Aus der Medizinischen Klinik mit Schwerpunkt Nephrologie und internistische Intensivmedizin der Medizinischen Fakultät der Charité–Universitätsmedizin Berlin

DISSERTATION

Structural and functional basis of Endothelin-1 type A receptor (ET_AR) activation

zur Erlangung des akademischen Grades
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ABBREVIATIONS

Ala	Alanine
AR	Adrenergic receptor
ASK1	Apoptosis-signal regulation kinase 1
Asn	Asparagine
AT_1R	Angiotensin II type 1 receptor
AT_1R-IgG	IgG targeting AT_1R
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CAMs	Constitutively active mutants
CCL 18	Chemokine ligand 18
cDNA	Complementary DNA
Conc.	concentration
Cys	Cysteine
DNA	Deoxyribonucleic acid
ECE	Endothelin-converting enzyme
ECL	Extracellular loop
ERK1/2	Extracellular signal-regulated kinases 1 and 2
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_AR	Endothelin-1 type A receptor
ET_AR-IgG	IgG targeting ET_AR
ET_BR	Endothelin-1 type B receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
Gln	Glutamine
Gly	Glycine
GPCR	G-protein coupled receptor
His	Histidine
HMEC	Human microvascular endothelial cell
IgG	Immunoglobulin class G

IL-8	Interleukin 8
JNK	c-Jun kinase
kD	Kilodalton
Lys	Lysine
M	Molar
MAPK	Mitogen-activated protein kinase
MEKK1	MAPK kinase kinase 1
mL	Milliliter
mRNA	Messenger RNA
NFAT	Nuclear factor of activated T-cells
nm	Nanometer
PAH	Pulmonary arterial hypertension
PBS	Dulbecco's phosphate buffered saline
PCR	Polymerase chain reaction
Phe	Phenylalanine
PKC	Protein kinase C
qRT-PCR	Quantitative Real Time-PCR
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RT	Reverse transcription
SD	Standard deviation
SEM	Standard error of the mean
SRF	Serum response factor
SSc	Systemic sclerosis
ssDNA	Single-strand DNA
TGF-β	Transforming growth factor beta
TM	Transmembrane
Trp	Tryptophan
TSH	Thyrotropin, thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
Tyr	Tyrosine
V	Voltage
WT	Wild type

α_{1a}AR	Alpha-1A adrenergic receptor
β_1AR	Beta-1 adrenergic receptor
β_2AR	Beta-2 adrenergic receptor
μg	Microgram
μL	Microliter

Abstract

Endothelin-1 type A (ET_AR) and B receptors (ET_BR) belong to the GPCR subfamily A and mediate the actions of their peptide agonist Endothelin-1 (ET-1). ET-1 is one of the most potent vasoconstrictors in the human organism and regulates blood pressure and local and systemic homeostasis. ET_AR can be activated not only by ET-1 but also by agonistic antibodies. As recently demonstrated, extracellular binding of agonistic autoantibodies targeting ET_AR (ET_AR-IgG) promotes downstream signaling through allosteric activation of the receptor, independent of ET-1. The resultant signaling induced severe renovascular disease, exemplified on systemic sclerosis related renal crisis (SSc). Despite their clinical relevance, the potential differences and similarities to natural ligand-mediated activation have not been studied yet.

To investigate relevant extracellular loops required for ET-1 and ET_AR-IgG binding and downstream signaling responses, each of the three extracellular loops (ECL) was individually mutated. Functional effects were assessed with two different assays. The first GPCR activation assay relied on the MMY yeast model, in which a single human GPCR controls yeast growth to assess the effects of the mutations on ET_AR-IgG-mediated ET_AR activation. In the second assay, luciferase reporter plasmids enabled monitoring of G-proteins binding to ET_AR in response to ET-1 and ET_AR-IgG.

Change of ECL3 structure resulted in the constitutive activation of ET_AR both in MMY yeast model and in luciferase reporter assays. Besides, mutating ECL1 to Alanine or replacing ECL2 demonstrated no effect on ET_AR-IgG -induced ET_AR activation in yeast GPCR activation assay. In addition, these mutations did not influence ET_AR-IgG -mediated $G_{q/11}$ and $G_{12/13}$ binding to ET_AR as revealed by luciferase reporter assays. On the contrary, results showed that intact structure of ECL1 and ECL2 is necessary for $G_{12/13}$ activation upon ET-1 stimulation.

This work demonstrates that unlike similar antibodies against other GPCRs, antibodies directed against Endothelin-1 type A receptor do not bind to the extracellular loops of the receptor; thereby eliciting intracellular signaling differences from the natural ligand. Elucidation of these epitopes

is a prerequisite for rational design of new potent and more precise pharmacological compounds to replace old, relatively inefficient ones.

Abstrakt

Endothelin-1 Typ A (ET_AR) und B Rezeptoren (ET_BR) gehören zur Unterfamilie A der GPCR und vermitteln die Signale ihres Peptidagonisten Endothelin-1 (ET-1). ET-1 ist einer der stärksten Vasokonstriktoren im menschlichen Organismus und reguliert Blutdruck und lokale und systemische Homöostase. ET_AR können nicht nur durch ET-1 aktiviert werden, sondern auch durch agonistische Antikörper. Wie vor kurzem gezeigt, induziert die extrazelluläre Bindung agonistischer Autoantikörper am ET_AR (ET_AR-IgG) downstream Signaling durch allosterische Rezeptoraktivierung unabhängig von ET-1. Dadurch können schwere renovaskuläre Erkrankungen ausgelöst werden, wie sich am Beispiel der renalen Krise bei Systemischer Sklerose (SSc) zeigt. Trotz ihrer klinischen Bedeutung, wurden die möglichen Unterschiede und Ähnlichkeiten zur Rezeptoraktivierung durch den natürlichen Liganden bisher noch nicht erforscht. Um die für die Bindung von ET-1 und der ET_AR-IgG entscheidenden extrazellulären Schleifen und intrazelluläre Signalantworten untersuchen zu können, wurden jede der drei extrazellulären Schleifen (ECL) einzeln mutiert. Funktionelle Effekte wurden mit zwei verschiedenen Assays überprüft. Der erste GPCR Aktivierungsassay beruhte auf einem MMY Hefemodell, bei dem ein einzelner humaner GPCR das Hefewachstum bestimmt. Beim zweiten Assay ermöglichte ein Luziferase-Reporterplasmidassay, die Bindung von G-Proteinen an den ET_AR nach Stimulation mit ET-1 und ET_AR-IgG zu monitoren.

Veränderungen an der Struktur der 3. ECL führten zur konstitutiven Aktivierung des ET_AR, sowohl im MMY Hefemodell, als auch im Luziferase-Reporterassay. Dagegen blieben die Mutation zu Alanin an der 1. ECL oder der Ersatz der 2. ECL ohne Wirkung auf die ET_AR-IgG -induzierte ET_AR Aktivierung im Hefe GPCR Aktivierungsassay. Darüberhinaus beeinflussten diese Mutation die ET_AR-IgG vermittelte Bindung von $G_{q/11}$ und $G_{12/13}$ an den ET_AR nicht, wie in Luziferase-Reporterassays gezeigt wurde. Im Gegensatz dazu zeigten die Ergebnisse, dass die intakten Strukturen der 1. ECL und 2. ECL für die $G_{12/13}$ Aktivierung durch ET-1 notwendig sind. Diese Arbeiten zeigen, dass anders als bei ähnlichen GPCR-Antikörpern, die gegen den Endothelin-1 type A Rezeptor gerichtete Antiköper nicht an die extrazellulären Rezeptorschleifen

binden. Dadurch ergeben sich Unterschiede zum intrazellulären Signaling des natürlichen Liganden. Die Aufklärung der Bindungsepitope ist die Voraussetzung für die Entwicklung neuer, präziser und wirksamerer Medikamente, um die alten, relativ ineffizienten Arzneien zu ersetzen.

1 Introduction

1.1 Endothelin-1 receptors

G-protein-coupled receptors (GPCR), also known as 7-transmembrane receptors (7-TM), comprise the largest receptor family and the most important drug targets, since more than 40% of currently used drugs are GPCR modulators. More than 800 human GPCRs are clustered into five different classes: rhodopsin, secretin, adhesion, glutamate and frizzled / taste2 [1]. GPCRs transduce signals elicited by their endogenous ligands, which range from vasoactive peptides, neurotransmitters, chemokines, hormones and fatty acids to physical stimuli such as light [2]. Endothelin receptors, Endothelin-1 type A (ET_AR) and type B (ET_BR) receptors, belong to the class A GPCRs and interact in a similar manner with their natural peptide agonist ET-1, but yield different signals with a sequence similarity of 63% [3]. The localization of the two receptors is different. ET_AR is localized in vascular smooth muscle, heart, lung and kidney, whereas ET_BR can be found in more tissues, mainly in the brain, kidney, liver and lung [4]. ET receptors are susceptible to various factors and can be up- or downregulated. Hypoxia, cAMP, epidermal growth factor upregulate the ET_AR production; Endothelins, Angiotensin II, platelet derived growth factor and TGF-β downregulate ET_AR expression [5].

