antibody against collagen III or fibronectin (1:1000, Biotrend, Cologne, Germany) for 1 hour at room temperature. After another 3 washes with 0.05% Tween in phosphate-buffered saline, horseradish peroxidase–conjugated secondary antibody (1:5000, Biotrend, Cologne, Germany) was applied for 1 hour at room temperature. After 3 washes with 0.05% Tween in phosphate-buffered saline, the wells were incubated with *o*-phenlyenediamine (Sigma-Aldrich); the reaction was stopped after 15 minutes with 1 N H_2SO_4, and the absorbance was measured at 490 nm. Cellular protein was determined by the BCA assay from Pierce. We tested the cross-reactivity of the primary antibodies using the collagen standards and did not observe any significant cross-reactivity.

Immunofluorescence

Cells were cultivated on glass coverslips, fixed with 4% formaldehyde (15 minutes), washed 3 times with PBS, and permeabilized with 0.5% Triton X-100. Subsequently, cells were incubated for 10 minutes in 1% SDS/PBS, followed by 10 minutes of 100 mmol/L glycine/PBS, and finally incubated for 20 minutes in 10% serum/1% BSA/PBS. After 60 minutes of incubation with primary antibody, the cells were washed 3 times with PBS and incubated for 45 minutes with anti-rabbit Alexa 568 secondary antibody or nonlabeled secondary antibody. After 3 additional washes, the cells were analyzed by fluorescence microscopy (Biozero, Keyence, Osaka, Japan) and confocal microscopy (Radiance 2000, Bio-Rad).

Materials

Unless otherwise stated, all other materials were from Sigma-Aldrich (Munich, Germany). Control Ringer solution was composed of (in mmol/L): NaCl 130.0, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 1.0, HEPES 10, and glucose 5 (pH 7.4 at 37°C), plus the respective vehicles (ethanol or DMSO ≤0.1%).

Statistics

The data are presented as mean values±SEM. Significance of difference was tested by paired or unpaired Student *t* test or ANOVA as applicable. Differences were considered significant if $P<0.05$. Cells from at least 3 mice per genotype, and 3 matching passages were used for each experimental series. Thus, all experiments were performed with cells from at least 3 different animals and 3 different passages. Cell from the 3 genotypes were treated in parallel for the various repetitions. N represents the number of cells or tissue culture dishes investigated.

Results

Basal Phenotype of $EGFR^{\Delta/\Delta\ VSMC}$ Animals

$EGFR^{\Delta/\Delta\ VSMC}$ animals showed no differences in body weight, systolic blood pressure (measured by tail-cuff or intravasal), blood glucose, or hematocrit compared with $EGFR^{+/+\ VSMC}$ animals (Supplemental Table I, available online at http://atvb.ahajournals.org). Aortae and coronary vessels of $EGFR^{\Delta/\Delta\ VSMC}$ animals showed signs of dilative remodeling (reduced wall/lumen ratio) and slight fibrosis (Supplemental Table II). Possible functional differences are currently under investigation (isolated aortic rings, in vivo reactivity to vasoactive substances) but were beyond the scope of the present study.

EGFR Expression and EGF Responsiveness

EGFR mRNA level was reduced to <10% in the aorta of $EGFR^{\Delta/\Delta\ VSMC}$ animals compared with $EGFR^{+/+\ VSMC}$ animals (Figure 1A). The same was true for isolated $VSMC^{EGFR-/-}$ compared with isolated $VSMC^{EGFR+/+}$. In liver, EGFR was not reduced in $EGFR^{\Delta/\Delta\ VSMC}$ animals (99±22% of $EGFR^{+/+\ VSMC}$ animals, n=9). On the protein level, EGFR

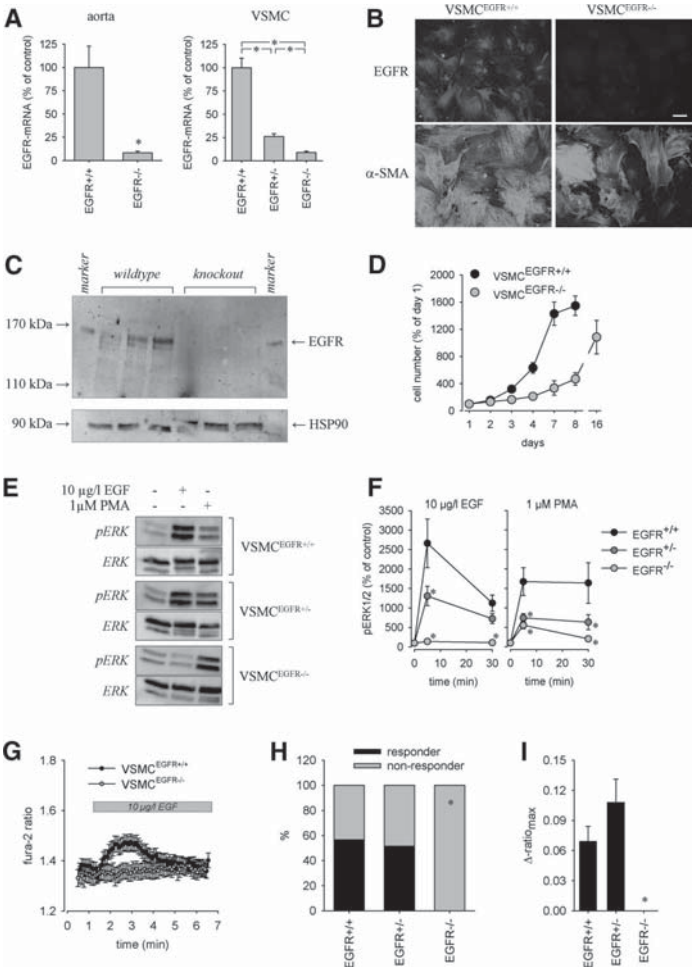

Figure 1. Knockout of EGFR in VSMC. A, Reduction of EGFR-mRNA to <10% in knockout aorta with connective tissue and endothelium removed and VSMC as determined by qPCR. Heterozygote VSMCs displayed an intermediate phenotype. N=9 and N=5 per group for aorta and VSMCs, respectively. B, Immunofluorescence analysis documented that EGFR protein (red) was virtually absent in knockout VSMCs. α-Smooth muscle actin (α-SMA) was used as a marker for VSMCs (green). Bar=10 μm. C, Western blot detection of EGFR expression. In knockout VSMC, no EGFR was detectable. Heat shock protein-90 Western blot served as loading control. D, Comparison of wild-type and knockout cell proliferation. N=4 for each value plotted. Starting cell density was 800±70 per cm^2 at day 1. E, Western blot detection of EGF-induced ERK1/2 phosphorylation (5 minutes of incubation). ERK phosphorylation was not induced by EGF exclusively in knockout VSMCs, which still partially responded to PMA. F, Summary of EGF-induced ERK1/2 phosphorylation shows a reduced response of knockout VSMCs to both EGF and PMA, with an intermediate response in heterozygous cells. N=5 to 7. *$P<0.05$ vs the respective control. G to I, EGF-induced Ca^{2+} signaling in VSMC. G, Original representative tracing of EGF-induced changes in fura-2 ratio. N=5 for each value plotted. The gray bar indicates exposure of the cultured cells to EGF, resulting in a peak response of wild-type VSMCs at 3 minutes, whereas knockout cells did not respond. H, Summary of EGF-induced Ca^{2+} signaling. Data show the percentage of cells responding with a Ca^{2+} signal to EGF, which is similar in wild-type and heterozygotes. I, Extent of changes in fura-2 ratios of cells responding with a Ca^{2+} signal to EGF. N=22 to 63. *$P<0.05$ vs the respective control.

was not detectable in $VSMC^{EGFR-/-}$, as shown by immunofluorescence images (Figure 1B) and by Western blot (Figure 1C). Staining for α-smooth muscle actin was not affected. Loss of EGFR also reduced cell proliferation in the presence of 10% serum (ie, when non-EGF stimuli were present), emphasizing the central role of EGFR (Figure 1D). EGF responsiveness (Figure 1E and 1F, incubation with 10 μg/L EGF) with respect to ERK1/2 phosphorylation was completely absent in $VSMC^{EGFR-/-}$. $VSMC^{EGFR+/-}$ showed a reduced response compared with $VSMC^{EGFR+/+}$. All 3 genotypes responded to phorbol-12-myristate-13-acetate (PMA) (protein kinase C [PKC] activation), which served as positive control, although the response was smaller in $VSMC^{EGFR+/-}$ and $VSMC^{EGFR-/-}$ compared with $VSMC^{EGFR+/+}$ (Figure 1F), suggesting that part of the effect of PKC on ERK1/2 phosphorylation is mediated by EGFR transactivation.[42] In $VSMC^{EGFR+/+}$, EGF elicited a slight and transient increase in cytosolic Ca^{2+}, as indicated by the increase in the fura-2 ratio (Figure 1G). The Ca^{2+} signal was similar in $VSMC^{EGFR+/-}$ but completely absent in $VSMC^{EGFR-/-}$ (Figure 1G to 1I). These data support the conclusion that EGFR was successfully deleted.

Cell Survival and Metabolism

We measured parameters of cell survival and metabolism known to be relevant for VSMC pathophysiology. LDH release (normalized to total cellular LDH) was significantly higher in $VSMC^{EGFR-/-}$ (Figure 2), indicating reduced viability. Furthermore, caspase-3 activity was elevated in $VSMC^{EGFR-/-}$ and $VSMC^{EGFR+/-}$ (Figure 2), indicating an enhanced basal apoptosis rate. Accordingly, protein content was reduced in $VSMC^{EGFR-/-}$. G6PD activity (Figure 2), rate limiting for NAPDH homeostasis and therefore reactive oxygen species defense, was significantly lower in $VSMC^{EGFR-/-}$. This was not the case for another enzyme of the pentose phosphate cycle, 6-PGDH

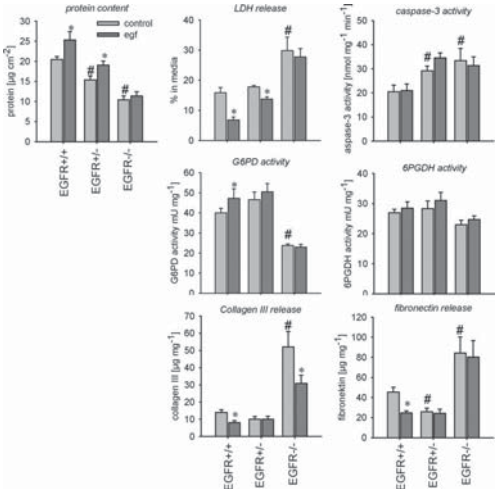

Figure 2. Cell survival and metabolism. Shown is a comparison of the different parameters in VSMC from all 3 genotypes and of their EGF responsiveness. N=22 to 60. #$P<0.05$ vs VSMC$^{E\text{-}GFR+/+}$, *$P<0.05$ vs the respective control (no EGF).

(Figure 2), excluding nonspecific effects. VSMC$^{EGFR-/-}$ accumulated more collagen III and fibronectin in the media compared with VSMC$^{EGFR+/+}$ (Figure 2), evidence of disturbed matrix homeostasis. mRNA levels for collagen III and fibronectin were also increased (Supplemental Table III). Differences in cell size were not observed (mean diameter of VSMC$^{EGFR+/+}$ 21.3±0.4 µm, n=19).

The responsiveness of these parameters to EGF was partially reduced in VSMC$^{EGFR+/-}$ and virtually abolished in VSMC$^{EGFR-/-}$ (Figure 2).

ET-1

ET-1, a ligand of the G-protein–coupled endothelin-1 receptors (ET_A, ET_B), induced a significant stimulation of ERK1/2 phosphorylation in VSMC$^{EGFR+/+}$ (Figure 3A, 100 nmol/L ET-1 for 5 minutes). This response was significantly reduced in VSMC$^{EGFR+/-}$ and VSMC$^{EGFR-/-}$ (Figure 3A), although ET-1 elicited a significant ERK1/2 phosphorylation in VSMC$^{EGFR+/-}$. These data support the hypothesis that ET-1 requires EGFR (transactivation) to activate ERK1/2 signaling. The functional relevance of this transactivation is indicated by the absence of a proliferative action of ET-1 in VSMC$^{EGFR-/-}$ in contrast to VSMC$^{EGFR+/+}$ (Supplemental Table IV).

ET_A/ET_B also couple to cellular Ca^{2+} signaling and induce Ca^{2+} release from cellular stores, as well as Ca^{2+} entry via the plasma membrane.[43] To test whether EGFR transactivation also has an impact on Ca^{2+} signaling, we compared ET-1-induced Ca^{2+} responses in the 3 genotypes. Figure 3B shows the typical response of VSMC$^{EGFR+/+}$ (mean±SEM of 7 cells) with a rapid peak (resulting predominantly from Ca^{2+} release from intracellular stores) and a subsequent plateau phase (resulting mainly from Ca^{2+} entry from the extracellular space). We analyzed the increase in fura-2 ratio compared with the mean of 10 control values before the addition of ET-1 during the peak phase (Δpeak) as well as during the plateau phase (Δplateau). In addition, we determined the relative number of cells responding to the stimulus (responders). As a positive control, we used 1 µmol/L thapsigargin, an inhibitor of the endoplasmic Ca^{2+}-ATPase,[44] and only cells showing an increase in intracellular Ca^{2+} on thapsigargin application were included for further analysis. The percentage of total responders (ie, cells responding with either peak or plateau), as well as peak and plateau responders, was significantly higher in VSMC$^{EGFR+/+}$ compared with the 2 other phenotypes (Figure 3C). Of

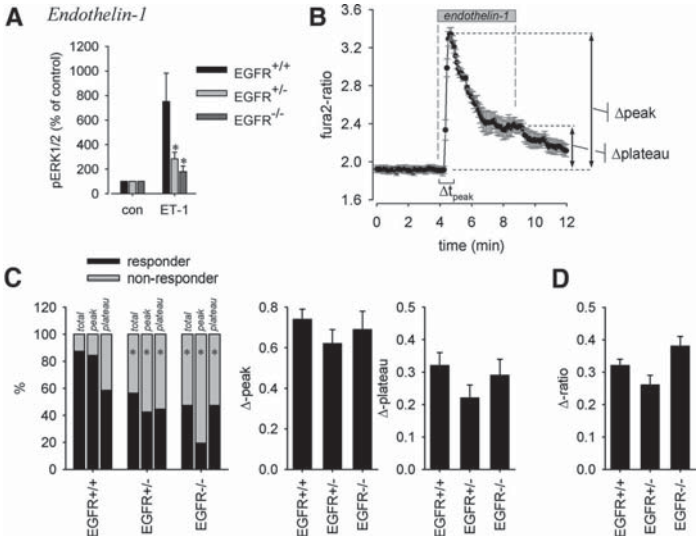

Figure 3. ET-1-induced signaling in VSMC. A, Summary of ET-1-induced ERK1/2 phosphorylation (5 minutes of incubation with 100 nmol/L ET-1). Values are expressed as percentage of the matched controls. N=5 to 7. B, Original tracing of ET-1-induced changes in fura-2 ratio. N=5 for each value plotted. C, Summary of ET-1-induced Ca^{2+} signaling. The left panel shows the percentage of cells responding with a Ca^{2+} signal to ET-1 (total indicates the percentage of cells with peak, plateau, or both; peak, percentage of cells showing at least a peak response; plateau, percentage of cells showing at least a plateau response). The right panels show the changes in fura-2 ratio. N=22 to 63. *$P<0.05$ vs the respective control. D, Summary of thapsigargin-induced Ca^{2+} signaling showing changes in the fura-2 ratio. N=40 to 77. *$P<0.05$ vs the respective control.

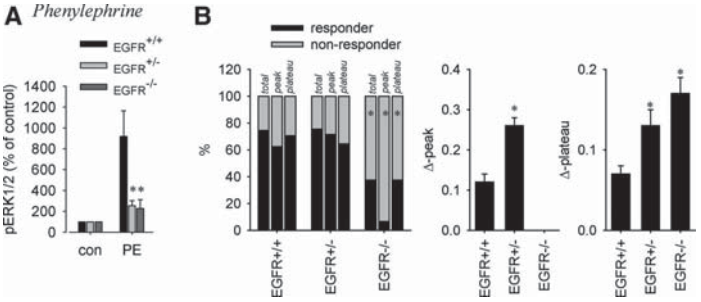

Figure 4. PE-induced signaling in VSMC. A, Summary of PE-induced ERK1/2 phosphorylation (5 minutes of incubation with 1 μmol/L PE). N=5 to 6. B, Summary of PE-induced Ca^{2+} signaling. The left panel shows the percentage of cells responding with a Ca^{2+} signal to PE (total indicates percentage of cells with peak, plateau, or both; peak, percentage of cells showing at least a peak response; plateau, percentage of cells showing at least a plateau response). The right panels show the changes in fura-2 ratio. N=34 to 79. *$P<0.05$ vs the respective control.

note, the percentage of peak responders was very low in $VSMC^{EGFR-/-}$. There was no significant difference in Δpeak or Δplateau when only the responders of the 3 genotypes were compared (Figure 3C). The time to peak (Δt_{peak}) was not different for the 3 genotypes (50±3 seconds, N=115). As shown in Figure 3D, there was no significant difference in the Ca^{2+} response elicited by thapsigargin for the different genotypes, arguing against a general effect of EGFR knockout on basic cellular Ca^{2+} handling.