1.1.1 Endothelin-1 type A receptor

The gene coding for the human ET_A receptor (*EDNRA*) is located on chromosome 4, which contains eight exons and seven introns [6]. The molecular weight of ET_AR is about 44 kDa, consisting of 427 amino acids; its affinity for ET-1 is higher than for ET-2 and ET-3. Figure 1 shows the amino acid sequence and structure of ET_AR. Mutation of a cluster of five Cys residues into either serine or alanine in the cytoplasmic tail did not alter the binding but completely blocked palmitoylation of ET_AR, which led to a failure of ET-1 to increase the cytoplasmic calcium [7]. Deletion of either the entire extracellular N-terminus or the intracellular C-terminus completely inactivated ET-1 binding [8].

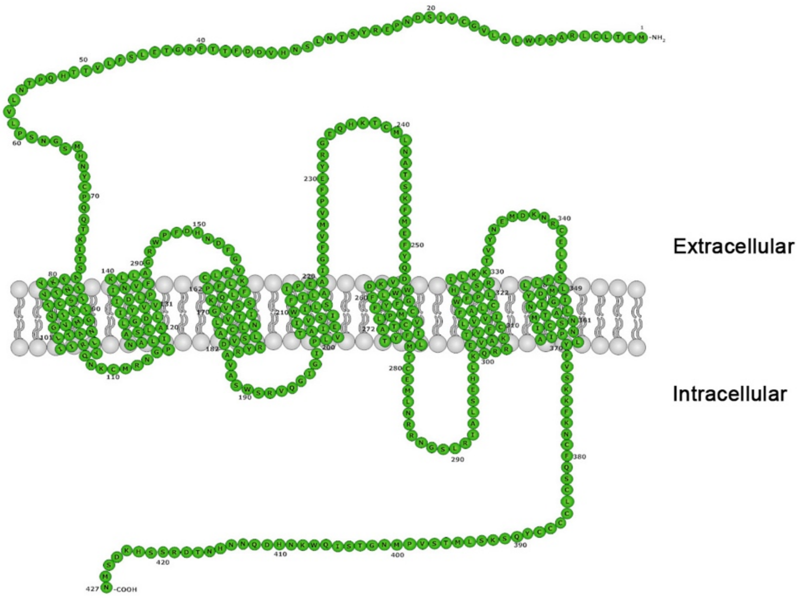

Figure 1. Schematic diagram of ET_AR.

Typical for a GPCR, the activation of ET_AR can initiate downstream physiological and pathophysiological processes. Human ET_AR can couple with $G_{q/11}$, G_s and $G_{12/13}$. ET_AR expressed at different levels in CHO cells provide evidence that the coupling of ET_AR to $G_{q/11}$ and $G_{12/13}$ differs depending on the expression level of the receptor, resulting in the activation of different signaling cascades [9]. The precise signaling pathways responsible for endothelin-induced vasoconstriction are still being actively studied. However, it is widely accepted that the activation of phospholipase C, production of inositol triphosphate and mobilization of calcium are the most relevant factors. Except for the effects on vascular tone, Endothelins also promote the growth and proliferation of vascular smooth muscle cells, which appears to be ET_AR-mediated, involving the activation of mitogen-activated protein (MAP) kinases and the transactivation of epidermal growth factor receptor [10].

In 2016, crystal structure and conformational changes of human Endothelin-1 type B Receptor (ET_BR) with and without its ligand ET-1 was reported [11]. However, the crystal structure of ET_AR remains unsolved. Due to the low expression levels and poor stability, crystallization and GPCRs structure determination studies are difficult to perform. Another way to study the structure of a GPCR is to change all amino acids of the GPCR individually or in a group. Amino acids are often replaced by Alanine, due to its high helix propensity and low probability of steric hindrance [12].

Such studies have been performed on ET_AR. Eighteen amino acids were mutated and analyzed for binding of Endothelin-1 and Bosentan, an inhibitor of both ET_A-and ET_BR. It has been observed that single point mutations differentially affect binding of agonist and antagonist [13]. In the N-terminus of ET_AR, two mutants containing D46N or R53Q substitutions showed dramatically reduced binding to ET-1, indicating that the substitutions of single amino acid alter the three-dimensional structure of the ligand-binding domain of the receptor [14]. Furthermore, it has been reported that Tyr129 is important for the peptide ligand affinity and selectivity of ET_AR [15].

1.1.2 Endothelin-1 type B receptor

The gene coding for the human ET_B receptor (*EDNRB*) is located on chromosome 13 and contains seven exons and six introns [16]. The molecular weight of ET_BR is 32 kDa and consists of 442 amino acids with same affinity for the three Endothelins. In 2016, Shihoya and co-workers reported the amino acid sequence, crystal structure and conformational changes of ET_BR in its ligand-free form and complexed with ET-1 [11]. When ET_BR interacts with ET-1, the N-terminus, the three extracellular loops and six transmembrane helices (2-7) are fully occupied. ET_BR has a fundamental role in renal, pulmonary, coronary, cerebral and systemic circulation. ET_BR in the endothelium mediates the release of vessel relaxants such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factors; it also plays an essential role in the clearance of ET-1 [17].

1.2 Endothelin-1, a natural peptide agonist of Endothelin-1 receptors

1.2.1 Synthesis of Endothelin-1

Endothelins are 21-amino acid peptides that are mainly produced in the endothelium and have a molecular weight of about 2.5 kDa. There are three isoforms (ET-1, ET-2 and ET-3), each with different gene and tissue distributions. In healthy individuals, there is a subtle balance between vasoconstriction and vasodilation, but when ET-1 is overexpressed, it can cause vascular diseases in various organ systems [18]. The gene coding for human ET-1 is located on chromosome 6 with a total length of 1246 base pairs. This gene product is a 212-amino acid preproET that is first converted intracellularly into an inactive pro-endothelin peptide, after the signal peptide has been removed, and then, in the case of ET-1, into the 38-amino acid long bigET. The bigET is then finally converted into the active form of the peptide and a C-terminal fragment by the endothelin-converting enzyme (ECE) [19] (Figure 2).

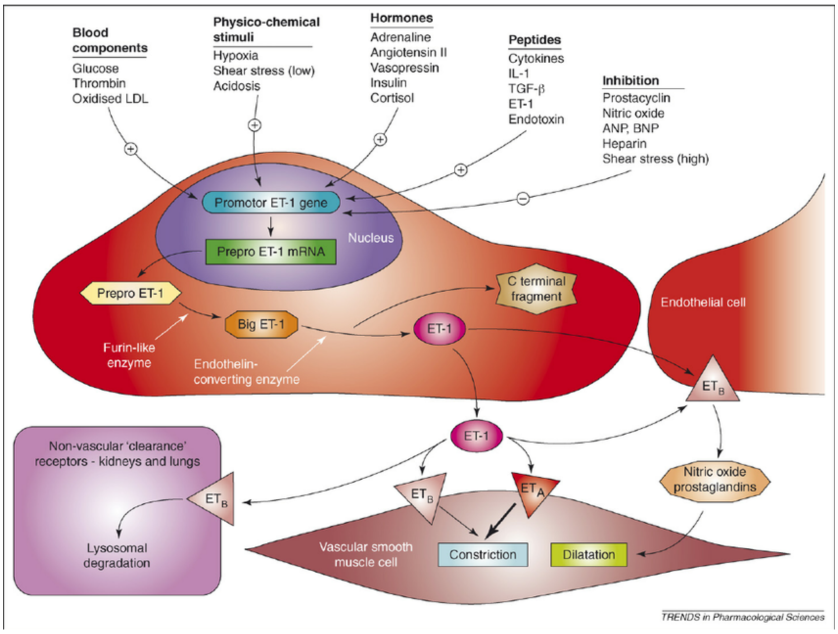

Figure 2. Pathways of endothelin-1 synthesis and sites of action [19]

Under normal physiological condition, the amount of circulating ET-1 is 0.26-5 ng/L [20], and ET-1 is eliminated from circulation with a half-life of one to seven minutes [21].