Phenylephrine

Phenylephrine (PE), a ligand of the G-protein–coupled α_1-adrenergic receptor, induced a significant stimulation of ERK1/2 phosphorylation in $VSMC^{EGFR+/+}$ (Figure 4A, 1 μmol/L PE for 5 minutes). This response was significantly reduced in $VSMC^{EGFR+/-}$ and $VSMC^{EGFR-/-}$ (Figure 4A), although PE still elicited a significant ERK1/2 phosphorylation compared with vehicle-treated cells. These data clearly support the hypothesis[12,45] that the major part of α_1-adrenergic receptor–induced ERK1/2 phosphorylation requires EGFR (transactivation). The functional relevance of this transactivation is indicated by the absence of a proliferative action of PE in $VSMC^{EGFR-/-}$ in contrast to $VSMC^{EGFR+/+}$ (Supplemental Table IV).

α_1-Adrenergic receptor also couples to cellular Ca^{2+} signaling and induces Ca^{2+} release from cellular stores, as well as Ca^{2+} entry via the plasma membrane.[46] As shown in Figure 4B, the percentage of responders was significantly reduced in $VSMC^{EGFR-/-}$ but not in $VSMC^{EGFR+/-}$. Similar to the situation for endothelin, virtually none of the $VSMC^{EGFR-/-}$ cells responded with a Ca^{2+} peak. Surprisingly, ΔCa^{2+} was larger in $VSMC^{EGFR-/-}$ and $VSMC^{EGFR+/-}$ responders compared with $VSMC^{EGFR+/+}$ responders (Figure 4B).

These findings raised the question of whether the difference in the fraction of responsive cells is due to the acute role of EGFR in Ca^{2+} signaling or results from altered developmental processes when EGFR is absent, possibly leading to a different expression pattern of Ca^{2+}-handling proteins. Therefore, we performed experiments determining the fraction of responders to ET-1 or PE in wild-type VSMC with and without the EGFR-kinase inhibitor AG1478 (100 nmol/L, present during the 60 minutes of preincubation, as well as the time of hormone application, N=58 to 101 for each condition). The fraction of responders to either hormone was not reduced in the presence of AG1478 (range, 92% to 98%), indicating that the probability of a Ca^{2+} response does not depend on acute EGFR transactivation but results from altered gene expression or adaptive processes. A more detailed investigation regarding the processes leading to these differences when EGFR is absent will be performed in future studies.

ATP

ATP, a ligand of the G-protein–coupled purinergic receptors, induced a significant stimulation of ERK1/2 phosphorylation in $VSMC^{EGFR+/+}$ (Figure 5A, 10 μmol/L ATP for 5 minutes). This response was significantly reduced in

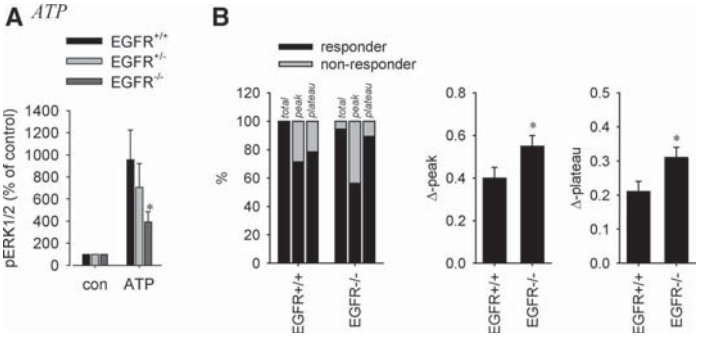

Figure 5. ATP-induced signaling in VSMC. A, Summary of ATP-induced ERK1/2 phosphorylation (5 minutes of incubation with 10 μmol/L ATP). N=5. B, Summary of ATP-induced Ca^{2+} signaling. The left panel shows the percentage of cells responding with a Ca^{2+} signal to ATP (total indicates percentage of cells with peak, plateau, or both; peak, percentage of cells showing at least a peak response; plateau, percentage of cells showing at least a plateau response). The right panels show the changes in fura-2 ratio. N=42 to 53. *$P<0.05$ vs the respective control.

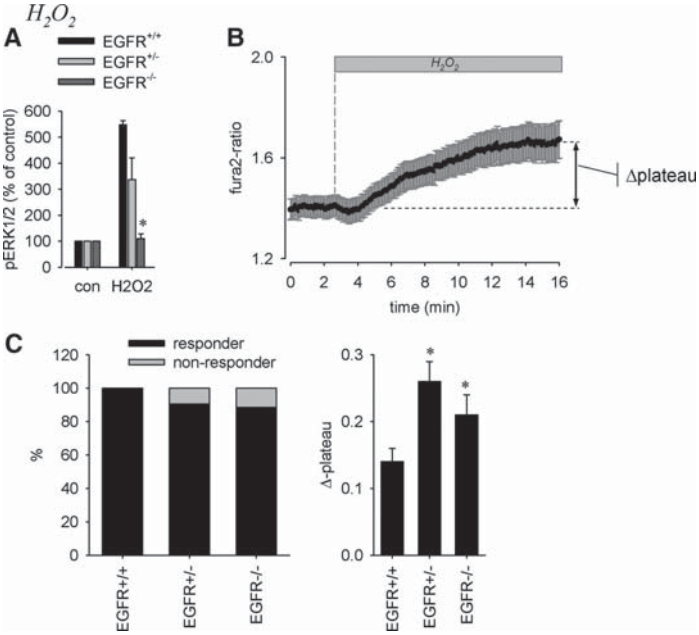

Figure 6. H_2O_2-induced signaling in VSMC. A, Summary of H_2O_2-induced ERK1/2 phosphorylation (30 minutes of incubation with 100 μmol/L H_2O_2). N=5. B, Original tracing of H_2O_2-induced changes in fura-2 ratio. N=5 for each value plotted. C, Summary of H_2O_2-induced Ca^{2+} signaling. The left panel shows the percentage of cells responding with a Ca^{2+} signal to H_2O_2. The right panel shows the changes in fura-2 ratio. N=11 to 35. *$P<0.05$ vs the respective control.

VSMC$^{EGFR-/-}$ but not in VSMC$^{EGFR+/-}$ (Figure 5A), although ATP still elicited a significant ERK1/2 phosphorylation in VSMC$^{EGFR-/-}$ compared with vehicle-treated cells. These data clearly support the hypothesis[12,45] that part of ATP-induced ERK1/2 phosphorylation requires EGFR (transactivation).

G-protein–coupled purinergic receptors also couple to cellular Ca^{2+} signaling and induce Ca^{2+} release from cellular stores, as well as Ca^{2+} entry via the plasma membrane.[47] As shown in Figure 5B, the percentage of responders was not significantly different in VSMC$^{EGFR-/-}$ compared with VSMC$^{EGFR+/+}$ (VSMC$^{EGFR+/-}$ were not tested). The percentage of VSMC$^{EGFR-/-}$ cells responding with a Ca^{2+} peak was lower, although not statistically significant. ΔCa^{2+} of responsive cells was larger in VSMC$^{EGFR-/-}$ compared with VSMC$^{EGFR+/+}$ (Figure 5B). Thus, ATP-induced Ca^{2+} signaling is modulated quantitatively by EGFR expression.

H_2O_2

Exposure to the reactive oxygen species H_2O_2 has been proposed to induce ERK1/2 phosphorylation as part of a cell protection response against radical stress.[12,48,49] As shown in Figure 6A, exposure to 100 μmol/L H_2O_2 for 30 minutes induced a significant stimulation of ERK1/2 phosphorylation in VSMC$^{EGFR+/+}$ and VSMC$^{EGFR+/-}$ but not in VSMC$^{EGFR-/-}$. In addition, H_2O_2 may disturb cellular Ca^{2+} homeostasis, most probably by enhanced Ca^{2+} entry.[50] In VSMC$^{EGFR+/+}$, 100 μmol/L H_2O_2 shifted baseline fura-2 ratio to higher values (Figure 6B). This response is typical for enhanced net Ca^{2+} influx. In VSMC$^{EGFR+/-}$ and VSMC$^{EGFR-/-}$, the percentage of responders was not significantly different from VSMC$^{EGFR+/+}$, but the Δplateau values were significantly higher. Thus, H_2O_2-induced changes of cytosolic Ca^{2+} do not require EGFR but seem to be restricted by EGFR signaling.

Discussion

Many studies on ErbB family functions focused on embryonic development, as well as cancer development. In recent years, however, ErbB1/EGFR has been studied more intensively with respect to its potential pathophysiological role for VSMC. It has been shown pharmacologically that several factors that are considered to be involved in vascular diseases transactivate EGFR and thereby initiate mitogen-activated protein kinase signaling.[51] Transactivation of EGFR was reported 15 years ago[52] and is now accepted as an important mechanism for signaling cross-talk that is essential to mediating critical functions of mediators and micromilieu parameters.[9] Thus, the mechanisms of EGFR transactivation, as well as its pathophysiological significance, are of major interest for vascular biology and medicine. EGFR transactivation seems to require intracellular second messenger activation, such as Ca^{2+}, PKC, or the generation of reactive oxygen species. Subsequently, cytosolic nonreceptor tyrosine kinase such as Src or protein tyrosine kinase-2 may phosphorylate and transactivate EGFR. Alternatively, transactivation is accomplished by metalloprotease-dependent EGFR/ErbB ligand production from its membrane-bound proform.[53] HB-EGF is a ligand with strong implications in vascular diseases. HB-EGF can stimulate growth and migration of VSMCs and is expressed in pathological vascular states such as atherosclerosis and restenosis after angioplasty.[51] In addition to HB-EGF and ErbB1/EGFR, other combinations of ErbB ligand-receptor complexes can also be activated by A

disintegrin and metalloproteinase-dependent shedding of ErbB ligands, leading to diverse arrays of downstream signal transduction pathways with potential relevance for vascular remodeling.

These findings implicate EGFR as an element of signaling convergence by which various vascular risk factors promote vascular remodeling.[6] Beyond that, the physiological significance of vascular EGFR expression and signaling is not known. Therefore, we currently do not know whether there is a yin-yang situation with respect to vascular EGFR, which might contribute to basic vascular homeostasis but could also be subject to "misuse" under certain pathophysiological conditions. In the present study, we investigated the consequence of cell-specific EGFR deletion in VSMC of mice with respect to the basal phenotype and signaling. Because general EGFR-knockout animals are not viable after birth,[54] this model for the first time allows genetic testing of the importance of VSMC EGFR.

Our data show, first of all, that although VSMC EGFR had been successfully deleted (in liver cells EGFR expression was not different from wild-type animals), the animals are viable for several weeks after birth. However, aorta and coronary arteries from $EGFR^{\Delta/\Delta\ VSMC}$ animals showed signs of dilative remodeling (reduced wall/lumen ratio) and slight fibrosis (enhanced collagen III mRNA and Sirius red staining). The morphological data on the vascular phenotype require further experimentation to obtain mechanistic insights and draw conclusion regarding the pathomechanisms and their consequences. At present we cannot exclude the possibility that the changes in wall/lumen ratio are in part the result of different vessel contraction at the time of fixation.

Further detailed phenotyping of the animals and isolated aortae is currently in progress. Net growth of $VSMC^{EGFR-/-}$ was slower, as shown by the growth curve (Figure 1D), and the lower protein amount per cm^2 in the Petri dish after 10 days (Figure 2), despite the same starting cell number and no major differences in cell size. The reduced net growth rate is in part due to an enhanced death rate, as indicated by the higher LDH release (necrosis) and caspase-3 activity (apoptosis). Because EGF reduced LDH release (antinecrotic effect) in $VSMC^{EGFR+/+}$ and $VSMC^{EGFR+/-}$, we conclude that the EGFR exerts a cytoprotective action in VSMC. Surprisingly, EGF did not affect caspase-3 activity in any of the 3 genotypes. Possibly, the antiapoptotic action of EGFR depends on the activation by other ligands or on transactivation. Cytoprotection by EGFR can be explained in part by the reduced pentose phosphate pathway activity (reduced G6PDH activity in $VSMC^{EGFR-/-}$). G6PD hydrogenase (H), which is known to be stimulated by EGF,[55,56] is the rate limiting enzyme of the pentose phosphate pathway supplying the cell with NADPH, an important intracellular reducing equivalent to protect against oxidative injury and a cofactor for nitric oxide synthase as well as for lipogenesis. In addition, this pathway supplies the cells with ribose 5-phosphate for nucleic acid synthesis. We assume that impaired protection against oxidative injury is the most probable explanation for enhanced cell death in $VSMC^{EGFR-/-}$. A nonspecific reduction in G6PDH activity can be excluded, as the activity of 6-PGDH (Figure 2) and cellular LDH (data not shown) were not significantly different from that of wild-type cells.

$VSMC^{EGFR-/-}$ showed an enhanced rate of extracellular collagen III and fibronectin accumulation, indicating an important role for EGFR in the control of basal matrix homeostasis. This conclusion is supported by the inhibitory effect of EGF on collagen III and fibronectin accumulation in $VSMC^{EGFR+/+}$. Thus, EGFR prevents excessive matrix deposition under physiological conditions, contributing to vascular wall tissue homeostasis. In $VSMC^{EGFR+/-}$ collagen III and fibronectin accumulation was significantly lower compared with $VSMC^{EGFR+/+}$, indicating a biphasic effect of EGFR on matrix homeostasis: low levels of EGFR are associated with a minimum of matrix deposition, which increases dramatically in the absence of EGFR but also rises when EGFR expression is elevated. This relation could explain why pathophysiological EGFR transactivation may contribute to vascular remodeling. Surprisingly, $VSMC^{EGFR-/-}$ responded to EGF with a partial reduction in collagen III accumulation. One explanation for this EGF responsiveness is the presence of ErbB-2/ErbB-3 heterodimers with high affinity for EGF,[57,58] comparable to EGFR. By qPCR analysis, ErbB-2 and -3 were detected in all 3 genotypes at comparable levels, whereas ErbB-4 was below the detection limit (data not shown). Of note, fibronectin accumulation of $VSMC^{EGFR-/-}$ did not respond to EGF, suggesting the existence of different EGF signaling pathways for the regulation of matrix homeostasis. Of course, this hypothesis awaits further testing in future studies. In summary, EGFR seems to play an important role for the maintenance of physiological cell and vascular tissue homeostasis, ie, exerts a beneficial vascular effect.

Our data on ERK1/2 phosphorylation show that EGFR contributes substantially to the effects of ET-1, α_1-adrenergic agonists (PE), ATP, reactive oxygen species (H_2O_2), and protein kinase C activation (PMA), supporting the concept of EGFR transactivation. ET-1, α_1-adrenergic agonists, and PKC activation seem to be more dependent on EGFR because their effect was also significantly reduced in $VSMC^{EGFR+/-}$. This was not the case for ATP and H_2O_2, although there was a tendency for reduced ERK1/2 phosphorylation in $VSMC^{EGFR+/-}$. The proliferative action of ET-1 and α_1-adrenergic agonists was completely abrogated in $VSMC^{EGFR-/-}$, confirming the functional relevance of EGFR transactivation.

The effect of H_2O_2 or ATP on cytosolic Ca^{2+} was not reduced by the absence of EGFR. On the contrary, the changes in cytosolic Ca^{2+} were higher in the absence of EGFR, suggesting a negative feedback loop involving EGFR. ET-1- and PE-induced responsiveness of Ca^{2+} signaling was affected by the EGFR genotype. In the case of ET-1, EGFR expression influenced the percentage of responsive cells, which was ≈90% in $VSMC^{EGFR+/+}$. Because only cells responding to thapsigargin were included in the analysis and the percentage was also reduced in $VSMC^{EGFR+/-}$, we can exclude a nonspecific effect of reduced cell viability. Although the immunoblot data contain no evidence for a residual EGFR expression in knockout cells, there remains the possibility for a subthreshold amount of EGFR in few cells, because by qPCR a residual amount of mRNA (less

than 10%) was detected. At present, it is not possible to distinguish experimentally between a small "contamination" of the primary culture with non-VSMC and a small residual amount of EGFR in VSMC. However, the fraction should be very small, as we did not observe EGF-sensitive knockout cells with respect to calcium signaling (Figure 1H). Possible EGFR-positive cells are not sufficient to explain the fraction of responders to PE or ET-1.

When the Ca^{2+} signals of ET-1-responsive cells were compared, no significant differences were observed. The percentage of PE-responsive cells was reduced only in $VSMC^{EGFR-/-}$. However, the Ca^{2+} signals of PE-responsive cells were enhanced in $VSMC^{EGFR+/-}$ and $VSMC^{EGFR-/-}$ compared with $VSMC^{EGFR+/+}$, also suggesting a negative feedback loop involving EGFR. At present, we have no proven explanation for the mechanisms behind this difference in Ca^{2+} signaling. The mechanism seems to have certain specificity, because Ca^{2+} handling was affected differently. An acute functional role of EGFR in Ca^{2+} signaling, as well as altered expression of Ca^{2+}-handling proteins, is conceivable. As the fraction of responders was not reduced by the acute addition of the EGFR inhibitor AG1478, we favor the hypothesis that the probability of a Ca^{2+} response does not depend on acute EGFR transactivation but results from altered gene expression or adaptive processes. Future studies will have to address this question in more detail.

Preliminary organ bath experiments with aortic ring preparations indicate that isometric force development during α_1-adrenergic stimulation is enhanced after repetitive hormone application in $VSMC^{EGFR-/-}$ compared with $VSMC^{EGFR+/+}$ (data not shown). Thus, the larger Ca^{2+} response may result in enhanced vasoconstrictor sensitivity and finally in augmented peripheral resistance, although altered receptor handling (ie, desensitization) cannot be excluded. On the other hand, with respect to the functional vascular relevance, it might be more relevant to consider the integrated calcium signal composed of the probability of a calcium signal (P_R=fraction of responders) and the magnitude of the signal (ΔCa^{2+}). The resulting integrated or average—and probably relevant for a vessel—signal is the product $P_R \times \Delta Ca^{2+}$. With respect to PE and ET-1, this average signal is lower in knockout cells compared with wild-type (compare P_R and ΔCa^{2+} in Figures 3C and 4B). Thus, from the integrated perspective, the data indicate a positive role for EGFR in mediating calcium signals, as also suggested for ang II signaling in afferent arterioles.[33] These hypotheses deserve more detailed investigation in the future.