1.2.2 Physiological effects of Endothelin-1

ET-1 affects virtually every system that regulates blood pressure and local and systemic homeostasis. It is the strongest vasoconstrictor yet identified, at least 10-fold stronger than Angiotensin-II and 100-fold stronger than Norepinephrine [20]. The constrictor response caused by ET-1 can last for a considerable period, of several hours [22]. ET-1 plays a major role in the regulation of vascular function. Under physiological conditions, ET-1 induces sustained vasoconstriction of smooth muscle cells [23]. ET-1 maintains the basic vascular tone, modulates endothelin biosynthesis, and regulates water balance [24]. In the endothelin system, ET-1 regulates multiple renal functions like renal blood flow, glomerular filtration rate, salt and water excretion, acid/base treatment and extracellular matrix accumulation [25]. ET-1 increases the release of superoxide anion products and cytokines, which contribute to the development of the inflammation [26], and it has also been shown to be involved in immune functions [27].

1.2.3 Structure of Endothelin-1

ET-1 adopts a bicyclic structure with two disulfide bonds consisting of four cysteines (Cys^1-Cys^{15} and Cys^3-Cys^{11}) (Figure 3); it has six hydrophobic amino acids at its C-terminus called hydrophobic tail, both of which are important for ET-1 to maintain its activity. It has been shown that some residues at positions 18, 19 and 21 are crucial for ET-1 binding to the receptors. The deletion or substitution of Trp21 leads to the loss of receptor binding and activation [28]. An α-helical structure is formed between the central residues and the N-terminus, which is firmly attached to the α-helix through the disulfide bonds [3]. The N-terminus and α-helical regions of ET-1 have a fairly stable conformation, while the C-terminus is flexible and has an extended conformation [29].

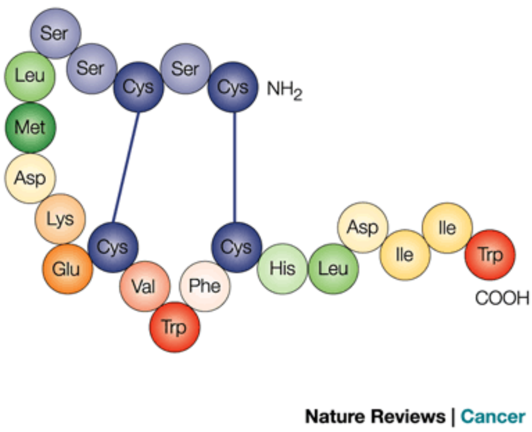

Figure 3. Amino-acid structure of ET-1 [30].

1.3 Autoantibodies targeting ET_AR

1.3.1 Autoantibodies targeting ET_AR in obliterative vasculopathies

ET_AR mediates the vasoconstrictive properties of ET-1 [31]. Functional autoantibodies have been involved in the pathogenesis of vascular lesions in renal and heart transplantation in the absence of donor specific-antibodies [32-34]. Banasik M *et al*. evaluated the presence of non-HLA antibodies (anti-Angiotensin II type 1 receptor (AT_1R) and/or anti-ET_AR) in 65 renal transplant patients. It was shown that 10.7% of the patients had a high level of non-HLA antibodies and graft loss in these patients was significantly higher. In another study conducted by the same group, the presence of ET_AR-IgG was evaluated in 116 renal transplant recipients. Anti-ET_AR antibodies were observed in 47.4% of the patients and the function of the renal transplant was significantly worse in these patients. Hiemann *et al.* tested the impact of antibodies targeting AT_1R and ET_AR in 30 heart transplant recipients. They demonstrated that elevated levels of antibodies against AT_1R and ET_AR were associated with cellular and antibody-mediated rejection. ET_AR-IgG have also been involved in the pathogenesis of systemic sclerosis (SSc) [35].

1.3.2 Autoantibodies targeting ET_AR in systemic sclerosis

1.3.2.1 Clinical manifestations

Systemic sclerosis (SSc), also known as Scleroderma, is a chronic autoimmune and vascular disease of systemic connective tissue. Pulmonary and renal involvements are the leading causes of death in patients with SSc [36]. About 5-10% of diffuse SSc patients present with internal organ lesions at some point during the course of the disease and patients with greater and rapid skin lesions have the highest risk of developing renal complications [37]. Although therapies can alleviate some of the symptoms, there are no specific medications or therapies to reverse or control the progression of the disease.

1.3.2.2 Pathogenesis and involvement of ET_AR-IgG

The difficulties in diagnosing and treating SSc are due to the complexity of the pathology of the disease, which involves interactions between the immune system, vascular system and connective tissue [38]. Extensive fibrosis, which is associated with diffuse small vessel vasculopathy, is unique and distinguishing in SSc [39]. Both vascular damage and activation of endothelial cells are prominent, and even occur before the fibrosis [40]. Microvasculature lesions involve mainly progressive thickening of the vessel wall, large gaps between endothelial cells, loss of vascular endothelium integrity, endometrial fibrosis, and hyperplasia, resulting in microthrombus formation leading to vascular occlusion and tissue hypoxia [41]. In addition, vascular repair and angiogenesis in SSc are found to be defective, leading to vasoconstriction and chronic clinical conditions in the affected area [42]. Several vasoactive peptides are involved in the pathogenesis of SSc, such as Endothelin-1[43]. Increased level of ET-1 has been reported in SSc patients [44] and elevated ET-1 levels are associated with renal failure [45]. Bosentan, an Endothelin-1 receptor blocker, can alleviate some of the vascular manifestations of SSc [46].

ET_AR-IgG were detected in most SSc patients with progressive vasculopathy; higher autoantibody levels were associated with more severe late complications and higher mortality [35]. This makes the detection and quantification of autoantibodies an important part of the diagnosis and treatment of SSc. ET_AR-IgG in SSc are not only associated with vascular complications, but may also be part of alloimmune and fibrotic complications that induce T cell migration and produce more interleukin 8 (IL-8) and chemokine ligand 18 (CCL18) [47]. *In vitro* ET_AR-IgG induced receptor-mediated ERK1/2 phosphorylation and increased TGF-β gene expression in HMECs [35]. ET_AR antibodies increase endothelial Ca^{2+} concentration and their action can be blocked *in vivo* by specific antagonist. In patient sera, anti-ET_AR IgG are more common in SSc-associated pulmonary hypertension than in other forms of pulmonary arterial hypertension (PAH) [48].

2 Hypothesis and Objectives

2.1 Hypothesis

Autoantibodies targeting the Endothelin-1 type A receptor (ET_AR) have been detected in systemic sclerosis (SSc) and contribute to the pathogenesis of the disease and its clinical severity. Both the receptor natural peptide agonist, Endothelin-1 (ET-1), and antibodies targeting ET_AR and isolated from SSc patients, ET_AR-IgG, activate ET_AR and trigger downstream intracellular signaling. However, binding sites and downstream intracellular signaling might differ substantially between patients´ ET_AR-IgG and natural peptide agonist. A thorough knowledge of the cellular mechanisms of antibody-mediated receptor activation is necessary to provide further insights for the development of therapeutic strategies to block specifically the action of the antibodies and to understand specific pathways. Hence, the study aim of this thesis was to test the hypothesis that ET_AR-IgG isolated from SSc patients bind differently to ET_AR as ET-1 with a binding occurring on the extracellular domains of the receptor and induce other cellular responses than the peptide agonist.

2.2 Objectives

1. To test the first hypothesis that ET_AR-IgG bind to extracellular domains of the receptor, different mutants of ET_AR disturbing the structure of one of the three extracellular loops had to be created. Thus, ECL1 and -3 were mutated to Alanine to suppress folding and charge. To study ECL2, a construct, where ECL2 of ET_AR has been replaced by Angiotensin II type 1 receptor (AT_1R) second extracellular loop, was already available in the laboratory [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy, http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000100230].

2. A GPCR activation assay already developed in the laboratory had to be optimized to study the binding of the antibodies to ET_AR and the receptor´s activation in presence or absence of the ECLs mutations.

3. To investigate ET_AR-IgG effects on downstream signaling in comparison to natural peptide agonist ET-1, human microvascular endothelial cells (HMEC-1) were chosen as a mammalian cell model and luciferase reporter assays were performed to determine specific G-protein involvements.

3 Materials and methods

3.1 Materials

3.1.1 Chemical substances

Chemicals and solutions	Manufacturer
1 kb DNA ladder	Thermo Fisher Scientific
2-Propanol	Carl Roth
3-amino-1,2,4-triazole (3-AT)	Sigma
5X Passive Lysis Buffer (PLB)	Promega
10X M-MuLV Reverse Transcriptase Buffer	New England BioLabs
10X Trypsin-EDTA	PAA
Agarose	Serva
Ampicillin	Alkom
Bacto Agar	BD
Bacto Peptone	BD
Bacto Trypton	BD
Bacto Yeast Extract	BD
Bis-Tris	AppliChem
Blasticidin S	InvivoGen
Dimethyl sulphoxide (DMSO)	Sigma Aldrich
dNTP mix	Thermo Fisher Scientific
Dulbecco's Modified Eagle's medium (DMEM) 4.5g/LGlucose	BioWest
DMEM 1.5 g/L Glucose	BioWest
Dulbecco's Phosphate Buffered Saline (PBS)	Biochrom

Chemicals and solutions	Manufacturer
Dextrose (D(+)-Glucose)	Applichem
Ethylenediamintetra-acetic acid (EDTA)	Carl Roth
Ethanol 99.8%	Carl Roth
Ethanol 96% (MEK, denaturated)	Herbeta Arzneimittel
FastStart Universal SYBR Green Master	Roche
Fetal calf serum (FCS)	Gibco
Fluorescein-D-glucopyranoside (FDGlu)	Invitrogen
Gelatin	Sigma
Glycerin	Carl Roth
hEGF	Sigma Aldrich
Hydrocortisone	Sigma Aldrich
L-Glutamine	PAA
L-arginine (HCl)	Applichem
L-aspartic acid	Applichem
L-glutamic acid (monosodium)	Applichem
L-lysine monohydrate	Applichem
L-methionine	Applichem
L-phenylalanine	Applichem
L-serine	Applichem
L-threonine	Applichem
L-tyrosine	Applichem
L-valine	Applichem
L-histidine	Applichem
Lithium acetate	Applichem
MCDB-131	c.c. pro GmbH

Chemicals and solutions	Manufacturer
Midori Green Advance DNA Stain	NIPPON Genetics
M-MuLV Reverse Transcriptase	New England BioLabs
NaCl Solution	Lonza
Oligo d(T) 16	Invitrogen
PEG 3350	Sigma Aldrich
Penicillin/Streptomycin (P/S)	PAA
RNase inhibitor	Thermo Fisher Scientific
SeaKem LE Agarose	Lonza
single-stranded DNA (ssDNA)	Sigma Aldrich
SOC Medium	Clontech
Sodium chloride	Carl Roth
Sodium hydroxide	Sigma Aldrich
Trypan blue	Sigma Aldrich
Tris	Carl Roth
Tris-HCl	Sigma Aldrich
Yeast nitrogen base without amino acids (AA)	Sigma Aldrich

3.1.2 Equipment

Equipment	Manufacturer
Applied Biosystems® 7500 Real-Time PCR System	Thermo Fisher Scientific
Axiovert 40 CFL Microscope	Carl Zeiss
Biofuge primo R	Thermo Fisher Scientific
Digital Heatblock II	VWR
FLUOstar OPTIMA Microplate Reader	BMG LABTECH

FRESCO 21 Centrifuge	Thermo Electron Corporation
HERA cell 240 Incubator	Thermo Electron Corporation
HERA safe Microbiological Safety Cabinet	Thermo Electron Corporation
Heraeus / BB 6220 CU O_2	Thermo Fisher Scientific
Incubator B28	BINDER
Incubating Orbital Shaker professional 3500	VWR
ND-1000 Spectrophotometer	VWR
Perfusor secura FT	Braun
Polymax 1040 T Platform Shaker	Heidolph
SUB Waterbath	Grant
T Professional BASIC XL 96 Thermocycler	Biometra
UV-transilluminator Gene Flash	SYNGENE

3.1.3 Kits

Kits	**Manufacturer**
Direct-zol™ RNA MiniPrep	Zymo Research
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific
HiTrap Protain G HP	GE Healthcare
In-Fusion ® HD Cloning Kit	Takara
Luciferase Assay System	Promega
NucleoBond®Xtra Midi/Maxi	Takara
NucleoBond®Xtra Midi/Maxi EF	Takara
PeqGOLD MicroSpin Cycle pure Kit	PEQLAB
Q5® Site-Directed Mutagenesis Kit	New England BioLabs
Xfect Transfection Reagent	Takara

3.1.4 Plasmid, bacteria, cell line, yeast strains and enzymes

Plasmid	**Manufacturer**
p426 GPD	GlaxoSmithKline
pcDNA3	Thermo Fisher Scientific
pGL4.30	Promega
pGL4.34	Promega

Bacteria	**Manufacturer**
NEB 5-alpha Competent *E.coli* Cells	New England BioLabs
Stellar competent Cells	Takara

Cell line	**Manufacturer**
Human Microvascular Endothelial Cells (HMEC-1)	Kindly given by Dr. H.D. Orzechowski

Yeast strains	**Manufacturer**
MMY 12	GlaxoSmithKline
MMY 14	GlaxoSmithKline

Enzymes and provided buffer	**Manufacturer**
*Hind*III-HF	New England BioLabs
*Xho*I	New England BioLabs
CutSmart Buffer	New England BioLabs

3.1.5 Agonist

Agonist	Manufacturer
Endothelin 1 human and porcine	Sigma Aldrich

3.1.6 Buffer recipes

Buffer	Reagent	Final conc.
3-AT	3-amino-1,2,4-triazole	1 M
	In H_2O	
50% PEG	PEG3350	50% m/v
	In H_2O	
	Filtered (0.45 µm)	
Ampicillin stock solution	Ampicillin powder	100 mg/mL
	In H_2O	
	Filter sterilized (0.22 µm)	
Binding buffer, pH 7.0	Na_2HPO_4	20 mM
	In H_2O	
	Filtered (0.45 µm)	
BU salts 10X, pH 7.0	$Na_2HP0_4 \cdot 7H2O$	7% m/v
	NaH_2PO_4	3% m/v
	In H_2O	
	Sterilized by autoclaving	
DNA sample loading buffer 6x	Glycerol	30% v/v
	Xylene cyanole	0.25% v/v
	Bromphenolblue	0.25% m/v

Buffer	Reagent	Final conc.
	In H_2O	
Elution buffer, pH 2.7	Glycin-HCl	0.1 M
	In H_2O	
	Filtered (0.45 µm)	
Gelatin stock solution	gelatin	2% m/v
	In PBS	
	Sterilized by autoclaving	
Histidine 100X	L-Histidine	2 mg/mL
	In H_2O	
	Filter sterilized (0.22 µm)	
LiAc	Lithium acetate	1 M
	In H_2O	
	Filter sterilized (0.22 µm)	
LiAc-TE	LiAc	0.1 M
	TE 10x	10%v/v
	In H_2O	
LiAc-PEG-TE	LiAc	0.1 M
	TE 10x	10%v/v
	50% PEG	80% v/v
Neutralization buffer, pH 9.0	Tris-HCl	1 M
	In H_2O	
	Filtered (0.45 µm)	
PBS (Ca^{2+}- /Mg^{2+} -free), pH 7.3	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	9 mM

Buffer	Reagent	Final conc.
	KH_2PO_4	2.3 mM
	In H_2O	
	Sterilized by autoclaving	
PLB 1X	PLB 5X	20%v/v
	In ddH_2O	
TBE buffer 1X, pH 8.0	Tris	89 mM
	Boric acid	89 mM
	EDTA	2 mM
	In H_2O	
TE buffer 10X	Tris-HCl, pH 7.5	0.1 M
	EDTA	0.01 M
	In H_2O	

3.1.7 Media

Bacteria media

Lysogeny broth (LB) medium	Conc.
Bacto tryptone	1% m/v
Bacto yeast extract	0.5% m/v
NaCl	1% m/v
	add Milli-Q water

Sterilize by autoclaving

LB agar	Conc.
Bacto tryptone	1% m/v
Bacto yeast extract	0.5% m/v

NaCl	1% m/v
Bacto agar	1.5% m/v
	add Milli-Q water

Sterilize by autoclaving

Cell culture media

Complete medium	**Conc.**
L-glutamine	10 mM
hEGF	10 ng/mL
Hydrocortisone	10 nM
FCS	5% v/v
Penicillin	100 U/mL
Streptomycin	100 µg/mL
	add MCDB-131

Starvation medium	**Conc.**
L-glutamine	10 mM
hEGF	10 ng/mL
Hydrocortisone	10 nM
FCS	0.5% v/v
Penicillin	100 U/mL
Streptomycin	100 µg/mL
	add MCDB-131

Yeast media

WHAUL powder	**Conc.**
L-arginine (HCl)	1.2 g

L-aspartic acid	6.0 g
L-glutamic acid (monosodium)	6.0 g
L-lysine	1.8 g
L-methionine	1.2 g
L-phenylalanine	3.0 g
L-serine	22.5 g
L-threonine	12 g
L-tyrosine	1.8 g
L-valine	9.0 g