In summary, our data confirm the importance of EGFR for ERK1/2 activation by a variety of unrelated stimuli. Furthermore, our data show that EGFR expression has a heterologous signaling role beyond mitogen-activated protein kinase activation and can modulate Ca^{2+} signaling. VSMC EGFR seems to be Janus-faced. Its absence makes cells more vulnerable and disturbs matrix homeostasis, leading to dilative remodeling. Overstimulation, on the contrary, promotes vascular fibrotic remodeling, as suggested by various reports in the literature. The model presented here offers the possibility to assess the contribution of VSMC EGFR to vascular remodeling induced by a variety of factors known to transactivate the EGFR.

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Disclosures

None.

References

1. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. 1999;402:884–888.
2. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001;2:127–137.
3. Singh AB, Harris RC. Autocrine, paracrine and juxtacrine signaling by EGFR ligands. *Cell Signal*. 2005;17:1183–1193.
4. Jones RB, Gordus A, Krall JA, MacBeath G. A quantitative protein interaction network for the ErbB receptors using protein microarrays. *Nature*. 2006;439:168–174.
5. Holbro T, Hynes NE. ERBB receptors: directing key signaling networks throughout life. *Annu Rev Pharmacol Toxicol*. 2004;44:195–217.
6. Dreux AC, Lamb DJ, Modjtahedi H, Ferns GAA. The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. *Atherosclerosis*. 2006;186:38–53.
7. Wetzker R, Bohmer FD. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol*. 2003;4:651–657.
8. Biscardi JS, Ishizawar RC, Silva CM, Parsons SJ. Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res*. 2000;2:203–210.
9. Carpenter G. Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. *J Cell Biol*. 1999;146:697–702.
10. Kagiyama S, Eguchi S, Frank GD, Inagami T, Zhang YC, Phillips MI. Angiotensin II-induced cardiac hypertrophy and hypertension are attenuated by epidermal growth factor receptor antisense. *Circulation*. 2002;106:909–912.
11. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res*. 2002;91:406–413.
12. Hao L, Nishimura T, Wo H, Fernandez-Patron C. Vascular Responses to α1-adrenergic receptors in small rat mesenteric arteries depend on mitochondrial reactive oxygen species. *Arterioscler Thromb Vasc Biol*. 2006;26:819–825.
13. Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corvoisier P, Violin JD, Wei H, Lefkowitz RJ, Rockman HA. β-Arrestin-mediated β_1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest*. 2007;117:2445–2458.
14. Hsieh HL, Sun CC, Wang TS, Yang CM. PKC-δ/c-Src-mediated EGF receptor transactivation regulates thrombin-induced COX-2 expression and PGE_2 production in rat vascular smooth muscle cells. *Biochim Biophysica Acta*. 2008;1783:1563–1575.
15. Flamant M, Tharaux PL, Placier S, Henrion D, Coffman T, Chatziantoniou C, Dussaule JC. Epidermal growth factor receptor transactivation mediates the tonic and fibrogenic effects of endothelin in the aortic wall of transgenic mice. *FASEB J*. 2003;17:327–329.
16. Northcott C, Florian JA, Dorrance A, Watts SW. Arterial epidermal growth factor receptor expression in deoxycorticosterone acetate-salt hypertension. *Hypertension*. 2001;38:1337–1341.
17. Chansel D, Ciroldi M, Vandermeersch S, Jackson LF, Gomez AM, Henrion D, Lee DC, Coffman TM, Richard S, Dussaule JC, Tharaux PL. Heparin binding EGF is necessary for vasospastic response to endothelin. *FASEB J*. 2006;20:1936–1938.
18. Morris JB, Pham TM, Kenney B, Sheppard KE, Woodcock EA. UTP transactivates epidermal growth factor receptors and promotes cardiomyocyte hypertrophy despite inhibiting transcription of the hypertrophic marker gene, atrial natriuretic peptide. *J Biol Chem*. 2004;279:8740–8746.
19. Mazak I, Fiebeler A, Muller DN, Park JK, Shagdarsuren E, Lindschau C, Dechend R, Viedt C, Pilz B, Haller H, Luft FC. Aldosterone

potentiates angiotensin II-induced signaling in vascular smooth muscle cells. *Circulation*. 2004;109:2792–2800.
20. Buchanan FG, Wang D, Bargiacchi F, DuBois RN. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem*. 2003;278:35451–35457.
21. Lemarie CA, Tharaux PL, Esposito B, Tedgui A, Lehoux S. Transforming growth factor-α mediates nuclear factor κB activation in strained arteries. *Circ Res*. 2006;99:434–441.
22. Kennedy DJ, Vetteth S, Periyasamy SM, Kanj M, Fedorova L, Khouri S, Kahaleh MB, Xie Z, Malhotra D, Kolodkin NI, Lakatta EG, Fedorova OV, Bagrov AY, Shapiro JI. Central role for the cardiotonic steroid marinobufagenin in the pathogenesis of experimental uremic cardiomyopathy. *Hypertension*. 2006;47:488–495.
23. Terzi F, Burtin M, Hekmati M, Federici P, Grimber G, Briand P, Friedlander G. Targeted expression of a dominant-negative EGF-R in the kidney reduces tubulo-interstitial lesions after renal injury. *J Clin Invest*. 2000;106:225–234.
24. Kagiyama S, Qian K, Kagiyama T, Phillips MI. Antisense to epidermal growth factor receptor prevents the development of left ventricular hypertrophy. *Hypertension*. 2003;41:824–829.
25. Bokemeyer D, Schmitz U, Kramer HJ. Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor. *Kidney Int*. 2000;58:549–558.
26. Thomas WG, Brandenburger Y, Autelitano DJ, Pham T, Qian H, Hannan RD. Adenoviral-directed expression of the type 1A angiotensin receptor promotes cardiomyocyte hypertrophy via transactivation of the epidermal growth factor receptor. *Circ Res*. 2002;90:135–142.
27. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat Med*. 2002;8:35–40.
28. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2000; 103:211–225.
29. Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol Syst Biol*. 2005; 1:msb4100014–msb41000E1.
30. Lovisolo D, Distasi C, Antoniotti S, Munaron L. Mitogens and calcium channels. *Physiology*. 1997;12:279–285.
31. Tinhofer I, Maly K, Dietl P, Hochholdinger F, Mayr S, Obermeier A, Grunicke HH. Differential Ca^{2+} signaling induced by activation of the epidermal growth factor and nerve growth factor receptors. *J Biol Chem*. 1996;271:30505–30509.
32. Han HJ, Park SH, Lee YJ. Epidermal growth factor regulates Ca^{2+} uptake in primary cultured renal proximal tubule cells: involvement of cAMP, PKC and $cPLA_2$. *Kidney Blood Press Res*. 2003;26:155–164.
33. Che Q, Carmines PK. Angiotensin II triggers EGFR tyrosine kinase-dependent Ca^{2+} influx in afferent arterioles. *Hypertension*. 2002;40: 700–706.
34. Natarajan A, Wagner B, Sibilia M. The EGF receptor is required for efficient liver regeneration. *Proc Natl Acad Sci U S A*. 2007;104: 17081–17086.
35. Holtwick R, Gotthardt M, Skryabin B, Steinmetz M, Potthast R, Zetsche B, Hammer RE, Herz J, Kuhn M. Smooth muscle-selective deletion of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. *Proc Natl Acad Sci U S A*. 2002;99:7142–7147.
36. Ray JL, Leach R, Herbert JM, Benson M. Isolation of vascular smooth muscle cells from a single murine aorta. *Methods Cell Sci*. 2001;23: 185–188.
37. Gekle M, Freudinger R, Mildenberger S, Silbernagl S. Aldosterone interaction with epidermal growth factor receptor signaling in MDCK cells. *Am J Physiol Renal Physiol*. 2002;282:F669–F679.
38. Schwerdt G, Holzinger H, Sauvant C, Königs M, Humpf HU, Gekle M. Long-term effects of ochratoxin A on fibrosis and cell death in human proximal tubule or fibroblast cells in primary culture. *Toxicology*. 2007; 232:57–67.
39. Leopold JA, Loscalzo J. Cyclic strain modulates resistance to oxidant stress by increasing G6PDH expression in smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 2000;279:H2477–H2485.
40. Esposito C, Fornoni A, Cornacchia F, Bellotti N, Fasoli G, Foschi A, Mazzucchelli I, Mazzullo T, Semeraro L, Dal Canton A. Cyclosporine induces different responses in human epithelial, endothelial and fibroblast cell cultures. *Kidney Int*. 2000;58:123–130.
41. Vesey DA, Cheung CWY, Cuttle L, Endre ZA, Gobe G, Johnson DW. Interleukin-1β induces human proximal tubule cell injury, α-smooth muscle actin expression and fibronectin production1. *Kidney International*. 2002;62:31–40.
42. Amos S, Martin PM, Polar GA, Parsons SJ, Hussaini IM. Phorbol 12-myristate 13-acetate induces epidermal growth factor receptor transactivation via protein kinase C+/c-Src pathways in glioblastoma cells. *J Biol Chem*. 2005;280:7729–7738.
43. Watts SW. Endothelin receptors: what's new and what do we need to know? *Am J Physiol Regul Integr Comp Physiol*. 2010;298: R254–R260.
44. Takemura H, Hughes AR, Thastrup O, Putney JWJ. Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J Biol Chem*. 1989;264:12266–12271.
45. Zhang H, Chalothorn D, Jackson LF, Lee DC, Faber JE. Transactivation of Epidermal Growth Factor Receptor Mediates Catecholamine-Induced Growth of Vascular Smooth Muscle. *Circ Res*. 2004; 95:989–997.
46. Zang Wj, Zacharia J, Lamont C, Wier WG. Sympathetically evoked Ca^{2+} signaling in arterial smooth muscle. *Acta Pharmacol Sin*. 2006;27: 1515–1525.
47. Burnstock G. Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol*. 2002;22:364–373.
48. Tabet F, Schiffrin EL, Touyz RM. Mitogen-activated protein kinase activation by hydrogen peroxide is mediated through tyrosine kinase-dependent, protein kinase C-independent pathways in vascular smooth muscle cells: upregulation in spontaneously hypertensive rats. *J Hypertens*. 2005;23:2005–2012.
49. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol*. 2000;20:2175–2183.
50. Hecquet CM, Malik AB. Role of H2O2-activated TRPM2 calcium channel in oxidant-induced endothelial injury. *Thromb Haemost*. 2009; 101:619–625.
51. Eguchi S, Frank GD, Mifune M, Inagami T. Metalloprotease-dependent ErbB ligand shedding in mediating EGFR transactivation and vascular remodelling. *Biochem Soc Trans*. 2003;31:1198–1202.
52. Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature*. 1996; 379:557–560.
53. Ohtsu H, Dempsey PJ, Eguchi S. ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *Am J Physiol Cell Physiol*. 2006;291:C1–C10.
54. Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science*. 1995;269:234–238.
55. Yoshimoto K, Nakamura T, Ichihara A. Reciprocal effects of epidermal growth factor on key lipogenic enzymes in primary cultures of adult rat hepatocytes. Induction of glucose-6-phosphate dehydrogenase and suppression of malic enzyme and lipogenesis. *J Biol Chem*. 1983;258: 12355–12360.
56. Molero C, Benito M, Lorenzo M. Glucose-6-phosphate dehydrogenase gene expression in fetal hepatocyte primary cultures under nonproliferative and proliferative conditions. *Exp Cell Res*. 1994;210:26–32.
57. Alimandi M, Wang LM, Bottaro D, Lee CC, Kuo A, Frankel M, Fedi P, Tang C, Lippman M, Pierce JH. Epidermal growth factor and betacellulin mediate signal transduction through co-expressed ErbB2 and ErbB3 receptors. *EMBO J*. 1997;16:5608–5617.
58. Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res*. 2003;284:54–65.

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Substance-specific importance of EGFR for vascular smooth muscle cells motility in primary culture

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ABSTRACT

Besides their importance for the vascular tone, vascular smooth muscle cells (VSMC) also contribute to pathophysiological vessel alterations. Various G-protein coupled receptor ligands involved in vascular dysfunction and remodeling can transactivate the epidermal growth factor receptor (EGFR) of VSMC, yet the importance of EGFR transactivation for the VSMC phenotype is incompletely understood. The aims of this study were (i) to characterize further the importance of the VSMC-EGFR for proliferation, migration and marker gene expression for inflammation, fibrosis and reactive oxygen species (ROS) homeostasis and (ii) to test the hypothesis that vasoactive substances (endothelin-1, phenylephrine, thrombin, vasopressin and ATP) rely differentially on the EGFR with respect to the abovementioned phenotypic alterations.
In primary, aortic VSMC from mice without conditional deletion of the EGFR, proliferation, migration, marker gene expression (inflammation, fibrosis and ROS homeostasis) and cell signaling (ERK 1/2, intracellular calcium) were analyzed.
VSMC-EGFR loss reduced collective cell migration and single cell migration probability, while no difference between the genotypes in single cell velocity, chemotaxis or marker gene expression could be observed under control conditions. EGF promoted proliferation, collective cell migration, chemokinesis and chemotaxis and leads to a proinflammatory gene expression profile in wildtype but not in knockout VSMC. Comparing the impact of five vasoactive substances (all reported to transactivate EGFR and all leading to an EGFR dependent increase in ERK1/2 phosphorylation), we demonstrate that the importance of EGFR for their action is substance-dependent and most apparent for crowd migration but plays a minor role for gene expression regulation.

1. Introduction

In 1996 Axel Ulrich and his colleagues discovered, that G-protein coupled receptors (GPCR) are able to activate receptor tyrosine kinases, especially the epidermal growth factor receptor (EGFR) and thereby acquire the potential to mediate effects like cell proliferation and migration [1,2]. To date, regarding G-protein coupled receptors there are three major signaling pathways activated: first, the classical pathways where binding of the receptor ligand causes G protein binding and subsequent alterations of effector molecule activity by Gα-subunits; second, the β-arrestin scaffold pathway activating multiple downstream signaling pathways; and third, the activation of receptor tyrosine kinases [3,4]. The activation of receptor tyrosine kinases by GPCRs has been identified as key events during hypertension and hyperglycemia [5,6]. In the development of atherosclerosis proliferation and migration of vascular smooth muscle cells (VSMC) probably involve growth factor receptors like those of the EGFR family [7]. It has been demonstrated in rats that the EGFR is involved in the early events of intima hyperplasia after balloon injury, where the main contribution of VSMC to the intima is due to increase in migration [8].

The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, forms homo- and heterodimers upon ligand binding with all EGF-receptor family members (EGFR, ErbB2, ErbB3 and ErbB4) [9,10]. These dimers activate important downstream signaling pathways involved in cell proliferation, differentiation and migration [11,12]. In addition to activation by classical ligands, EGFR can also be transactivated through a cross-talk with other receptor-initiated pathways [9,13], possibly also promoting the aforementioned effects. Among the substances discussed to transactivate the EGFR are vasopressin (ADH) [14,15], α_1- and β-adrenergic agonists [16–20], thrombin [21,22], endothelin-1 [23–25] and purinergic receptor ligands [26]. Thus, EGFR – via transactivation – has the potential to mediate signaling of non-EGFR ligands and thereby serve as a heterologous transducer of cellular signaling. Pathophysiological effects of EGFR include cell transformation and tumorigenesis as well as parainflammatory dysregulation of tissue homeostasis leading for example to vascular dysfunction, fibrosis and ROS dysbalance [18].

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To analyze the function of the EGFR family members several gain- and loss-of-function mutants have been generated [27,28], but the main research on EGFR in the vascular system has been performed using EGFR inhibitors, like AG1478 [29,30]. This is due to the fact that global EGFR-knockout mice die shortly after birth [31]. Therefore we generated a mouse model with a deletion of the EGFR in vascular smooth muscle cells (VSMC) [32]. These mice show a reduced wall-to-lumen ratio in aortas [32] and an impaired activation of ERK1/2 in isolated VSMC upon stimulation with different vasoactive substances [33].

We have already demonstrated that in vascular smooth muscle cells, isolated from the $EGFR^{\Delta/\Delta VSMC\&CM}$ knockout mice calcium and ERK 1/2 signaling induced by different vasoactive stimuli are affected differentially by EGFR deletion [33]. The aim of the present study was to characterize specifically the role of EGFR (ErbB1) on VSMC proliferation and migration as well as on the expression of marker genes for inflammation, fibrosis and ROS balance using cells from a genetic knockout model and compare them with cells from respective wildtype mice. In particular we analyzed the importance of EGFR for of the impact of vasoactive substances supposed to transactivate the EGFR on the abovementioned parameters [23].

While the mechanism of transactivation and the intracellular signaling cascade have been the subject of extensive investigation, the cellular effects of EGFR transactivation by the different substances have not been compared in that detail. Herein we aim to analyze if the EGFR is necessary for alterations in the cellular phenotype of VSMC. Additionally, we aim to elucidate, if the change in VSMC phenotype elicited by the EGFR is independent of the substance transactivating the EGFR. To evaluate this question we incubated VSMC with or without EGFR-deletion with EGF, endothelin-1 (ET-1), thrombin, vasopressin, phenylephrine (PE) or ATP and studied their impact on vascular smooth muscle cell proliferation, migration and marker gene expression. The well described EGFR-induced signaling cascades were not in the focus of our study.

2. Materials and methods

2.1. Generation of $EGFR^{\Delta/\Delta\ VSMC}$ mice and VSMC culture

Generation of the mice and baseline phenotype were described before as well as the preparation of the VSMC [32,33] and are described again in brief in the supplementary material for convenience. Mice were kept in the facilities of the University of Halle-Wittenberg in accordance with institutional policies and federal guidelines according to the Landesverwaltungsamt Sachsen-Anhalt, Halle, Germany.

Primary culture of VSMC from 4- to 5-month-old mice ($EGFR^{flox/flox} \times SM22\text{-}Cre^{+/-} \rightarrow VSMC^{\Delta/\Delta EGFR}$ = KO; $EGFR^{flox/flox} \times SM22\text{-}Cre^{-/-} \rightarrow VSMC^{+/+EGFR}$ = WT) was performed as described before [33,34]. Cells from 3 to 7 mice per experiment were cultured in MEM with 10% fetal calf serum (FCS) and SMC-Growth medium and passages from 2 to 10 were employed in the individual experiments. Only in the single cell tracking experiments just two different knockout cell lines were used.

2.2. Western blot

Cells were lysed with Cell Signaling Technologies (CST) lysis buffer. Proteins were separated by 12% SDS–PAGE for detection of pERK and ERK, transferred to nitrocellulose membranes and incubated either with anti-phospho-ERK and anti-ERK antibodies (1:1000 each, Cell signaling technologies). Protein bands were detected by HRP-conjugated secondary antibody (anti rabbit IgG, Biotrend, Cologne, Germany) [33].

2.3. Determination of cytosolic Ca^{2+}

Cytosolic free calcium was determined using the Ca^{2+} sensitive dye fura-2 (Molecular Probes, Leiden, Netherlands) as described previously [33,35] with an inverted Axiovert 100 TV microscope (400× magnification, oil immersion; Zeiss, Oberkochen, Germany) and an automatic filter change device (Hamamatsu, Herrsching, Germany). At the end of each experiment thapsigargin (SERCA inhibitor) was applied as a positive control and only cells responding to thapsigargin were included for further analysis.

2.4. Gene expression

Gene expression analysis was carried out as described before [32,33]. RNA was isolated using the RNeasy Mini Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. Subsequently residual remaining DNA was digested with DNase I. Reverse transcription was performed with 1 μg RNA and random primers using qScript from Quanta Biosciences according to the manufacturer's protocol. Additionally each sample was analyzed without reverse transcription. The signals obtained without RT were neglectable (<1%). Finally, real-time amplification was performed with the Stratagene Mx3005P using the Platinum SYBR Green kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The primers used are given in Supplementary Table 1. qPCR efficiency was >90%. All primers for genes of interest are intron-spanning and have been validated by RT-PCR and gel electrophoresis. Furthermore, the primers were validated by melting curve analysis. The relative expression of the two genes of interest was calculated according to the $2^{-\Delta\Delta Ct}$ method, using the 18S signal for normalization. Each sample was analyzed as triplicate. All values are expressed as mean difference to respective control or wildtype (−ΔΔct(treatment-control) or −ΔΔct(KO-WT)) with standard error of mean (SEM).

2.5. Cell proliferation

Cells were plated on a 24-well dish, given the possibility to seed for 24 h, and then the cells in a marked square micrometer were counted. Afterwards cells were starved for 24 h, counted again and incubated for 24 h with either starvation medium without supplements (control), 10% FCS (equals normal growth medium), 10 μg/l EGF (Millipore, Billerica, USA), 100 nM ADH, 0.5 U/ml thrombin, 100 nM endothelin-1, 1 μM phenylephrine or 10 μM ATP in starvation medium. The proliferation was calculated as percentage of cell increase within 24 h; this was normalized to the increase of cell number in wells under control conditions. The same substance concentrations were employed in all experiments.

2.6. Gap closure (reconstitution) assay

Cells were counted and plated in 24-well dishes, with an IBIDI insert for injury assay according to the manufacturer's protocol. Twenty-four hours after seeding the cells were starved for the next 24 h. The inlet was removed and the cells were incubated under the same conditions as mentioned above. Photos were taken immediately after removal of the inlet, after 4, 24 and 48 h. To improve the images we enhanced the contrast in the pictures, reduced the RGB-color image to a grayscale image and used the tool “hard mix” in the blending tools menu of the computer program Adobe Photoshop CS3. To ensure that the same area was measured, characteristic marks were placed on the outside of the tissue culture dish before the experiment. At the edges of these marks vertical bars were placed and the empty area between these bars was measured (Supplementary Figs. S1 and S2). Wound healing is given as the percentage of the re-covered area at each time point, relative to the initially not covered area (0 h). Data were calculated according to the formula: $(((\text{not covered area})_{t0} - (\text{not covered area})_{tx})/(\text{not covered area})_{t0} * 100) - 100$. As in this actual assay no wound/scratch is

formed, the assay will be further on named reconstitution or gap closure assay in this article.

2.7. Boyden chamber

Cells were counted and 100,000 cells were added on a filter with 5 μm pores. Cells were allowed to attach for 6 h and were starved afterwards for 24 h. After starvation cells were stimulated as mentioned under "cell proliferation" for 24 h with different substances in the bottom compartment and starvation medium in the upper compartment. The medium in the upper compartment was changed every 4 h to maintain the concentration gradient. After 24 h the cells in the upper compartment were removed with a cotton swap and the filter was washed three times with PBS. The cells on the bottom side of the filter membrane were stained with trypan blue, lysed and the lysate was measured in a Tecan sunrise fluorescence reader (Tecan, Maennedorf, Swiss).

2.8. Single cell tracking experiments

Cells were counted and seeded either on IBIDI μ- object slides (ibidi GmbH, Martinsried, Germany) or Falcon object slides (Corning, Tewksbury, USA) for 24 h, afterwards cells were starved for 24 h. Single cell tracking experiments were performed on a ZEISS AxioObserver A1 (Carl Zeiss, Jena, Germany) with a magnification of ×20 at brightfield illumination. Every 5 min a picture was taken by the VisiView software (Visitron Systems GmbH, Puchheim, Germany), for 2 h under control conditions and 4 h under stimulation either with 10 μg/l EGF or 100 nM ADH. Precaution was taken that the same cells could be tracked for the whole time. During the experiment the cells were placed in an IBIDI stage top incubator (ibidi GmbH, Martinsried, Germany) connected to a active gas mixer (the brick, life technologies services, Basel, Switzerland) at a temperature of 37 °C, filled with moisturized gas (5% CO_2, 19% O_2 and 76% N_2, flow 10 l/h). Cell movement was analyzed using the ImageJ software and the manual tracking tool.

Cell movement must not be continuous and therefore gross velocity depends on at least two parameters: the fraction of an observation period in which the cells do not move (migration probability, P_m) and the mean net velocity obtained from the periods when the cells are moving (Supplementary Fig. S3).

2.9. Materials

Unless otherwise stated all materials were purchased from Sigma, Munich, Germany. Control Ringer solution was composed of (mmol/l): NaCl 130.0, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 1.0, HEPES 10 and glucose 5 (pH 7.4 at 37 °C), plus the respective vehicles (ethanol or DMSO ≤1‰).

2.10. Statistics

The data are presented as fold-change ± standard error of mean (SEM). Significance of difference was tested by unpaired Student's *t*-test. Differences were considered significant if $p < 0.05$. Regarding gene expression data two-way ANOVA was performed to detect significant interactions between the four treatment groups. *p*-Values for the interaction test are given in separate columns in Supplementary Table S3. No significant interaction between the groups could be observed ($p < 0.05$). Cells from at least three different mice per genotype and three different matching passages were used for each experimental series. Thus, all experiments were performed with cells from at least three different animals. Cells from the two genotypes were treated in parallel for the various repetitions. *N* represents the number of cells or tissue culture dishes investigated.

3. Results

3.1. Control conditions and effects of FCS

In the absence of serum cells were quiescent and did not proliferate (WT: −11.3 ± 5.7%, KO: −7.1 ± 4.7% of cells compared to time point at the beginning of the experiment). VSMC from wildtype and knockout animals show no difference in cell proliferation upon stimulation with 10% FCS (Fig. 1A), nor could we observe differences in apoptosis or necrosis (Caspase-3 activity and LDH release, Supplementary Fig. S4). Performing a gap closure (reconstitution) assay to monitor collective cell migration we observed a significant increase in recovered area after 4 h in WT cells under control conditions (Fig. 1B/C) compared to the initial area in the same well. After 48 h 73 ± 7% of the initial area was covered with WT cells, whereas there was no significant gap closure at any time by KO cells under these conditions (Fig. 1F). Since we did not observe any proliferation under control conditions or after 4 h (Fig. 1E) the gap closure of WT cells cannot be attributed to proliferation but results from collective cell migration. These data are in good agreement with the known role of EGF in collective migration during e.g. epidermal regeneration [36]. In the next step we performed single cell tracking experiments over a period of 6 h. There was no statistically significant difference in gross velocity nor net velocity (V_{net}) between the genotypes under control conditions, yet the KO cells showed a significant lower migration probability (P_m) compared to WT cells (Supplementary Table S2), which seems to be evened out by a higher net velocity in trend. To analyze if EGFR knockout cells have the potential to follow a chemical gradient we performed a modified Boyden chamber assay. There was no difference in unstimulated cells (WT: 0.35 ± 0.07 trypan blue O.D., KO: 0.30 ± 0.03 trypan blue O.D., data is given as mean ± SEM, $N = 6–7$ filter/group).

The impact of EGFR on gene expression was analyzed by evaluating the mRNA levels of marker genes for fibrosis (Col1a1, Col3a1, Fn-1, Serpine-1), inflammation (IL-6, SPP-1, MCP-1, c-Jun, TGF-b, NFκB1) and ROS homeostasis (NOX-1, NOX4, p22phox). EGFR deletion had no major impact on the expression of these genes (Supplementary Table S3) in unstimulated cells.

To verify that the knockout cells are still capable of proliferation and migration comparable to wildtype cells, we incubated both cell types with medium containing 10% FCS. We chose FCS as a positive control, as it increases cell proliferation and migration via various signalling pathways, thereby compensating for the deletion of EGFR in knockout cells. Upon addition of FCS cell proliferation was stimulated to virtually the same extent in wildtype and knockout cells (Fig. 1A). Addition of 10% FCS led to a significant reduction in cell free area to a similar extend in both phenotypes after 24 and 48 h compared to the respective controls (Fig. 1B, Supplementary Figs. S1 and 2). After 48 h there was no further difference between the genotypes. These data show that KO cells have not lost migratory capacity *per se*. In WT and KO cells transmigration was stimulated by 10% FCS to a similar extend (Fig. 1D).

3.2. Effects of epidermal growth factor

The EGFR is reduced in knockout cells below 10% compared to wildtype cells, as shown before and ERK1/2 signaling as well as Ca^{2+}-signaling is impaired [33]. As we report for the first time on the effect of a targeted, genomic deletion of the EGFR on VSMC migration, proliferation and marker gene expression, we tested, additionally to the five different vasoactive substances, also the effect of direct EGFR activation by EGF. After 24 h starvation, addition of 10 μg/l EGF increased VSMC proliferation of WT but not of KO cells (Fig. 2A). There was no difference in apoptosis or necrosis between the genotypes or due to EGF exposure (Supplementary Fig. S4).

In wildtype but not in KO cells 10 μg/l EGF enhanced gap closure, an effect detectable already within the first 4 h and even more pronounced after 48 h (Fig. 2B, C, E, and F). Since an effect of EGF on proliferation

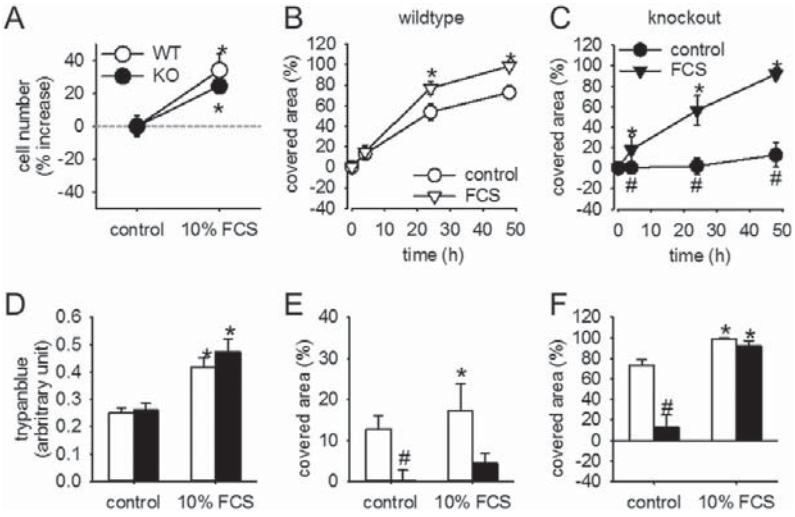

Fig. 1. Impact of EGFR in VSMC on proliferation, transmigration and reconstitution. A) Cells in a marked square millimeter were counted after 24 h starvation and were either further starved for 24 h or incubated with normal cell medium or starvation medium supplemented with 10% FCS. $^{*}p < 0.05$ vs. respective control, $n = 6$–13 experiments/group. B, C) After 24 h starvation injury assay inlet was removed and a photograph was taken of the cell free area. Afterwards the cells were either starved for 48 more hours or incubated with starvation medium supplemented either with 10% FCS. The cell-free area was repeatedly measured after 4, 24 and 48 h. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 6$–17 experiments/group. D) After seeding and 24 h starvation either starvation medium or starvation medium supplemented with 10% FCS was added to the lower compartment of the Boyden chamber. Medium in the upper compartment was changed every 4 h to maintain the gradient. After 24 h cells on the filter were removed; cells under the filter were stained with trypan blue and lysed. Migration is given as fold-change trypan blue staining compared to control cells. $^{*}p < 0.05$ vs. respective control, $n = 12$–18 wells/group. Reconstitution after 4 h (E) or 48 h (F) under control conditions or stimulation with 10% FCS is depicted. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 6$–17 experiments/group.

within 4 h is highly unlikely, these data already indicate that EGF stimulates chemokinesis via the EGFR. To distinguish between accelerated motion and directed movement in single cells Boyden chamber assays (directed movement) were performed over 24 h and single cell tracking experiments (accelerated motion) over 6 h. Ten micrograms per liter EGF increased the directed movement of WT cells in the Boyden chamber (Fig. 2D). Additionally, EGF increased gross velocity, in part due to an enhanced P_m and an increase in V_{net} (Supplementary Table S4), in WT VSMC. There was no effect of EGF on KO cells.

To analyze the effect of activated EGFR on the expression of marker genes for inflammation, fibrosis and ROS homeostasis, we stimulated wildtype and knockout cells for 48 h with 10 μg/l EGF. We observed an increase in the expression of SPP-1 and MCP-1 in wildtype but not in knockout cells. By contrast, EGF reduced the expression of TGF-β slightly in EGFR knockout but not in wildtype cells. This apparently paradoxical behavior can be explained by the fact that EGF also binds to and activates ErbB3 and ErbB4 [10] — especially ErbB2/3-heterodimers. Supposing a neutralizing antagonistic action of EGFR-containing dimers and ErbB2/3-heterodimers with respect to TGF-β-expression, the ErbB2/3-effect would be unveiled in the absence of EGFR. Of course this hypothesis will need further substantiation in the future.

The above data confirm that activation of the EGFR via its ligand EGF promotes a proinflammatory phenotype in VSMC. The expression of Col3a1 was stimulated slightly in wildtype and knockout cells to the same extend, indicating that this effect is also not mediated by the EGFR but possibly also by Erb2/3-dimers. The expression of marker genes for ROS homeostasis was not affected by EGF (Supplementary Fig. S5).

3.3. Effects of thrombin

Incubation with 0.5 U/ml thrombin enhanced proliferation (Fig. 3A, Supplementary Fig. S4) and collective migration (Fig. 3B, C, E, and F) in wildtype but not in knockout cells, while thrombin had no effect on transmigration neither in wildtype nor in knockout cells (Fig. 3D).

Regarding gene expression thrombin increased the expression of marker genes for inflammation (Fig. 3G) and fibrosis (Fig. 3H), independent of EGFR expression. Thrombin has no major effect on the expression of marker genes for ROS homeostasis in wildtype and knockout animals, except a small decrease of p22phox expression in knockout cells (Supplementary Fig. S5).

As we already reported [33], at least some of the intracellular signalling of EGFR in VSMC is mediated via the ERK 1/2 signaling pathway. Therefore we analyzed thrombin-induced ERK1/2 signaling. Upon incubation of VSMC from WT or KO animals ERK 1/2 phosphorylation increased independent of the genotype (WT: 403 ± 49%, KO: 461 ± 48% of control, $N = 3$–6 per group) after 5 min of incubation. We therefore conclude that the alterations in cell migration are most probably not mediated via differences in the ERK 1/2 pathway.

In summary, EGFR seems to be important for thrombin-induced migration and proliferation but not for thrombin-induced regulation of gene expression.