40% Glucose	**Conc.**
Dextrose	40% m/v

Filtered (0.45 µm)

YNB 10X	**Conc.**
yeast nitrogen base without AA	6.7% m/v

Filter sterilized (0.22 µm)

WHAUL medium, pH 7.0	**Conc.**
WHAUL powder	1.1 g
	Milli-Q water 850 mL

Sterilize by autoclaving

Supplemented with

YNB 10X	10%v/v
40% glucose	5%v/v

WHAUL agar, pH 7.0	**Conc.**
WHAUL powder	1.1 g
Bacto agar	20 g
	Milli-Q water 850 mL
Sterilize by autoclaving	
Supplemented with	
Histidine 100X	10% v/v
YNB 10X	10% v/v
40% glucose	5% v/v

FDGlu assay medium, pH 7.0	**Conc.**
BU salts 10X	10%v/v
FDGlu	10 μM
3-AT	2 mM
	add WHAUL medium

YPD medium	**Conc.**
Bacto peptone	2% m/v
Bacto yeast extract	1% m/v
dextrose	2% m/v
	add Milli-Q water
Sterilize by autoclaving	

YPD agar	**Conc.**
Bacto peptone	2% m/v

Bacto yeast extract	1% m/v
dextrose	2% m/v
Bacto agar	2% m/v
	add Milli-Q water

Sterilize by autoclaving

3.1.8 Primers

Primers for mutagenesis experiments were designed with the NEBaseChanger^{TM} software online. All primers were provided by Biolegio (the Netherlands). Primers used to generate the constructs are listed in Table 1, primers for subcloning in Table 2, primers for sequencing in Table 3 and primers for quantitative Real Time-PCR (qRT-PCR) in Table 4.

Constructs name	**5'- to -3'**
ET_AR ECL1 Ala	F: tgccgctgccgctgccGTATTTCTTTGCAAGCTGTTC R: gcagcagccgcggccgcAGCCAGCAGCTTAAATAC
ET_AR ECL3 Ala	F: gcggccgcagctgcagcagctAGTTTCTTACTGCTCATGG R: ggccgctgcggcagccgcagcTTTCTTCAATATACGGCTTAAG
ET_AR with ECL2 of AT_1R	F: TACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTCCCGGATGTAAAGGACTGGTGGCTCTTCG R: ATATTGGTGTTCTCAATGAAAAATACATTTCGATGGATCGCTTCAGGAATGGCCAG

Table 1. Primers for constructs generation

Sub-cloning primer	**5'- to -3'**
ET_AR pcDNA3	F: AGGGAGACCCAAGCTTATGGAAACCCTTTGCCTCA R: TAGATGCATGCTCGAGTCAGTTCATGCTGTCCTTAT

Table 2. Primer for subcloning

Sequencing primers	**5'- to -3'**
p426GPD (from nucleotides 4025 to 4046)	Forward: TTGACCCACGCATGTATCTATC
pcDNA cmv (from nucleotides from 769 to 789)	Forward: CGCAAATGGGCGGTAGGCGTG
ET_AR (from nucleotides 446 to 465)	Forward: ATCACAATGACTTTGGCGTA
ET_AR (from nucleotides 715 to 734)	Forward: TGTATGCTCAATGCCACATC

Table 3. Primers for sequencing

Primers for qRT-PCR	**Sequence**
Human EDNRA se	gATAgCCAgTCTTgCCCTTg
Human EDNRA ase	CAgAggTTgAggACggTgAT
Yeast GAPDH se	AGACTGTTGACGGTCCATCC
Yeast GAPDH ase	CAACAGCGTCTTCGGTGTAA

Table 4. Primers for quantitative Real Time-PCR

3.2 Methods

3.2.1 Generation of constructs

3.2.1.1 Preparation of the linearized vector by restriction enzyme digestion

One µg of the plasmid was linearized using two appropriate restriction enzymes in the requested digestion buffer in a 50 µL reaction. Samples were incubated at 37°C for three hours and digestion was verified on an agarose gel.

3.2.1.2 Agarose gel electrophoresis

0.9% agarose gel was prepared in 1X TBE. 5µL of Midori Green was added to every 100mL of gel. The solid gel was placed into a gel electrophoresis apparatus filled with 1X TBE. Samples were mixed with 6X agarose gel loading buffer to 1X final. DNA marker (1 kb DNA ladder) was run together with the samples as a molecular weight indicator. Samples were migrated at 100 V for 45 minutes and examined under ultraviolet light at 302 nm using an UV-transilluminator.

3.2.1.3 Purification of digested vector

The digested plasmid was purified with a PeqGOLD MicroSpin Cycle-pure kit according to the manufacturer's instructions. After purification, the vector was free of enzymes and ready to be directly used for ligation. The concentration was measured using a NanoDrop® Spectrophotometer.

3.2.1.4 PCR amplification of target fragment

To subclone a PCR product into the digested plasmid, In-Fusion cloning kit was used according to the manufacturer's instructions. Amplification of the DNA target was performed with CloneAmp HiFi PCR Premix, which contains dNTPs and optimized buffer, allowing rapid set-up of PCR reactions. The PCR reaction components are listed in Table 5 and the reaction conditions are shown in Table 6. PCR primers were designed for the target gene with 5' 15-bp extensions that are homologous to the ends of the linearized vector (Table 2). The PCR product was verified on an agarose gel.

Reagent	**Volume/Quantity**	**Final conc.**
CloneAmp HiFi PCR Premix	12.5µL	1X
10 µM Forward Primer	5 pmol	0.2 µM
10 µM Reverse Primer	5 pmol	0.2 µM
Template	< 100 ng	20 ng
Sterilized distilled water	up to 25 µL	

Table 5. Master Mix components

Temperature	**Time**	**Amplification cycle**
98°C	10 sec	
60°C	15 sec	X 35 cycles
72°C	30 sec	

Table 6. Reaction conditions

3.2.1.5 Set up of In-Fusion cloning reaction

The In-Fusion HD Cloning kit enables fast, directional cloning of one fragment of DNA into any vector. The PCR product generated in the previous step was first treated with the Cloning Enhancer in order to be purified. The In-Fusion Enzyme was then used to fuse the DNA fragment and the linearized vectors effectively and precisely by recognizing 15-bp overlaps at their ends. The reaction components are listed in Table 7 and the procedures were performed according to manufacturer's instructions.

Component	**Volume**
5X In-Fusion HD Enzyme Premix	2 µL
Linearized Vector	1µL
Cloning Enhancer Treated PCR Insert	1µL
dH_2O	6 µL

Table 7. In-Fusion cloning reaction

3.2.1.6 Bacterial transformation

Foreign DNA can be introduced into bacteria by transformation; this enables plasmid amplification and an easy collection. By exposing competent cells to a 42°C heat shock, a pressure difference between the outside and the inside of the cell is created. This leads to the formation of pores in the membrane, through which supercoiled plasmid DNA can easily enter. Followed by incubation on ice, the cell wall is closed and the plasmids are kept inside. Stellar™ Competent Cells (Table 8) were used for the transformation. 2.5 µL of the cloning product was added to 50 µL of bacteria and the transformation steps were performed according to the manufacturer's instructions. The vectors employed contained an ampicillin resistant gene, conferring antibiotic resistance to all bacteria containing the plasmid, allowing them to grow on ampicillin plates (100 µg/mL). Plates were incubated at 37°C overnight.

Bacteria	Provider	Genotype
Stellar™Competent Cells	Takara	*F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZ Δ M15, Δ (lacZYA-argF) U169, Δ (mrr-hsdRMS-mcrBC), Δ mcrA, λ -*

Table 8. Bacteria genotype

3.2.1.7 Mini-prep

A pre-culture of LB medium containing 100 µg/mL ampicillin was inoculated with a single colony picked from the LB-Ampicillin agar plate containing the bacteria transformants. 3 mL of the pre-culture was centrifuged at room temperature. All steps were carried out according to the GeneJET Plasmid Miniprep Kit protocol. The nucleic acid concentration was measured by NanoDrop® Spectrophotometer. The plasmids were stored at -20°C.

3.2.1.8 Screening for positive plasmids

Plasmids isolated by mini-prep were digested by the two restriction enzymes used to linearize the vector in a 25 µL reaction. Samples were incubated at 37°C for three hours and then run on an agarose gel. Plasmids showing two bands corresponding to the molecular size of the vector and the insert were considered positive.

3.2.1.9 DNA sequencing and alignment

The plasmids potentially containing the insert were sequenced by the company LGC Genomics GmbH using appropriate primers (Table 3). The resulting sequences were analyzed by using the DNASTAR MegAlign 14 software.