3.4. Effects of ADH

ADH (100 nM) did not increase proliferation of vascular smooth muscle cells neither in WT nor in KO cells (Fig. 4A, Supplementary Fig. S4), but increased gap closure in WT cells already after 4 h (Fig. 4B) as well as after 24 and 48 h compared to control conditions (Fig. 4E and F). In KO cells ADH did not increase gap closure at any time point compared to control conditions, indicating that the EGFR is required for ADH induced collective migration (Fig. 4C, E and F). In single cell tracking experiments stimulation with ADH had no effect on the velocity of WT or KO cells (WT: 106 ± 9% of control; KO: 113 ± 16% of control; data from 4 preparations with 6–14 cells each). In the Boyden chamber assay ADH did not increase migration over 24 h neither in

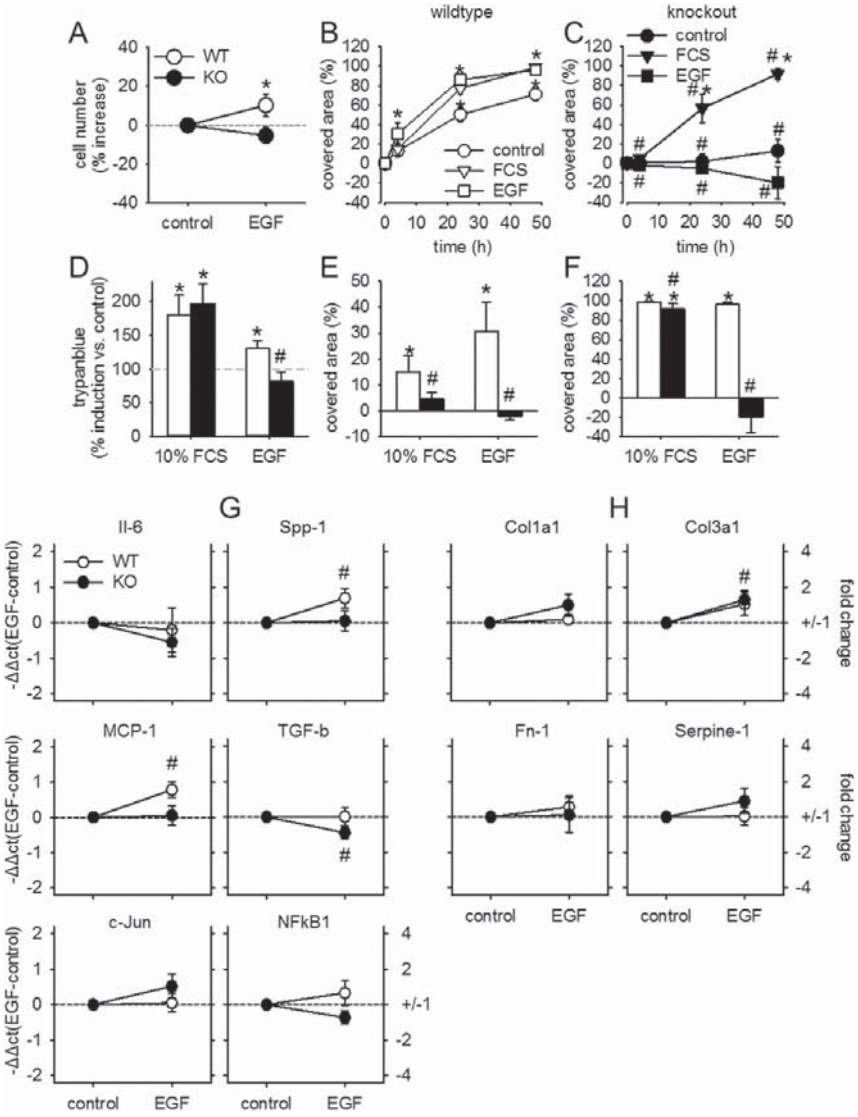

Fig. 2. Impact of EGF-activated (10 μg/l) EGFR on proliferation, transmigration, reconstitution and marker gene expression. A) Cells in a marked square millimeter were counted after 24 h starvation and were either further starved for 24 h or incubated with normal cell medium or starvation medium supplemented with EGF. $^{*}p < 0.05$ vs. respective control, $n = 5$–13 experiments/group. B, C) After 24 h starvation injury assay inlet was removed and a photograph was taken of the cell free area. Afterwards the cells were either starved for 48 more hours or incubated with starvation medium supplemented either with EGF. The cell-free area was repeatedly measured after 4, 24 and 48 h. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 3$–17 experiments/group. D) After seeding and 24 h starvation either starvation medium, growth medium or starvation medium supplemented with EGF was added to the lower compartment of the Boyden chamber. Medium in the upper compartment was changed every 4 h to maintain the gradient. After 24 h cells on the filter were removed, cells under the filter were stained with trypan blue and lysed. Migration is given as fold-change trypan blue staining compared to control cells. $^{*}p < 0.05$ vs. respective control, $n = 6$–18 wells/group. Reconstitution after 4 h (E) or 48 h (F) stimulation either with 10% FCS or EGF is depicted. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 3$–17 experiments/group. Marker gene expression for inflammation (G) and fibrosis (H) was evaluated by qRT-PCR. $^{*}p < 0.05$ vs. respective control, $^{\#}p < 0.05$ vs. respective wildtype cells, $n = 6$–20 cell preparations/group.

WT nor in KO cells (Fig. 4D). Thus, single cell migration was not ADH-sensitive under our experimental conditions.

There was no change in the expression of marker genes for fibrosis, inflammation or ROS homeostasis after 48 h incubation with ADH (Fig. 4G and H, Supplementary Fig. S5).

Incubation of VSMC with ADH induced a transient increase in ERK1/2 phosphorylation after 5 min in WT but not in KO cells (WT: 182 ± 36%, KO: 129 ± 29% of control, $N = 3$–8 per group). ERK1/2 phosphorylation returned to baseline levels within 30 min. By contrast to the ERK1/2 signaling branch, ADH-induced Ca^{2+}-signaling (i.e. the increase in fura-2-

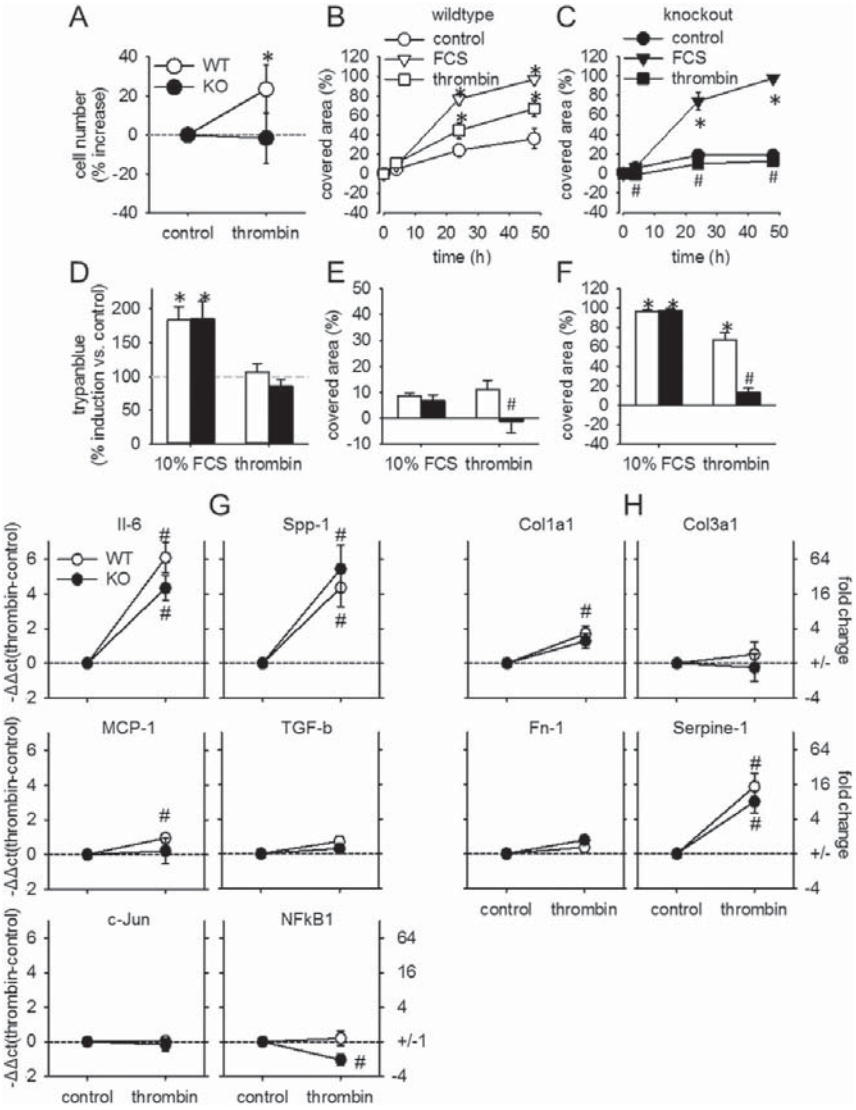

Fig. 3. Effect of thrombin-transactivated (0.5 U/ml) EGFR on VSMC proliferation, transmigration, reconstitution and marker gene expression. A) Cells in a marked square millimeter were counted after 24 h starvation and were either further starved for 24 h or incubated with normal cell medium or starvation medium supplemented with thrombin. $^{*}p < 0.05$ vs. respective control, $n = 6$–13 experiments/group. B, C) After 24 h starvation injury assay inlet was removed and a photograph was taken of the cell free area. Afterwards the cells were either starved for 48 more hours or incubated with starvation medium supplemented either with thrombin. The cell-free area was repeatedly measured after 4, 24 and 48 h. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 5$–11 experiments/group. D) After seeding and 24 h starvation either starvation medium, growth medium or starvation medium supplemented with thrombin was added to the lower compartment of the Boyden chamber. Medium in the upper compartment was changed every 4 h to maintain the gradient. After 24 h cells on the filter were removed, cells under the filter were stained with trypan blue and lysed. Migration is given as fold-change trypan blue staining compared to control cells. $^{*}p < 0.05$ vs. respective control, $n = 5$–18 wells/group. Reconstitution after 4 h (E) or 48 h (F) stimulation either with 10% FCS or thrombin is depicted. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 5$–11 experiments/group. Marker gene expression for inflammation (G) and fibrosis (H) was evaluated by qRT-PCR. $^{*}p < 0.05$ vs. respective control, $^{\#}p < 0.05$ vs. respective wildtype cells, $n = 4$–20 cell preparations/group.

ratio) was not reduced in KO cells (Δfura-2-ratio$_{WT} = 0.44 \pm 0.05$, Δfura-2-ratio$_{KO} = 0.94 \pm 0.13$, $N = 31$–87).

In summary, EGFR seems to be important for ADH-induced collective migration.

3.5. Effects of endothelin-1

Incubation with endothelin-1 (100 nM) did not affect proliferation of wildtype or knockout cells (Fig. 5A, Supplementary Fig. S4). ET-1

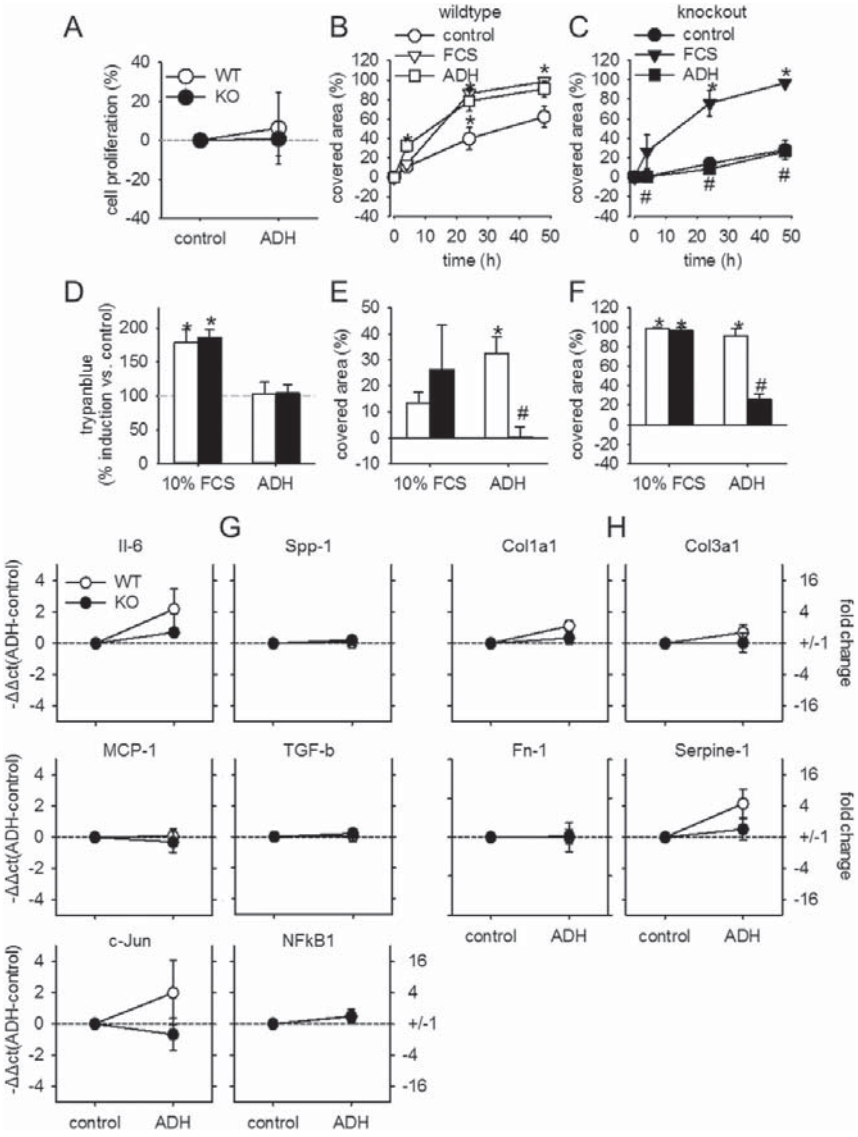

Fig. 4. Effect of ADH-transactivated (100 nM) EGFR on VSMC proliferation, transmigration, reconstitution and marker gene expression. A) Cells in a marked square millimeter were counted after 24 h starvation and were either further starved for 24 h or incubated with normal cell medium or starvation medium supplemented with ADH. $^{*}p < 0.05$ vs. respective control, $n = 4$–13 experiments/group. B, C) After 24 h starvation injury assay inlet was removed and a photograph was taken of the cell free area. Afterwards the cells were either starved for 48 more hours or incubated with starvation medium supplemented either with ADH. The cell-free area was repeatedly measured after 4, 24 and 48 h. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 8$–14 experiments/group. D) After seeding and 24 h starvation either starvation medium, growth medium or starvation medium supplemented with ADH was added to the lower compartment of the Boyden chamber. Medium in the upper compartment was changed every 4 h to maintain the gradient. After 24 h cells on the filter were removed, cells under the filter were stained with trypan blue and lysed. Migration is given as fold-change trypan blue staining compared to control cells. $^{*}p < 0.05$ vs. respective control, $n = 8$–18 wells/group. Reconstitution after 4 h (E) or 48 h (F) stimulation either with 10% FCS or ADH is depicted. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 8$–14 experiments/group. Marker gene expression for inflammation (G) and fibrosis (H) was evaluated by qRT-PCR. $^{*}p < 0.05$ vs. respective control, $^{\#}p < 0.05$ vs. respective wildtype cells, $n = 3$–5 cell preparations/group.

increased gap closure in wildtype cells as compared to control conditions after 48 h (Fig. 5B and F), while in KO cells gap closure after 48 h was virtually not affected by ET-1 (Fig. 5C and F). A significant difference in gap closure (i.e. collective migration) between wildtype and knockout cells treated with ET-1 exists at 4 h, 24 h and 48 h (Fig. 5B and C). There was no significant impact of ET-1 in the Boyden chamber assay compared between cells from the two different genotypes (Fig. 5D).