3.2.1.10 Midi-prep

The NucleoBond® Xtra Midi/Maxi and Endotoxin Free (EF) kits were used to extract a large amount of plasmid DNA for mammalian cell and yeast experiments. After sequencing, one single positive colony was incubated in LB medium with 100 µg/mL ampicillin for eight hours. This pre-culture was then diluted 1 to 1000 into 100 mL LB medium containing 100 µg/mL ampicillin and incubated overnight at 37°C under shaking. The High-copy protocol was performed according to manufacturer's instructions. After elution and precipitation, plasmid DNA was dissolved in Tris buffer. The nucleic acid concentration was measured by NanoDrop® Spectrophotometer. The plasmids were stored at -20°C for further use.

3.2.1.11 Bacterial glycerol stock

Glycerol stocks enable long-term storage of bacteria. A pre-culture of LB medium containing 100 µg/mL ampicillin was inoculated with the colony to be preserved and shaken six hours at 37°C. 250 µL of the pre-culture was added to 750 µL of 60% glycerol and gently mixed. Then the glycerol stock was frozen at -80°C.

3.2.2 Generation of mutated constructs

3.2.2.1 Site-directed mutagenesis

Mutated constructs were generated using the Q5® Site-Directed Mutagenesis Kit according to the manufacturer's instructions. Briefly, exponential amplification of cDNA fragment encoding ET_AR was performed with Q5 Hot Start High-Fidelity DNA Polymerase along with specific primers designed using NEBaseChanger (Table 1). The forward primer was designed to incorporate the desired nucleotide change in its center, and also to include at least 10 complementary nucleotides on the 3' side of the mutation. The reverse primer was designed to anneal back to back at the 5' ends of the two primers. The experimental conditions of PCR are listed in Tables 9, 10 and 11. The PCR products were then treated with the Kinase-Ligase-*Dpnl* (KLD) enzyme, which enables a quick circularization and template removal at room temperature (Table 12). Transformation into high-efficiency NEB 5-alpha-Competent E. *coli* ensures high numbers of transformants on LB-Ampicillin plates. The transformation was performed using NEB 5-alpha-Competent *E.coli* (Table 13). The procedure was the same as described in 2.2.1.6.

Component	**25**µL RXN	**Final conc.**
Q5 Hot Start High-Fidelity 2X Master Mix	12.5µL	1X
10 µM Forward Primer	1.25µL	0.5 µM

Component	**25**μL RXN	**Final conc.**
10 μM Reverse Primer	1.25μL	0.5 μM
Template DNA	1μL	1-25 ng
Nuclease-free water	9.0μL	

Table 9. Reaction components

Construct	**DNA template**	**DNA quantity**
ET_AR ECL1 Ala	p426 GPD ET_AR wild type plasmid	10 ng
ET_AR ECL3 Ala	p426 GPD ET_AR wild type plasmid	10 ng
ET_AR with ECL2 of AT_1R	p426 GPD ET_AR wild type plasmid	10 ng

Table 10. Components of PCR reactions for the different constructs

Construct	**Second denaturation**	**Annealing**	**Elongation step**
ET_AR ECL1 Ala	98°C 10 s	67.6°C 30 s	72°C 6min
ET_AR ECL3 Ala	98°C 10 s	60°C 30 s	72°C 6min
ET_AR with ECL2 of AT_1R	98°C 10 s	70°C 30 s	72°C 4min

Table 11. PCR conditions for the different constructs

Components	**Volume**	**Final conc.**
PCR Product	1 μL	
2X KLD Reaction Buffer	5 μL	1X
10X KLD Enzyme Mix	1 μL	1X
Nuclease-free Water	3 μL	

Table 12. KLD treatment components

Bacteria	**Provider**	**Genotype**
NEB 5-alpha Compent *E.coli*	NEB	*fhuA2, Δ(argF-lacZ) U169, phoA, glnV44, Φ80 Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*

Table 13. Bacteria genotype

After transformation, clones were amplified and sequenced as described in 2.2.1.7 to 9. A midi-prep was performed to produce appropriate quantities of the plasmids as described in 2.2.1.10.

3.2.3 Patient IgG isolation

The isolation of immunoglobulins class G (IgG) was carried out from plasma of patients suffering from systemic sclerosis. These patients were tested positive for the presence of antibodies targeting ET_AR (ET_AR-IgG). Plasma was filtered through 0.45 µm filters before use. HiTrap Protein G HP columns packed with Protein G Sepharose™ High Performance, which can strongly bind to the Fc region of IgG were used for the isolation. After the plasma was diluted 1:2 with binding buffer run through the column, the ET_AR-IgG solution was eluted, neutralized and dialyzed against WHAUL medium or DMEM low glucose, depending on whether the stimulation was performed on yeasts or mammalian cells. After dialysis, aliquots of the IgG were sent to Labor Berlin and the concentrations were measured by ELISA.

3.2.4 GPCR activation assay in yeast

The yeast GPCR activation assay in yeast is well-established as an experimental system for the detection of receptor activation [49] [50] [51]. Mating in haploid yeast cells is stimulated by pheromone binding to GPCRs. The MMY yeast strains provided by the GlaxoSmithKline Company were modified to express chimeric G-proteins, in which the five C-terminal residues of Gpa1p were replaced by the corresponding sequence of the human G_α subunits (Table 14). Furthermore, these yeasts do not express the yeast GPCR anymore but depend on the expression of a human GPCR, able to bind to the yeast chimeric G-protein in order to grow.

Strain	**Genotype**
MMY11	*MAT***a** *his3 leu2 trp1 ura3 can1 gpa1Δ::ADE2 far1Δ::ura3 sst2Δ::ura3Δ fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2Δ::G418^R* [49]
MMY14	MMY11 *TRP1::Gpa1/*$G_\alpha^{q(5)}$ [51]

Table 14. Genotypes of yeast strains used for GPCR activation assay

3.2.4.1 Yeast transformation

The procedures are similar to the ones described by Dr. med. Nan Zhu in her thesis [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type

A receptors in the context of obliterative vasculopathy, http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000100230]. Briefly, yeasts were recovered from -80 °C and spread on a YPD plate. A single colony was put into YPD medium and incubated overnight at 30°C, as a pre-culture. On the next day, 100mL of YPD medium was inoculated and the yeasts were harvested after two to three hours. Transformation was performed using the lithium acetate (LiAc)/ single-stranded DNA (ssDNA)/ polyethylene glycol (PEG) method. Transformed yeast cells were plated on WHAUL-His plates and incubated for three or four days.

3.2.4.2 Measuring gene expression in yeast

Yeast transformation with different amounts of plasmid was performed. Colonies were picked after three or four days and incubated in 3 mL YPD medium at 30°C overnight under shaking. Yeast samples were obtained on the next day by centrifugation. The total RNA was extracted using Direct-zol™ RNA MiniPrep, which can provide a streamlined method for purification of up to 100 µg per prep of high-quality RNA directly from samples. The RNA concentration was measured by NanoDrop® Spectrophotometer. Reverse Transcription (RT)-PCR was performed to obtain cDNA, the RT-PCR components are shown in Table 15. Quantitative Real Time-PCR (qRT-PCR) using Applied Biosystems® 7500 qRT-PCR System was performed, components are shown in Table 16. The amount of fluorescence released during amplification cycle is proportional to the relative quantity of mRNA initially present.

Content	**Volume (1X) / Amount**
10X M-MuLV Reverse transcriptase Buffer	5µL
dNTP mix 10 mM	8µL
RNase inhibitor	1µL
M-MuLV Reverse transcriptase 200,000U/mL	0.25µL
Oligo	1µL
RNA	500ng
Total	50µL

Table15. RT-PCR system

Content	Volume (1X)
SYBR Green	4µL
Primer se 10 µM	0.5µL
Primer ase 10 µM	0.5µL
ddH2O	7µL
cDNA	1µL
Total	13µL

Table16. qRT-PCR system

3.2.4.3 Yeast GPCR assay

Four days after transformation, four clones from transformed yeasts were picked-up and transferred individually into WHAUL medium containing Histidine and incubated overnight at 30°C under shaking. Each pre-cultured clone was then added to FDGlu assay medium (lacking Histidine) containing different concentrations of ET_AR-IgG (0, 0.5, 1.0 and 1.5 mg/mL) in 96-well plates. 3-AT in the FDGlu assay medium is a competitive inhibitor of the *HIS3* enzyme which can regulate background activity caused by leakiness of the reporter or constitutive activity of the GPCR. Plates were protected from light and incubated at 30°C under shaking. After incubation for 16 hours, fluorescent measurement was performed at an excitation of 485 nm and emission of 535 nm.