Regarding gene expression ET-1 had no impact on the expression of marker genes for inflammation, fibrosis or ROS homeostasis neither in

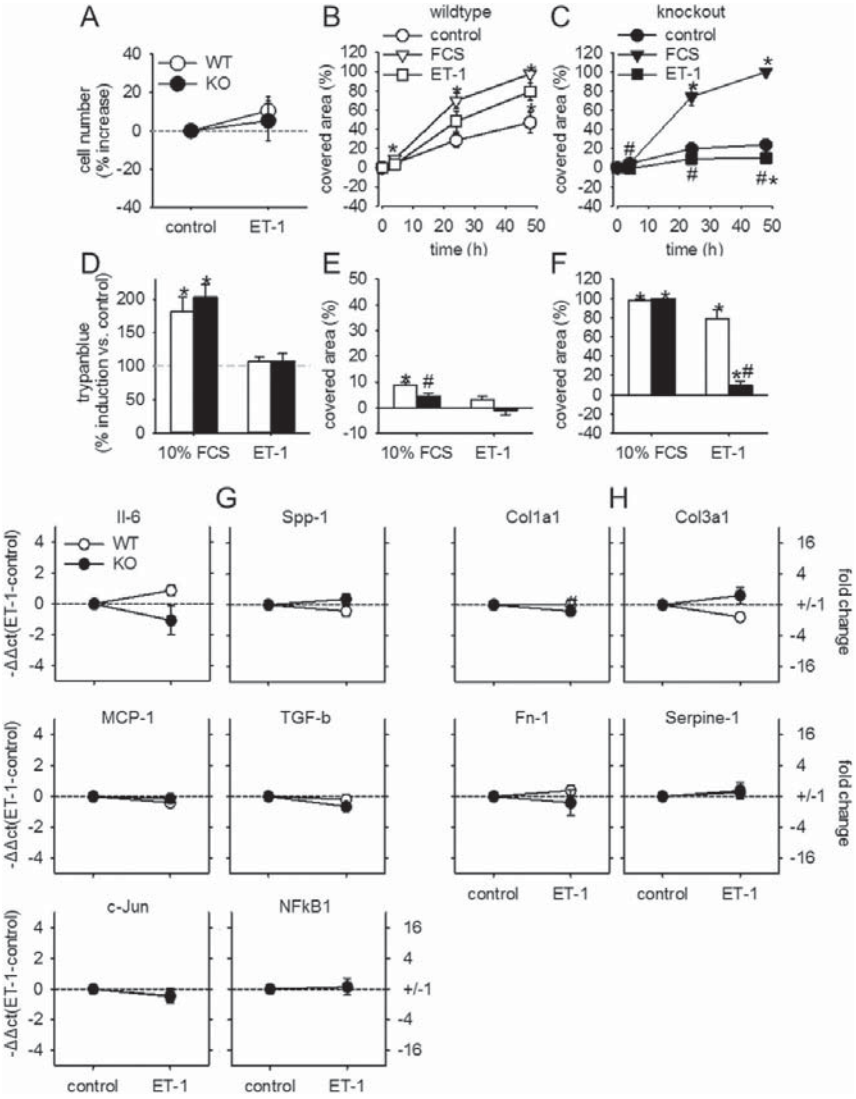

Fig. 5. Effect of endothelin-transactivated(100 nM) EGFR on VSMC proliferation, transmigration, reconstitution and marker gene expression. A) Cells in a marked square millimeter were counted after 24 h starvation and were either further starved for 24 h or incubated with normal cell medium or starvation medium supplemented with endothelin-1. $^{*}p < 0.05$ vs. respective control, $n = 5$–13 experiments/group. B, C) After 24 h starvation injury assay inlet was removed and a photograph was taken of the cell free area. Afterwards the cells were either starved for 48 more hours or incubated with starvation medium supplemented either with endothelin-1. The cell-free area was repeatedly measured after 4, 24 and 48 h. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 6$–10 experiments/group. D) After seeding and 24 h starvation either starvation medium, growth medium or starvation medium supplemented with endothelin-1 was added to the lower compartment of the Boyden chamber. Medium in the upper compartment was changed every 4 h to maintain the gradient. After 24 h cells on the filter were removed, cells under the filter were stained with trypan blue and lysed. Migration is given as fold-change trypan blue staining compared to control cells. $^{*}p < 0.05$ vs. respective control, $n = 8$–18 wells/group. Reconstitution after 4 h (E) or 48 h (F) stimulation either with 10% FCS or ET-1 is depicted. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 6$–10 experiments/group. Marker gene expression for inflammation (G) and fibrosis (H) was evaluated by qRT-PCR. $^{*}p < 0.05$ vs. respective control, $^{\#}p < 0.05$ vs. respective wildtype cells, $n = -20$ cell preparations/group.

wildtype nor in knockout cells under cell culture conditions (Fig. 5G and H, Supplementary Fig. S5). NOX-4 expression, as the only exception, was reduced in VSMC of both genotypes upon ET-1 stimulation.

In summary, EGFR seems to be important for ET-1-induced collective migration.

3.6. Effects of phenylephrine

Phenylephrine (1 μM) exerted no effect on proliferation, gap closure or migration in the Boyden chamber assay in wildtype or knockout cells (Fig. 6A–F, Supplementary Fig. S4).

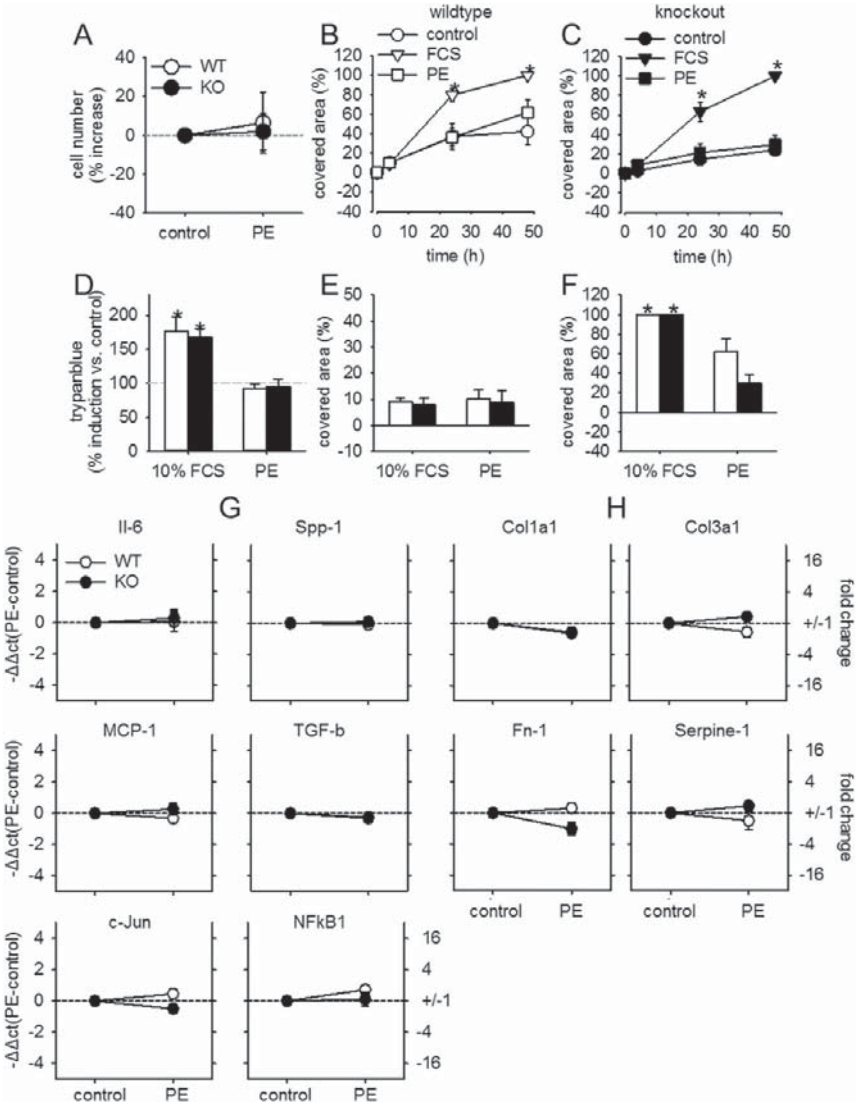

Fig. 6. Effect of phenylephrine-transactivated (1 μM) EGFR on VSMC proliferation, transmigration, reconstitution and marker gene expression. A) Cells in a marked square millimeter were counted after 24 h starvation and were either further starved for 24 h or incubated with normal cell medium or starvation medium supplemented with phenylephrine. $^{*}p < 0.05$ vs. respective control, $n = 6$–13 experiments/group. B, C) After 24 h starvation injury assay inlet was removed and a photograph was taken of the cell free area. Afterwards the cells were either starved for 48 more hours or incubated with starvation medium supplemented either with phenylephrine. The cell-free area was repeatedly measured after 4, 24 and 48 h. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 4$–7 experiments/group. D) After seeding and 24 h starvation either starvation medium, growth medium or starvation medium supplemented with phenylephrine was added to the lower compartment of the Boyden chamber. Medium in the upper compartment was changed every 4 h to maintain the gradient. After 24 h cells on the filter were removed, cells under the filter were stained with trypan blue and lysed. Migration is given as fold-change trypan blue staining compared to control cells. $^{*}p < 0.05$ vs. respective control, $n = 9$–18 wells/group. Reconstitution after 4 h (E) or 48 h (F) stimulation either with 10% FCS or PE is depicted. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 4$–7 experiments/group. Marker gene expression for inflammation (G) and fibrosis (H) was evaluated by qRT-PCR. $^{*}p < 0.05$ vs. respective control, $^{\#}p < 0.05$ vs. respective wildtype cells, $n = 4$–20 cell preparations/group.

Phenylephrine did not affect the expression of marker genes for inflammation (Fig. 6G), fibrosis (Fig. 6H) or ROS homeostasis (Supplementary Fig. S5).

In summary, phenylephrine does not affect VSMC phenotype in culture, although it activated its classical signaling pathways [33] under the same conditions.

3.7. Effects of ATP

Regarding proliferation, there was no significant impact of ATP (10 μM) of VSMC(Fig. 7A, Supplementary Fig. S4). This was also true for gap closure experiments and the Boyden chamber assay (Fig. 7B–F).

When wildtype cells were incubated with ATP the expression of c-Jun was reduced, while the expression of Col3a1 was increased (Fig. 7G and H, Supplementary Fig. S5). Both effects were not detectable in KO cells. No further alterations of ATP on the expression of marker genes could be observed.

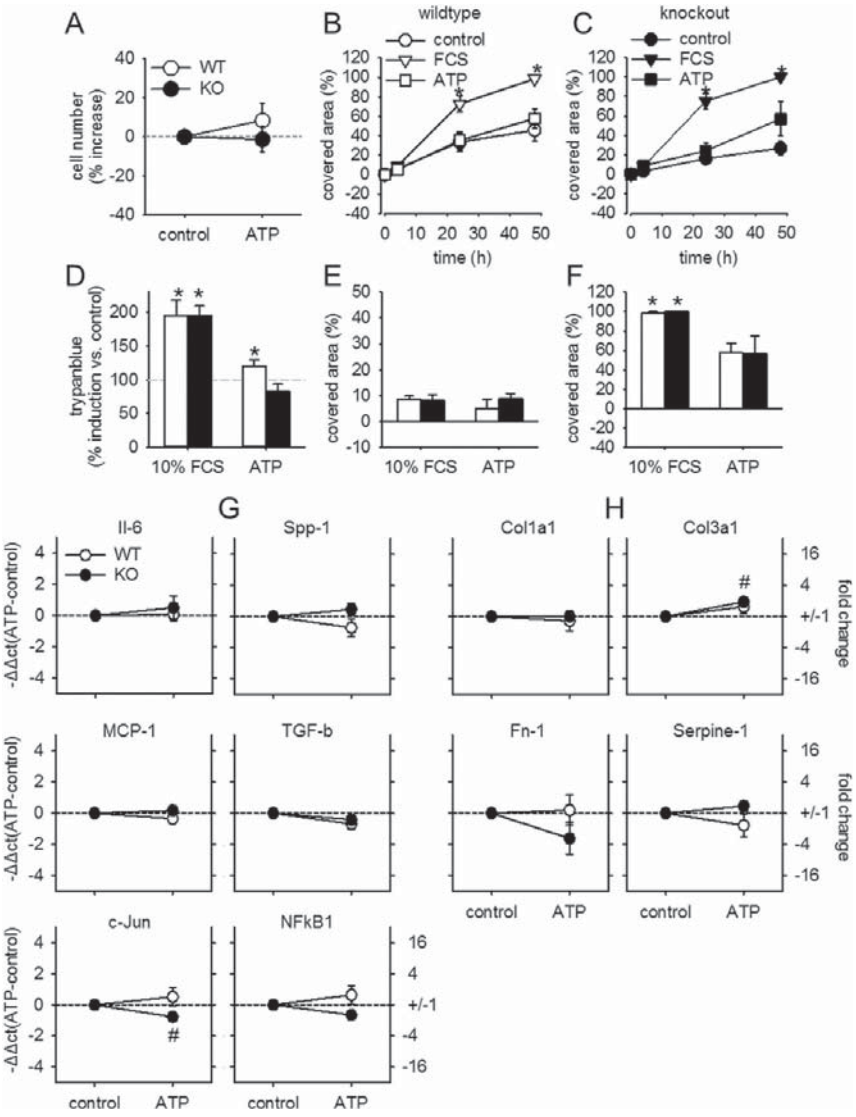

Fig. 7. Effect of ATP-transactivated (10 μM) EGFR on VSMC proliferation, transmigration, reconstitution and marker gene expression. A) Cells in a marked square millimeter were counted after 24 h starvation and were either further starved for 24 h or incubated with normal cell medium or starvation medium supplemented with ATP. $^{*}p < 0.05$ vs. respective control, $n = 6$–13 experiments/group. B, C) After 24 h starvation injury assay inlet was removed and a photograph was taken of the cell free area. Afterwards the cells were either starved for 48 more hours or incubated with starvation medium supplemented either with ATP. The cell-free area was repeatedly measured after 4, 24 and 48 h. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 7$–9 experiments/group. D) After seeding and 24 h starvation either starvation medium, growth medium or starvation medium supplemented with ATP was added to the lower compartment of the Boyden chamber. Medium in the upper compartment was changed every 4 h to maintain the gradient. After 24 h cells on the filter were removed, cells under the filter were stained with trypan blue and lysed. Migration is given as fold-change trypan blue staining compared to control cells. $^{*}p < 0.05$ vs. respective control, $n = 9$–18 wells/group. Reconstitution after 4 h (E) or 48 h (F) stimulation either with 10% FCS or ATP is depicted. Reconstitution is given as percentage of re-covered area at the given time point. $^{*}p < 0.05$ vs. respective control, $n = 7$–9 experiments/group. Marker gene expression for inflammation (G) and fibrosis (H) was evaluated by qRT-PCR. $^{*}p < 0.05$ vs. respective control, $^{\#}p < 0.05$ vs. respective wildtype cells, $n = 4$–20 cell preparations/group.

In summary, ATP does not affect VSMC phenotype in culture, although it activated its classical signaling pathways [33] under the same conditions.

4. Discussion

VSMC can switch between a contractile phenotype, considered quiescent, and phenotypes of increased proliferation, migration, synthetic and inflammatory capacity [37]. This ability to change the phenotype is considered to be a prerequisite for vascular remodeling processes involved in vascular development, repair and adaptation to chronically altered hemodynamics [38]. In the last years there have been several reports (reviewed in Refs. [39,40]) demonstrating the role of the EGFR for vasoactive substance mediated changes in VSMC function or phenotype with at least partially contradictory results. These studies have mainly been undertaken using pharmacological inhibition of the EGFR. Herein we present for the first time a study with VSMC from a genetic EGFR deletion model analyzing key features of VSMC phenotypic switch like VSMC proliferation, migration and gene expression upon a variety of pathophysiological relevant vasoactive stimuli in dependence of the EGFR.

4.1. EGFR and EGF

Proliferation and migration of VSMC are important processes involved in vascular remodeling and atherosclerotic lesion formation [7]. EGFR has been reported to have an impact on VSMC cell proliferation [33,41] and migration [42]. Mice with a deletion of the EGFR in VSMC show a dilated vascular phenotype with a reduced vascular wall thickness in aortas and reduced diastolic blood pressure [32]. To ensure that, although lacking the EGFR, basic features of proliferation and migration are unaltered, VSMC were incubated with FCS. FCS stimulated proliferation, crowd migration as well as single cell migration in both cell types to the same extent. We thereby conclude that the EGFR is not essential for VSMC proliferation, crowd or single cell migration. If the EGFR would be essential, FCS acting via a plethora of signaling molecules would not be able to restore the phenotype of EGFR deletion. Because EGFR acts as a signaling platform enabling the interaction of various molecules, it is of interest if the lack of EGFR *per se*, i.e. without exogenous addition of mediators, modulates phenotypic changes of VSMC.

As the VSMC were quiescent after 24 h in starvation medium the impact of EGFR deletion on proliferation *per se* could not be evaluated in these experiments. Deletion of the EGFR does not alter VSMC single cell migration substantially, as determined in the Boyden chamber chemotaxis and in single cell tracking experiments. In the single cells tracking experiments with starvation medium we observed that migration probability was reduced by 20% in the KO cells. This did not translate into a reduced gross migration velocity. Because V_{net} (i.e. velocity in the non-resting period) is in tendency lower in WT as compared to KO cells, the difference of gross velocity between the genotypes did not reach statistical significance. P_m or alterations in P_m possibly reflect the extend of tumbling periods, which in primordial germ cells serve the adjustment of cell polarization and migration direction [43]. To our knowledge we are the first to describe this impact of the EGFR on single cell behavior in VSMC.

By contrast to single cell migration, collective migration was reduced significantly in VSMC from KO animals. In the gap closure assay a mixture of migration, especially chemokinesis, and proliferation is measured [44]. In *Drosophila melanogaster* a uniformly activated EGFR inhibits migration of border cells [45,46]. By creating a gap, the cells at the edge would experience a lower concentration of EGFR ligands being shed from the cell surface in an auto- or paracrine fashion. This would abolish uniformity of EGFR activation and thereby inhibition of migration, too. For VSMC this would imply an increase in VSMC migration towards the "disturbed" vessel site upon injury either in settings of e.g. wounds, bypass surgery or balloon catheterization.

Under control conditions marker gene expression for inflammation, fibrosis and ROS homeostasis was not affected by EGFR deletion. Of course, further analysis on the possible impact of EGFR on other proinflammatory or fibrotic marker proteins or ROS generation should be performed in future studies, to determine also alterations in posttranscriptional gene regulation.

In conclusion, VSMC-EGFR in the absence of exogenous stimuli does not impact proliferation, chemotaxis or the expression of marker genes for fibrosis, inflammation or ROS homeostasis, as tested, but, possibly via its function as a signal integrator [40], it increases single cell and collective migration. This could lead to an increase in VSMC migration into the vascular intima if the EGFR abundance is increased, as suggested during aging. Of course, the data from cells in primary culture have to be confirmed in further *in vivo* experiments.

After analyzing the effect of EGFR *per se*, we investigated the effect of EGF-activated EGFR on VSMC. We tested the effect of EGF on VSMC proliferation, migration and gene expression. Our data show that the EGFR is responsible for EGF-induced effects in VSMC.

In this study we confirm that EGF-activated EGFR induces VSMC proliferation, single cell migration, chemotaxis as well as collective migration. A confounding effect by proliferation in the gap closure assays can be excluded under control conditions as well as during the first 4 h in the presence of EGF since there is no relevant proliferation under these conditions. These findings are in good agreement with data from other groups demonstrating that EGF increases gap closure and that the effect is abolished by EGFR inhibition with AG1478 [47,48]. Furthermore, it has been shown *in vivo* that blockage of the EGFR by AG1478 in carotid arteries reduces intimal thickening after balloon injury [8]. In contrast to these studies, we now demonstrate for the first time in a genetic model unequivocally the importance of EGFR for VSMC migration. By which downstream signaling mechanisms EGFR influences VSMC migration has to be evaluated. One possibility is the activation of the focal adhesion kinase (FAK) pathway. It has been demonstrated that EGF increases the turnover of focal adhesions and that EGFR, EBP50 and FAK build a signaling complex, whereby EGFR increases the phosphorylation of FAK at tyrosine-925, enhancing the ability of FAK to turnover focal adhesions [49], thereby increasing migration.