3.2.5 Luciferase reporter assay

Activation of $G_{q/11}$ or $G_{12/13}$ can be monitored with the binding of specific transcription factors, NFAT and SRF to the luciferase promoter.

3.2.5.1 Human microvascular endothelial cells (HMEC-1)

HMEC-1 cells were grown in flasks with complete growth medium in a humidified atmosphere with 5% CO_2 at 37 °C. T75 flasks and 24 wells plates were pre-coated with 0.2% gelatin. The cells were passaged at 90 % confluence and used to seed 24 wells plates for the experiments. Cells were briefly washed once with PBS, then deattached with 1X Trypsin added into the flask for five minutes. Complete medium was used to deactivate the enzyme. After centrifugation and resuspension in complete medium, the cells were counted with a Neubauer cell chamber in the presence of Trypan blue to assess their viability. To further cultivate the cells, 1.5 million cells were seeded into a T75 flask. For experimental purposes, 50,000 cells per well were added into each well of the 24-well coated plates.

3.2.5.2 Transient transfection

After seeding, the cells were allowed to grow for four days before the transfection was performed. HMEC-1 cells were transfected transiently with 250 ng of two plasmids, a pcDNA3 plasmid enabling the expression of the wild-type or mutated ET_A receptor and a plasmid containing a reporter specific to NFAT or SRF. Transfection was performed in starvation medium using the Xfect^{TM} Transfection Reagent following the manufacturer's instructions. 16 hours later, medium was changed to complete medium.

3.2.5.3 Cell stimulation

To study the functionality of the receptors, ET-1 and ET_AR-IgG were used to stimulate the transfected cells. 48 hours after transfection, the medium was changed to starvation medium. One hour later, cells were treated with ET-1 (100 nM) or ET_AR-IgG (1.0 mg/mL) or DMEM Low Glucose as controls.

3.2.5.4 Luciferase measurement

After six hours of stimulation, cells were washed once in PBS and lysed with 1X PLB for 15 minutes under shaking. Luciferase quantity was assessed with the Luciferase Reporter Kit according to the manufacturer's instructions and measured in a FLUOstar microplate reader using the OPTIMA software.

3.2.6 Statistical analysis

Data are expressed as mean ± SEM, *n* stands for the number of independent experiments. Comparison between wild type non-stimulated and other groups were analyzed by Wilcoxon test; the other comparisons were performed by Mann-Whitney U test. Differences were considered significant when one-tailed tests *p* value was smaller than 0.05. GraphPad Prism software version 5.01 was used for the calculations.

4 Results

4.1 Generation of human Endothelin-1 type A receptor mutants

The extracellular loops (ECL) of ET_AR are potential binding sites for patients' antibodies, thus mutants of the ECL regions of ET_AR to Alanine were generated to test their involvement in the receptor immune activation.

4.1.1 Generation of yeast expressing ET_A wild-type and mutant receptor

The p426 GPD plasmid allows the expression of a gene of interest in yeasts. The ampicillin resistance gene expressed by the plasmid enables selection in *E. coli* (Figure 4). Three constructs had been previously generated in the p426 GPD plasmid in the lab: the human wild-type ET_AR (ET_AR WT), a mutant in which the first ECL of human ET_AR was substituted for Alanine (ET_AR ECL1 Ala) and a mutant where ET_AR second ECL was replaced by the second ECL of AT_1R (ET_AR with ECL2 of AT_1R, [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy, http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000100230]).

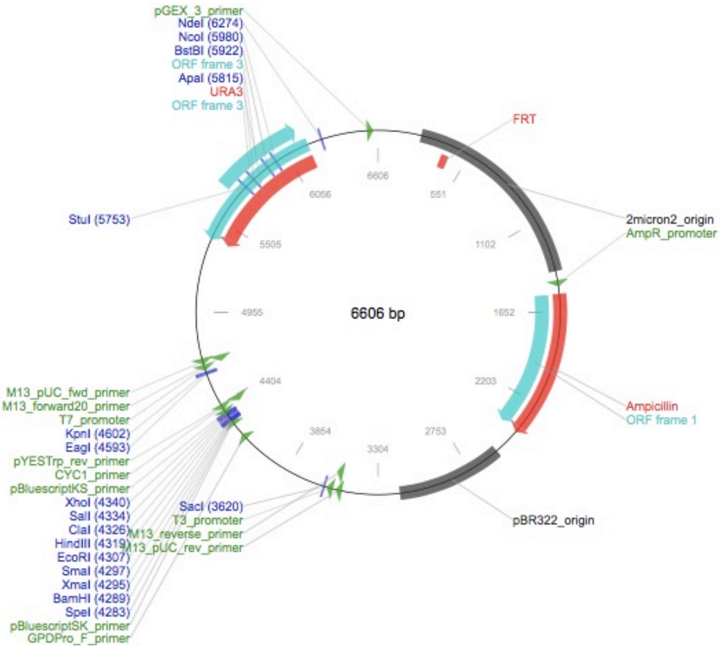

Figure 4. Map of p426 GPD (6606 base pairs).

In order to mutate the third extracellular loop of ET_AR to Alanine, a site-directed mutagenesis was realized. PCR amplification was performed using the p426 GPD ET_AR WT construct as template and primers were specially designed for site-directed mutagenesis (Table 1). After transformation, the plasmid was purified and DNA was sequenced. As shown in figure 5, the third ECL of ET_AR was correctly substituted to Alanine.

A

ET_AR WT ECL3	ACTGTGTATAACGAGATGGACAAGAACCGATGTGAATTACTT
ET_AR ECL3 Ala	GCTGCGGCTGCCGCAGCGGCCGCGGCCGCAGCTGCAGCAGCT

B

ET_AR WT ECL3	TVYNEMDKNRCELL
ET_AR ECL3 Ala	AAAAAAAAAAAAAA

Figure 5. Mutation of ET_AR ECL3 to Alanine. (A) DNA Sequence of the third ECL of ET_AR wild type and mutant. (B) Amino acid translation of the sequences. Mismatches are shown in red.

4.1.2 Generation of mammalian cells expressing ET_A wild-type and mutant receptor

The pcDNA3 plasmid (Figure 6) was used to express the human Endothelin-1 type A receptor in mammalian cells. The ampicillin resistance gene in the plasmid allows selection in *E. coli*.

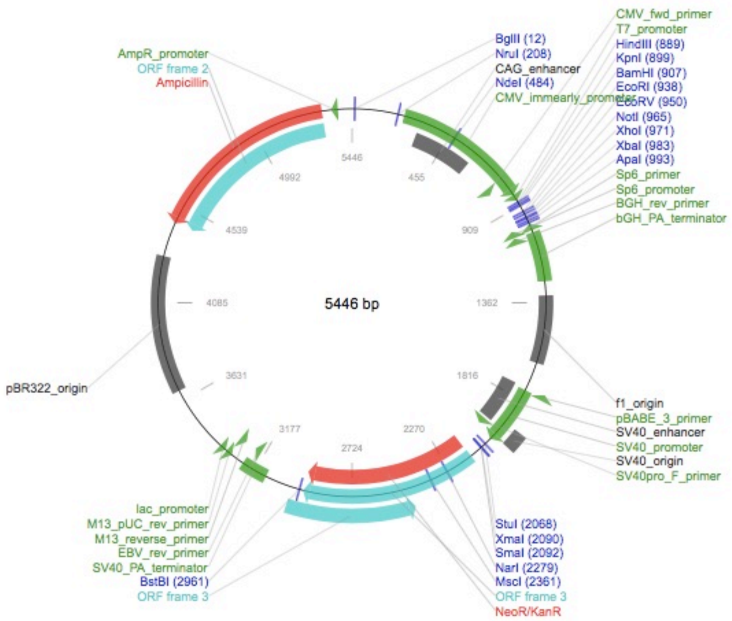

Figure 6. Map of the pcDNA3 plasmid (5446 base pairs).

*Hind*III and *Xho*I restriction enzymes were used to linearize the plasmid. PCR primers were designed with 5' 15-bp extensions homologous to the ends of the linearized vector and p426 GPD ET_AR constructs were used as templates. The appropriate insert and the linearized plasmid were ligated and transformed into *E. coli*.

4.1.2.1 Generation of pcDNA3 ET_AR WT

Digestion of pcDNA3 ET_AR WT with the two cloning enzymes, *Hind*III and *Xho*I, should produce three fragments, one 82 base pair (bp)-long fragment and one 5364 bp-long corresponding to the pcDNA3 plasmid, one 1281 bp-long fragment corresponding to human ET_AR. Figure 7 shows plasmids of ten ampicillin resistant colonies obtained after cloning. All plasmids showing two bands of the right size were considered positive as the 82 bp-long fragment was too small to be detected.