Upon addition of EGF, the mRNA expression of SPP-1 and MCP-1 increases in wildtype but not in knockout cells while the expression of TGF-β is reduced in knockout cells but not in wildtype. These alterations in gene expression could represent the switch to a more inflammatory phenotype, that is associated with ageing and atherosclerosis [50,51]. The underlying signaling cascade leading to the increase or respectively decrease of the proinflammatory cytokines by EGFR also needs to be evaluated. A possible mechanism under observation for the increase in gene expression is the impact of CCAAT/enhancer-binding proteins. These transcription factors are activated by the EGFR [52]. In mice with a heterozygous deletion of myocardin, presumably also involved in EGFR signaling cascade [26], these transcription factors are upregulated and responsible for enhanced cytokine expression in arteries upon high fat diet treatment [53]. Interestingly, the increase in TGF-β expression seems to be outbalanced by other signaling cascades induced by EGF, presumably ErbB2/3 or ErbB2/4 dimer activation. For a further insight into the underlying mechanisms an analysis of the levels of relevant proteins and possible their modifications is required.

In summary, EGF – via the EGFR – stimulates VSMC proliferation, influences net migration velocity, migration probability, directed movement and collective movement and supports a pro-inflammatory phenotype. These effects potentially support vascular remodeling, e.g. during atherosclerotic processes or ageing *in vivo*, and might be the explanation for the dilated vascular phenotype in the $EGFR^{\Delta/\Delta VSMC\&CM}$ mice [32].

4.2. EGFR and vasoactive substances

The EGFR can be transactivated by various vasoactive substances and thereby contribute in part their cellular effects. In VSMC the EGFR impacts different intracellular signaling cascades. ERK 1/2 phosphorylation upon PE, ET-1 or ATP stimulation is reduced if the EGFR is lacking [33]. In contrast intracellular Ca^{2+} release depends only for PE and ET-1 on EGFR [33]. A similar effect can be observed in cardiomyocytes. Phenylephrine and UTP both increase ERK 1/2 phosphorylation via the EGFR, but only PE increases the transcription of the atrial natriuretic peptide [26]. Therefore we conclude that ERK 1/2 activation is not sufficient to predict EGFR mediated effects upon substance application. In consequence, downstream effects of EGFR transactivation need to be evaluated for each substance separately. Consequently, we aimed to answer the following questions with this study: (i) Which effects of thrombin, endothelin-1, phenylephrine, ATP and vasopressin regarding VSMC migration and gene expression are dependent on EGFR? (ii) Do all these substances elicit the same effects via transactivation of the EGFR?

4.2.1. Thrombin

In our model EGFR expression is required for thrombin-induced proliferation and collective migration (reconstitution assay). The increase in gap closure may be in part the result of increased proliferation. The data on migration suggest that EGFR supports the wound healing action of thrombin in injured vessels. Because thrombin did not affect single cell migration in the Boyden chamber in wildtype cells a potential contribution to chemokinesis and chemotaxis could not be determined under these conditions.

Furthermore, thrombin stimulated the expression of marker genes for inflammation and fibrosis, namely IL-6, SPP-1, Col1a1 and Serpine 1 virtually independent of EGFR-expression. The effect on MCP-1-expression was only modest, but seems to require EGFR. This is in agreement with the results from EGF treated cells, where we also observe an increase in MCP-1 expression in wildtype but not in knockout cells. Surprisingly, thrombin slightly reduced the expression of NFkB1 and p22phox in knockout but not in wildtype cells. Probably thrombin acts on the expression of these two genes in a stimulatory EGFR-dependent and an inhibitory EGFR-independent pathway in parallel. In the absence of EGFR the inhibition is unmasked.

4.2.2. ADH

Besides its role in volume homeostasis, ADH functions as a growth factor inducing hypertrophy and growth of different cell types, e.g. VSMC[54] via the V_{1A} receptor ($V_{1A}R$) [55]. In many cardiovascular diseases the plasma concentration of ADH is elevated leading to the hypothesis that the mitogenic action of ADH may contribute to atherosclerosis [56]. Stimulation of the $V_{1A}R$ can transactivate the EGFR and thereby potentially exert a proliferative effect [15,57]. In contrast to some other reports [58,59], in our model ADH did not increase cell number, arguing against a net-proliferative action, although the classical intracellular signaling pathways (ERK1/2 and/or calcium) are activated in WT cells, confirming the ADH responsiveness (lack of ERK1/2-phosphorylation in KO cells shows that ADH action requires in part EGFR also in our model system). In most reports the mitogenic potential of ADH is assessed by [^{3}H]-thymidine incorporation or cell cycle analysis [58,59]. By contrast, in rat aortic smooth muscle cells it was shown that ADH induces cell hypertrophy but not hyperplasia, as assessed by [^{35}S]methionine incorporation and unchanged cell number upon ADH stimulation [60]. ADH stimulated collective migration in an EGFR-dependent manner but not single cell migration of VSMC. These results are in agreement with data from Chiu et al. [61] showing that ADH stimulates wound closure in intestinal epithelial cells, depending on the EGFR. With respect to VSMC it is known that a transient activation of ERK1/2 is sufficient to enhance cell motility whereas prolonged activation is required for proliferation [62]. Thus, the transient effects on ERK1/2 phosphorylation can explain the ability of ADH to enhance cell motility and its inability to enhance proliferation.

Interestingly, although having an impact on collective migration ADH does not influence the expression of marker genes for inflammation, fibrosis or ROS homeostasis, as chosen in this study. As we observed an effect of ADH on migration, ERK-phosphorylation and Ca^{2+}-homeostasis we exclude the possibility that the appropriate receptor is lacking, as we could detect $V_{1A}R$-mRNA via real-time qRT-PCR(data not shown).

4.2.3. Endothelin-1

The activation of ET_A-receptors has been implicated in VSMC proliferation, cell growth and adhesion [63,64]. We could demonstrate in our cell culture model that ET-1 increases ERK-1/2 phosphorylation in an EGFR dependent fashion [33].

In the present study there was no effect of ET-1 on VSMC proliferation, neither from wildtype nor from knockout animals. This is apparently different to observations in rat aortic vascular smooth muscle cells, where ET-1 induced cell proliferation and cell cycle progression in an ERK 1/2 dependent fashion [65], yet Jia et al. [65] supplemented the starvation media with 0.25% of FBS and therefore did not study the effect of plain ET-1, in contrast to our study. At present we cannot draw a final conclusion with regard to a potential role of EGFR for a potential costimulatory effect of ET-1 on proliferation in the presence of other growth factors. According to the literature endothelin-1 does not impact single cell migration or chemotaxis [66], but increases crowd migration [67], effects we confirm with this study, and to our knowledge we are the first to demonstrate that this effect depends on EGFR. The underlying mechanisms need to be evaluated further. Regarding marker gene expression endothelin-1 exerted only minor effects in the absence of costimulatory agents.

4.2.4. Phenylephrine

Phenylephrine is a selective α_1-adrenergic receptor agonist. It has been reported that α_1-adrenergig receptors stimulate proliferation [68], migration [69] and gene expression [70] of VSMC. These effects of α_1-adrenergic receptor agonists have been reported to be partially mediated by the EGFR and subsequent phosphorylation of mitogen-activated kinases [20,71,72]. In VSMC from mice with a deletion with the EGFR we could demonstrate that ERK 1/2 phosphorylation and intracellular Ca^{2+}-increase depends at least partially on the EGFR [33]. In this study we observed no alteration of VSMC proliferation or migration upon stimulation with phenylephrine and only minor alterations in marker gene expression. From the tested genes only the expression of c-Jun by PE seems to be EGFR dependent. To oppose norepinephrine oxidization some authors add ascorbic acid to the cell culture media [69]. In the presented analysis, the media was without supplementation of ascorbic acid or fetal calf serum, perhaps explaining the observed differences.

4.2.5. ATP

Seye et al. have shown that the upregulation of purinergic receptors in VSMC of arteries leads to an increase in neointima formation [73,74]. Other studies reported a stimulatory effect of ATP on VSMC migration and a chemotactic and chemokinetic effects at least by UTP [75]. The intracellular mechanism by which ATP stimulates the phenotypic alterations of VSMC is still under discussion. There are studies demonstrating that EGFR is necessary for ERK 1/2 phosphorylation upon ATP stimulation [33,76,77]. In this study we demonstrate that ATP does not influence VSMC proliferation and crowd migration, but does increase transmigration, thus chemotaxis. These results are partially different from findings reported in the literature. To evaluate if the effect depends on the dose [76] or the source of the VSMC [78] further studies are needed. The increase in transmigration is most probably due to an activation of P_2Y-receptors, which allow extracellular nucleotides to act as directional cues for VSMC [79].

Upon incubation with ATP only minor changes in gene expression occur. There is a reduction in c-Jun expression in VSMC from knockout animals, an effect that has to our knowledge not been reported until to date.

5. Conclusion

We investigated the functional contribution of vascular smooth muscle cell EGFR to phenotypic alterations. With this study we demonstrate for the first time, that (i) EGFR without additional activation contributes to VSMC phenotype, because in the absence of EGFR cells are more of a "quiescent" phenotype. (ii) EGF-activated EGFR promotes the proliferative, migratory and proinflammatory phenotype, thereby presumably contributing to vascular pathology and age related remodeling *in vivo*. Furthermore, (iii) the EGFR requirement for phenotypic alterations elicited by different vasoactive substances differs with respect to the substances tested and the pathological events. Modulation of the expression of marker genes for fibrosis and reactive oxygen generation is virtually independent of EGFR expression. Proinflammatory marker gene expression was affected by EGFR in some isolated cases. (iv) By contrast, cell migration was affected negatively by EGFR deletion for all efficacious substances (ADH, ET-1, thrombin, EGF). (v) Thus, EGFR seems to be more important for pathological cell movement than for gene expression regulation. The evaluation of the underlying mechanisms as well as the *in vivo* impact will be the scope of future studies. Additionally further species, e.g. human, and VSMC from different origin, e.g. mesenteric arteries, need to be tested.

Abbreviations

ADH	antidiuretic hormone, vasopressin
AG1478	EGFR inhibitor
ATP	adenosinetriphosphate
c-Jun	c-Jun-N-terminal kinase
Cre	causes recombination-recombinase
Col1a1	collagen, type I, α1
col3a1	collagen, type III, α1
EBP50	ezrin-radixin-moesin-binding phosphoprotein 50
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ErbB	avian erythroblastosis oncogene B
ERK 1/2	extracellular signal-regulated kinase 1/2
ET-1	endothelin-1
FCS	fetal calf serum
FAK	focal adhesion kinase
flox	flanked by loxP
Fn-1	fibronectin 1
GPCR	G-protein coupled receptor
h	hour
HRP	horseradish peroxidase
IL-6	interleukin-6
JNK	c-Jun N-terminal kinase
KO	knockout
MCP-1	monocyte chemoattractant protein 1
NFkB1	nuclear factor NF-κ-B p105 subunit
NOX-1	NADPH oxidase 1
NOX-4	NADPH oxidase 4
PBS	phosphate buffered saline
PE	phenylephrine
P_m	migration probability
p22phox	NADPH oxidase subunit *p22phox*
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
RT	reverse transcription
SEM	standard error of mean
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SM22	transgelin
SPP-1	secreted phosphoprotein 1
TGF-b	transforming growth factor β
UTP	uridintriphosphat
U/ml	units per milliliter
VSMC	vascular smooth muscle cells
V_{net}	net velocity
$V_{1A}R$	ADH receptor type 1 A
WT	wildtype
18S	18S ribosomal RNA

Competing interests

The authors declare that there are no competing interests.

Author contributions

B.S. and M.G. drafted and designed the study. S.R., S.M., D.B., and C.H. acquired data. B.S., G.S., D.B., C.H. and M.G. analyzed and interpreted the data. B.S. and M.G. drafted the article. M.G. was responsible for financial funding.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2016.03.017.

References

[1] H. Daub, C. Wallasch, A. Lankenau, A. Herrlich, A. Ullrich, Signal characteristics of G protein-transactivated EGF receptor, EMBO J. 16 (1997) 7032–7044.
[2] H. Daub, F.U. Weiss, C. Wallasch, A. Ullrich, Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors, Nature 379 (1996) 557–560.
[3] P.J. Little, GPCR responses in vascular smooth muscle can occur predominantly through dual transactivation of kinase receptors and not classical Galphaq protein signalling pathways, Life Sci. 92 (2013) 951–956.
[4] N. Prenzel, E. Zwick, H. Daub, M. Leserer, R. Abraham, C. Wallasch, A. Ullrich, EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF, Nature 402 (1999) 884–888.
[5] P.R. Nagareddy, F.L. Chow, L. Hao, X. Wang, T. Nishimura, K.M. Macleod, J.H. McNeill, C. Fernandez-Patron, Maintenance of adrenergic vascular tone by MMP transactivation of the EGFR requires PI3K and mitochondrial ATP synthesis, Cardiovasc. Res. (2009).
[6] P.R. Nagareddy, K.M. Macleod, J.H. McNeill, GPCR agonist-induced transactivation of the EGFR upregulates MLC II expression and promotes hypertension in insulin-resistant rats, Cardiovasc. Res. 87 (2010) 177–186.
[7] R. Ross, The pathogenesis of atherosclerosis: a perspective for the 1990s, Nature 362 (1993) 801–809.
[8] A.K. Chan, A. Kalmes, S. Hawkins, G. Daum, A.W. Clowes, Blockade of the epidermal growth factor receptor decreases intimal hyperplasia in balloon-injured rat carotid artery, J. Vasc. Surg. 37 (2003) 644–649.
[9] P.O. Hackel, E. Zwick, N. Prenzel, A. Ullrich, Epidermal growth factor receptors: critical mediators of multiple receptor pathways, Curr. Opin. Cell Biol. 11 (1999) 184–189.
[10] Y. Yarden, M.X. Sliwkowski, Untangling the ErbB signalling network, Nat. Rev. Mol. Cell Biol. 2 (2001) 127–137.
[11] T. Holbro, N.E. Hynes, ErbB receptors: directing key signaling networks throughout life, Annu. Rev. Pharmacol. Toxicol. 44 (2004) 195–217.
[12] C. Liebmann, EGF receptor activation by GPCRs: an universal pathway reveals different versions, Mol. Cell. Endocrinol. 331 (2011) 222–231.
[13] R. Wetzker, F.D. Bohmer, Transactivation joins multiple tracks to the ERK/MAPK cascade, Nat. Rev. Mol. Cell Biol. 4 (2003) 651–657.
[14] T. Du, D. Song, H. Li, B. Li, L. Cai, L. Hertz, L. Peng, Stimulation by vasopressin of ERK phosphorylation and vector-driven water flux in astrocytes is transactivation-dependent, Eur. J. Pharmacol. 587 (2008) 73–77.

[15] L.Q. Fuentes, C.E. Reyes, J.M. Sarmiento, C.I. Villanueva, C.D. Figueroa, J. Navarro, C.B. Gonzalez, Vasopressin up-regulates the expression of growth-related immediate-early genes via two distinct EGF receptor transactivation pathways, Cell. Signal. 20 (2008) 1642–1650.
[16] L. Hao, T. Nishimura, H. Wo, C. Fernandez-Patron, Vascular responses to alpha1-adrenergic receptors in small rat mesenteric arteries depend on mitochondrial reactive oxygen species, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 819–825.
[17] R.M. Touyz, Mitochondrial redox control of matrix metalloproteinase signaling in resistance arteries, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 685–688.
[18] C.E. Pullar, R.R. Isseroff, The beta 2-adrenergic receptor activates pro-migratory and pro-proliferative pathways in dermal fibroblasts via divergent mechanisms, J. Cell Sci. 119 (2006) 592–602.
[19] T. Noma, A. Lemaire, S.V. Naga Prasad, L. Barki-Harrington, D.G. Tilley, J. Chen, P. Le Corvoisier, J.D. Violin, H. Wei, R.J. Lefkowitz, H.A. Rockman, Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection, J. Clin. Invest. 117 (2007) 2445–2458.
[20] N. Ulu, H. Gurdal, S.W. Landheer, M. Duin, M.O. Guc, H. Buikema, R.H. Henning, alpha1-Adrenoceptor-mediated contraction of rat aorta is partly mediated via transactivation of the epidermal growth factor receptor, Br. J. Pharmacol. 161 (2010) 1301–1310.
[21] H.L. Hsieh, W.H. Tung, C.Y. Wu, H.H. Wang, C.C. Lin, T.S. Wang, C.M. Yang, Thrombin induces EGF receptor expression and cell proliferation via a PKC(delta)/c-Src-dependent pathway in vascular smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 1594–1601.
[22] H.L. Hsieh, C.C. Sun, T.S. Wang, C.M. Yang, PKC-delta/c-Src-mediated EGF receptor transactivation regulates thrombin-induced COX-2 expression and PGE(2) production in rat vascular smooth muscle cells, Biochim. Biophys. Acta 1783 (2008) 1563–1575.
[23] D. Chansel, M. Ciroldi, S. Vandermeersch, L.F. Jackson, A.M. Gomez, D. Henrion, D.C. Lee, T.M. Coffman, S. Richard, J.C. Dussaule, P.L. Tharaux, Heparin binding EGF is necessary for vasospastic response to endothelin, FASEB J. 20 (2006) 1936–1938.
[24] M. Flamant, S. Placier, C. Dubroca, B. Esposito, I. Lopes, C. Chatziantoniou, A. Tedgui, J.C. Dussaule, S. Lehoux, Role of matrix metalloproteinases in early hypertensive vascular remodeling, Hypertension 50 (2007) 212–218.
[25] C. Northcott, J.A. Florian, A. Dorrance, S.W. Watts, Arterial epidermal growth factor receptor expression in deoxycorticosterone acetate-salt hypertension, Hypertension 38 (2001) 1337–1341.
[26] J.B. Morris, T.M. Pham, B. Kenney, K.E. Sheppard, E.A. Woodcock, UTP transactivates epidermal growth factor receptors and promotes cardiomyocyte hypertrophy despite inhibiting transcription of the hypertrophic marker gene, atrial natriuretic peptide, J. Biol. Chem. 279 (2004) 8740–8746.
[27] M. Sibilia, R. Kroismayr, B.M. Lichtenberger, A. Natarajan, M. Hecking, M. Holcmann, The epidermal growth factor receptor: from development to tumorigenesis, Differentiation 75 (2007) 770–787.
[28] D. Meyer, C. Birchmeier, Multiple essential functions of neuregulin in development, Nature 378 (1995) 386–390.
[29] R.M. Touyz, M. Cruzado, F. Tabet, G. Yao, S. Salomon, E.L. Schiffrin, Redox-dependent MAP kinase signaling by Ang II in vascular smooth muscle cells: role of receptor tyrosine kinase transactivation, Can. J. Physiol. Pharmacol. 81 (2003) 159–167.
[30] K.J. Elliott, A.M. Bourne, T. Takayanagi, A. Takaguri, T. Kobayashi, K. Eguchi, S. Eguchi, ADAM17 silencing by adenovirus encoding miRNA-embedded siRNA revealed essential signal transduction by angiotensin II in vascular smooth muscle cells, J. Mol. Cell. Cardiol. (2013) 1–7.
[31] M. Sibilia, E.F. Wagner, Strain-dependent epithelial defects in mice lacking the EGF receptor, Science 269 (1995) 234–238.
[32] B. Schreier, S. Rabe, B. Schneider, M. Bretschneider, S. Rupp, S. Ruhs, J. Neumann, U. Rueckschloss, M. Sibilia, M. Gotthardt, C. Grossmann, M. Gekle, Loss of epidermal growth factor receptor in vascular smooth muscle cells and cardiomyocytes causes arterial hypotension and cardiac hypertrophy, Hypertension 61 (2013) 333–340.
[33] B. Schreier, M. Dohler, S. Rabe, B. Schneider, G. Schwerdt, S. Ruhs, M. Sibilia, M. Gotthardt, M. Gekle, C. Grossmann, Consequences of epidermal growth factor receptor (ErbB1) loss for vascular smooth muscle cells from mice with targeted deletion of ErbB1, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 1643–1653.
[34] J.L. Ray, R. Leach, J.M. Herbert, M. Benson, Isolation of vascular smooth muscle cells from a single murine aorta, Methods Cell Sci. 23 (2001) 185–188.
[35] M. Gekle, R. Freudinger, S. Mildenberger, S. Silbernagl, Aldosterone interaction with epidermal growth factor receptor signaling in MDCK cells, Am. J. Physiol. Ren. Physiol. 282 (2002) F669–F679.
[36] P. Friedl, D. Gilmour, Collective cell migration in morphogenesis, regeneration and cancer, Nat. Rev. Mol. Cell Biol. 10 (2009) 445–457.
[37] G.K. Owens, M.S. Kumar, B.R. Wamhoff, Molecular regulation of vascular smooth muscle cell differentiation in development and disease, Physiol. Rev. 84 (2004) 767–801.
[38] T. Althoff, S. Offermanns, G-protein-mediated signaling in vascular smooth muscle cells — implications for vascular disease, J. Mol. Med. 9 (2015) 1.
[39] N. Makki, K.W. Thiel, F.J. Miller Jr., The epidermal growth factor receptor and its ligands in cardiovascular disease, Int. J. Mol. Sci. 14 (2013) 20597–20613.
[40] B. Schreier, M. Gekle, C. Grossmann, Role of epidermal growth factor receptor in vascular structure and function, Curr. Opin. Nephrol. Hypertens. 23 (2014) 113–121.
[41] Y.A. Li, L.O. Levesque, M.B. Anand-Srivastava, Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR, Am. J. Physiol. Heart Circ. Physiol. 299 (2010) H1959–H1967.
[42] A.C. Dreux, D.J. Lamb, H. Modjtahedi, G.A. Ferns, The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis, Atherosclerosis 186 (2006) 38–53.
[43] S. Hashim, Y. Li, M.B. Anand-Srivastava, Small cytoplasmic domain peptides of natriuretic peptide receptor-C attenuate cell proliferation through gialpha protein/MAP kinase/PI3-kinase/AKT pathways, Am. J. Physiol. Heart Circ. Physiol. 291 (2006) H3144–H3153.
[44] F. Entschladen, T.L. Drell, K. Lang, K. Masur, D. Palm, P. Bastian, B. Niggemann, K.S. Zaenker, Analysis methods of human cell migration, Exp. Cell Res. 307 (2005) 418–426.
[45] A. Bianco, M. Poukkula, A. Cliffe, J. Mathieu, C.M. Luque, T.A. Fulga, P. Rorth, Two distinct modes of guidance signalling during collective migration of border cells, Nature 448 (2007) 362–365.
[46] P. Duchek, P. Rorth, Guidance of cell migration by EGF receptor signaling during drosophila oogenesis, Science 291 (2001) 131–133.
[47] S.M. Nichol, E. Roztocil, M.G. Davies, Urokinase-induced smooth muscle cell responses require distinct signaling pathways: a role for the epidermal growth factor receptor, J. Vasc. Surg. 41 (2005) 672–681.
[48] X.P. Yang, M.J. Zhu, N. Sreejayan, J. Ren, M. Du, Angiotensin II promotes smooth muscle cell proliferation and migration through release of heparin-binding epidermal growth factor and activation of EGF-receptor pathway, Mol. Cells 20 (2005) 263–270.
[49] G.J. Song, K.L. Leslie, S. Barrick, S. Bougoin, J.M. Taboas, A. Bisello, EBP50 promotes focal adhesion turnover and vascular smooth muscle cells migration, J. Mol. Cell. Cardiol. 53 (2012) 809–819.
[50] A.K. Uryga, M.R. Bennett, Ageing induced vascular smooth muscle cell senescence in atherosclerosis, J. Physiol. (2015) n/a.
[51] E.G. Lakatta, So! What's aging? Is cardiovascular aging a disease? J. Mol. Cell. Cardiol. 83 (2015) 1–13.
[52] D. He, V. Natarajan, R. Stern, I.A. Gorshkova, J. Solway, E.W. Spannhake, Y. Zhao, Lysophosphatidic acid-induced transactivation of epidermal growth factor receptor regulates cyclo-oxygenase-2 expression and prostaglandin E(2) release via C/EBPbeta in human bronchial epithelial cells, Biochem. J. 412 (2008) 153–162.
[53] M. Ackers-Johnson, A. Talasila, A.P. Sage, X.C. Long, I. Bot, N.W. Morrell, M.R. Bennett, J.M. Miano, S. Sinha, Myocardin regulates vascular smooth muscle cell inflammatory activation and disease, Arterioscler. Thromb. Vasc. Biol. 35 (2015) 817–828.
[54] C. Serradeillegal, B. Bourrie, D. Raufaste, P. Carayon, C. Garcia, J.P. Maffrand, G. Lefur, P. Casellas, Effect of a new, potent, nonpeptide V-1 A vasopressin antagonist, Sr 49059, on the binding and the mitogenic activity of vasopressin on Swiss 3 T3 cells, Biochem. Pharmacol. 47 (1994) 633–641.
[55] T.A. Koshimizu, K. Nakamura, N. Egashira, M. Hiroyama, H. Nonoguchi, A. Tanoue, Vasopressin V1A and V1B receptors: from molecules to physiological systems, Physiol. Rev. 92 (2012) 1813–1864.
[56] Z. Ye, Z. Ali, G.G. Klee, T.H. Mosley Jr., I.J. Kullo, Associations of candidate biomarkers of vascular disease with the ankle-brachial index and peripheral arterial disease, Am. J. Hypertens. 26 (2013) 495–502.
[57] P.M. Ghosh, M. Mikhailova, R. Bedolla, J.I. Kreisberg, Arginine vasopressin stimulates mesangial cell proliferation by activating the epidermal growth factor receptor, Am. J. Physiol. Ren. Physiol. 280 (2001) F972–F979.
[58] C. Serradeillegal, J.M. Herbert, C. Delisee, P. Schaeffer, D. Raufaste, C. Garcia, F. Dol, E. Marty, J.P. Maffrand, G. Lefur, Effect of Sr-49059, a vasopressin V-1 A antagonist, on human vascular smooth-muscle cells, Am. J. Physiol. Heart Circ. Physiol. 268 (1995) H404–H410.
[59] M. Reichman-Fried, S. Minina, E. Raz, Autonomous modes of behavior in primordial germ cell migration, Dev. Cell 6 (2004) 589–596.
[60] A.A.T. Geisterfer, G.K. Owens, Arginine vasopressin-induced hypertrophy of cultured rat aortic smooth-muscle cells, Hypertension 14 (1989) 413–420.
[61] T. Chiu, S.S. Wu, C. Santiskulvong, P. Tangkijvanich, H.F. Yee Jr., E. Rozengurt, Vasopressin-mediated mitogenic signaling in intestinal epithelial cells, Am. J. Physiol. Cell Physiol. 282 (2002) C434–C450.
[62] W.T. Gerthoffer, Mechanisms of vascular smooth muscle cell migration, Circ. Res. 100 (2007) 607–621.
[63] A. Bouallegue, G.B. Daou, A.K. Srivastava, Endothelin-1-induced signaling pathways in vascular smooth muscle cells, Curr. Vasc. Pharmacol. 5 (2007) 45–52.
[64] A. Bouallegue, G.B. Daou, A.K. Srivastava, Nitric oxide attenuates endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 in vascular smooth muscle cells by a cGMP-dependent pathway, Am. J. Physiol. Heart Circ. Physiol. 293 (2007) H2072–H2079.
[65] L. Jia, R. Wang, D.D. Tang, Abl regulates smooth muscle cell proliferation by modulating actin dynamics and ERK1/2 activation, Am. J. Physiol. Cell Physiol. 302 (2012) C1026–C1034.
[66] D.F. Meoli, R.J. White, Endothelin-1 induces pulmonary but not aortic smooth muscle cell migration by activating ERK1/2 MAP kinaseThis article is one of a selection of papers published in the two-part special issue entitled 20 years of endothelin research Can. J. Physiol. Pharmacol. 88 (2010) 830–839.
[67] V.A. Rufanova, A. Alexanian, T. Ostendorf, D. Bokemeyer, S. Prosser, B. Miller, A. Sorokin, Endothelin signaling via guanine exchange factor C3G in renal glomerular mesangial cells, Can. J. Physiol. Pharmacol. 88 (2010) 808–816.
[68] Z.W. Hu, X.Y. Shi, R.Z. Lin, J. Chen, B.B. Hoffman, alpha1-adrenergic receptor stimulation of mitogenesis in human vascular smooth muscle cells: role of tyrosine protein kinases and calcium in activation of mitogen-activated protein kinase, J. Pharmacol. Exp. Ther. 290 (1999) 28–37.
[69] H. Zhang, C.S. Facemire, A.J. Banes, J.E. Faber, Different alpha-adrenoceptors mediate migration of vascular smooth muscle cells and adventitial fibroblasts in vitro, Am. J. Physiol. Heart Circ. Physiol. 282 (2002) H2364–H2370.
[70] M.T. Piascik, D.M. Perez, alpha1-Adrenergic receptors: new insights and directions, J. Pharmacol. Exp. Ther. 298 (2001) 403–410.
[71] N. Ulu, R.H. Henning, S. Guner, T. Zoto, B. Duman-Dalkilic, M. Duin, H. Gurdal, Intracellular transactivation of epidermal growth factor receptor by α1A-adrenoceptor Is mediated by phosphatidylinositol 3-kinase independently of activation of extracellular signal regulated kinases 1/2 and serine–threonine kinases in Chinese hamster ovary cells, J. Pharmacol. Exp. Ther. 347 (2013) 47–56.

[72] H. Zhang, D. Chalothorn, L.F. Jackson, D.C. Lee, J.E. Faber, Transactivation of epidermal growth factor receptor mediates catecholamine-induced growth of vascular smooth muscle, Circ. Res. 95 (2004) 989–997.
[73] C.I. Seye, Q. Kong, L. Erb, R.C. Garrad, B. Krugh, M. Wang, J.T. Turner, M. Sturek, F.A. Gonzalez, G.A. Weisman, Functional P2Y2 nucleotide receptors mediate uridine 5′-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries, Circulation 106 (2002) 2720–2726.
[74] C.I. Seye, N. Yu, R. Jain, Q. Kong, T. Minor, J. Newton, L. Erb, F.A. Gonzalez, G.A. Weisman, The P2Y2 nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells, J. Biol. Chem. 278 (2003) 24960–24965.
[75] H. Chaulet, C. Desgranges, M.A. Renault, F. Dupuch, G. Ezan, F. Peiretti, G. Loirand, P. Pacaud, A.P. Gadeau, Extracellular nucleotides induce arterial smooth muscle cell migration via osteopontin, Circ. Res. 89 (2001) 772–778.
[76] C.C. Lin, W.N. Lin, S.E. Cheng, W.H. Tung, H.H. Wang, C.M. Yang, Transactivation of EGFR/PI3K/Akt involved in ATP-induced inflammatory protein expression and cell motility, J. Cell. Physiol. 227 (2012) 1628–1638.
[77] R. Ginnan, P.J. Pfleiderer, K. Pumiglia, H.A. Singer, PKC-delta and CaMKII-delta 2 mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle, Am. J. Physiol. Cell Physiol. 286 (2004) C1281–C1289.
[78] D. Erlinge, H. Yoo, L. Edvinsson, D.J. Reis, C. Wahlestedt, Mitogenic effects of ATP on vascular smooth muscle cells vs. other growth factors and sympathetic cotransmitters, Am. J. Physiol. Heart Circ. Physiol. 265 (1993) H1089–H1097.
[79] N. Yu, L. Erb, R. Shivaji, G.A. Weisman, C.I. Seye, Binding of the P2Y2 nucleotide receptor to filamin A regulates migration of vascular smooth muscle cells, Circ. Res. 102 (2008) 581–588.

7. Thesen

1. Der epidermale Wachstumsfaktorrezeptor (EGFR) wird in allen Zellen des kardiovaskulären Systems exprimiert.

2. Der EGFR in Kardiomyozyten verhindert das exzessive Wachstum des Herzens, vermutlich durch Beeinflussung der ROS-Homöostase.

3. Wird der EGFR embryonal in Kardiomyozyten deletiert entwickelt sich eine Herzhypertrophie mit erhaltener fraktioneller Verkürzung und ohne signifikante Zunahme der Fibrose.

4. Durch embryonale Deletion des EGFR in Kardiomyozyten oder glatten Gefäßmuskelzellen (VSMC) konnte keine Veränderung der Morphologie der Herzklappen beobachtet werden.

5. Der EGFR ist beteiligt an der Aufrechterhaltung der Blutgefäßwandhomöostase und des basalen Blutdruckes.

6. Deletion des EGFR in VSMC führt nicht zu einer generellen Störung der pharmakomechanischen oder elektromechanischen Kopplung. Auch die Vasodilatation ist durch die Deletion des EGFR in VSMC nicht gestört.

7. Der EGFR in glatten Gefäßmuskelzellen vermittelt einen Teil der vasokonstriktorischen Effekte von Angiotensin II aber scheinbar nicht die von Endothelin-1.

8. Der EGFR beeinflusst die akute Wirkung von Angiotensin II durch eine Reduzierung der homologen Desensitivierung des Angiotensin II-Rezeptors.

9. Durch seinen Beitrag zur chronischen Blutdruckbelastung des Organismus, fördert der EGFR alterns-abhängige Umbauprozesse des Herz-Kreislaufsystems.

10. Der EGFR vermittelt einen Teil der schädlichen Wirkung von inadäquat hohen Plasmaaldosteronkonzentrationen auf das Herz von männlichen Mäusen.

11. Der EGFR selber ist notwendig für die Aufrechterhaltung des kontraktilen Phänotyps von VSMC.

12. Aktivierung des EGFR durch seinen Liganden EGF fördert die Proliferation, Einzelzellmigration, Migration im Zellverbund und die Expression von proinflammatorischen Genen.

13. Der EGFR in VSMC ist wichtig für die ERK 1/2 Aktivierung durch Endothelin-1, Phenylephrin, ATP, Thrombin, Antidiuretisches Hormon und Wasserstoffperoxid. Die intrazelluläre Ca^{2+}-Freisetzung bei Stimulation mit den unterschiedlichen Substanzen wird unterschiedlich durch den EGFR beeinflusst.

14. Die Effekte der Transaktivierung des EGFR hängen von der transaktivierenden Substanz ab. In der Summe scheint der EGFR durch die Transaktivierung jedoch hauptsächlich die Migration im Zellverbund zu fördern und weniger die Expression von Markergenen für Fibrose und Entzündung.

Anhang

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die hier vorliegende Habilitationsschrift selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Ein Habilitationsverfahren wurde an keiner anderen Universität eröffnet oder beantragt.

Frühere Habilitationsversuche sind nicht unternommen worden.

Halle (Saale), den 14.05.2017

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