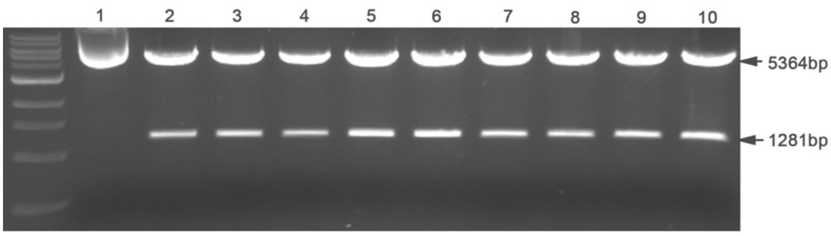

Figure 7. Double digestion of ten ampicillin-resistant colonies. Plasmids 2 to 10 are positive.

In order to verify the absence of mutations, the positive plasmids were sequenced and aligned with the sequence of the template. Two positive plasmids without any mutations were chosen to perform the luciferase reporter assays.

4.1.2.2 Generation of pcDNA3 ET_AR ECL1 Ala

The p426 GPD ET_AR ECL1 Ala construct was used as template to sub-clone the ET_AR ECL1 Ala fragment to pcDNA3. Six colonies obtained after transformation were picked and verified by double digestion, as done for wild type ET_AR. All colonies showed two bands, corresponding respectively to the linearized pcDNA3 plasmid and the insert (Figure 8). One clone was completely sequenced (Figure 9) and was used in further cell experiments.

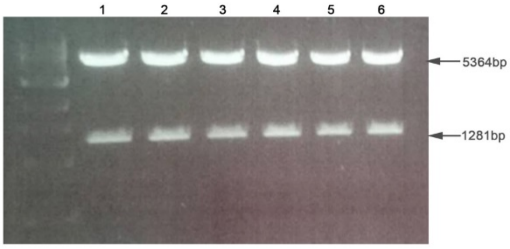

Figure 8. Double digestion of plasmids from colonies potentially containing the pcDNA3 ET_AR ECL1 Ala plasmid.

A

ET_AR WT ECL1 GGGCGCTGGCCTTTTGATCACAATGACTTTGGC

ET_AR ECL1 Ala GCGGCCGCGGCTGCTGCTGCCGCTGCCGCTGCC

B

ET_AR WT ECL1 GRWPFDHNDFG

ET_AR ECL1 Ala AAAAAAAAAAA

Figure 9. Mutation of ET_AR ECL1 to Alanine. (A) Sequence of the first ECL of ET_AR wild type and mutant. (B) Amino acid translation of the sequences. Mismatches are shown in red.

4.1.2.3 Generation of pcDNA3 ET_AR with ECL2 of AT_1R and pcDNA3 ET_AR ECL3 Ala

These sub-cloning experiments were made using the same protocol as that used for the ECL1 mutant. In each case, from the positive plasmids found after double digestion, one was completely sequenced (Figure 10 and 11) and used for luciferase assays if it was free of mutations.

A

ET_AR WT ECL2 ATTGGCTTCGTCATGGTACCCTTTGAATATAGGGGTGAACAGCATAAAACCTGTATGCTCAATGCCACATCAAAATTCATGGAGTTCTACCAA

ET_AR with ECL2 of AT_1R ATCCATCGAAATGTATTTTTCATTGAGAACACCAATATTACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTCCCG

B

ET_AR WT ECL2 IGFVMVPFEYRGEQHKTCMLNATSKFMEFYQ

ET_AR with ECL2 of AT_1R IHRNVFFIENTNITVCAFHYESQNSTLP

Figure 10. Mutation of ET_AR with ECL2 of AT_1R. (A) Sequence of the second ECL of ET_AR and AT_1R wild type. (B) Amino acid translation of the sequences.

A

ET_AR WT ECL3 ACTGTGTATAACGAGATGGACAAGAACCGATGTGAATTACTT

ET_AR ECL3 Ala GCTGCGGCTGCCGCAGCGGCCGCGGCCGCAGCTGCAGCAGCT

B

ET_AR WT ECL3 TVYNEMDKNRCELL

ET_AR ECL3 Ala AAAAAAAAAAAAAA

Figure 11. Mutation of ET_AR ECL3 to Alanine. (A) DNA Sequence of the third ECL of ET_AR wild type and mutant. (B) Amino acid translation of the sequences. Mismatches are shown in red.

4.2 Effects of the structure of the extracellular loops on ET_AR activation

In order to study the influence of the structure of each extracellular loop on ET_AR activation, a GPCR activation assay already established for previous yeast batches in the lab was applied [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy, http://www.diss.fu-berlin.de/diss/receive/FUDISS_ thesis_000000100230]. This assay had to be first optimized for novel experimental conditions.

4.2.1 Optimization of the GPCR activation assay

In the MMY yeast model, the yeast's growth depends on the activation of the expressed human GPCR. Dependent on the kind of receptor transformed, several parameters had to be optimized.

4.2.1.1 Optimization of MMY yeast transformation

The first step was to improve MMY yeast transformation, first, by determining the optimal plasmid amount. Total RNA extracted from transformed or non-transformed yeast cells was subjected to RT-PCR to obtain cDNA. Then, quantitative RT-PCR was performed to determine the human ET_AR wild-type expression level. After normalization to yeast GAPDH, the ET_AR expression level was calculated as relative to control (non-transformed yeasts). Results showed that transformation with 0.5-µg plasmid achieved the lowest expression, 3-µg the best (Figure 12A). Then, optimal incubation times after transformation were investigated to obtain high ET_AR transcription levels. As shown in figure 12B, yeasts transformed with 1-µg plasmid and incubated for four days had the highest receptor expression level. Therefore, this condition was used in further experiments.

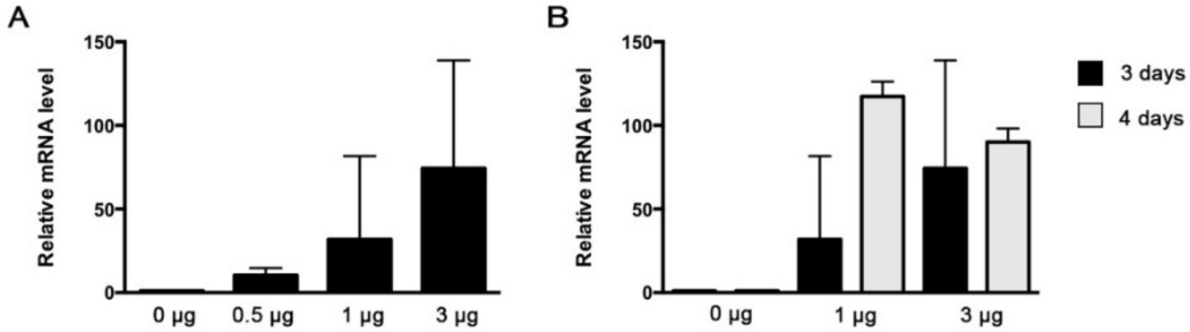

Figure 12. ET_AR wild-type transcription level in yeast (A) Yeasts were transformed with different amounts of plasmid and incubated for three days. (B) Yeasts were transformed with different amounts of plasmid and incubated for three or four days after transformation. Values are from three colonies and presented as mean ± SD.

4.2.1.2 Optimization of the GPCR assay

Three parameters were further modulated to optimize the GPCR assay: the first is 3-AT (3-amino triazole), which is a competitive inhibitor of the *HIS3* protein moderating background yeast growth caused by leakiness of the receptor or constitutive activity of the GPCR [51]. The second, the way to inoculate the yeast cultures before the assay and the third parameter is the seeding density of the yeast cells into the assay medium, which can also influence the outcome of the assay. All three parameters were tested but only the way of inoculation of the yeast cultures showed effects.

For the first inoculation method, single colonies were picked with a sterile toothpick 72 hours after transformation, streaked onto a WHAUL+His plate for further 24 hours and then scraped and inoculated into 200 µL of WHAUL+His medium. For the second method, transformed yeast clones were picked after four days and single colonies were directly inoculated into 100 µL of WHAUL+His. In each case, yeast cells grown overnight in WHAUL+His were stimulated with ET-1 in increasing concentrations: 0, 10^{-7}, $5*10^{-7}$ and 10^{-6} M. As shown in figure 13, the second inoculation way gave the strongest fluorescence intensity and showed a dose-dependent activation of ET_AR upon stimulation with ET-1.

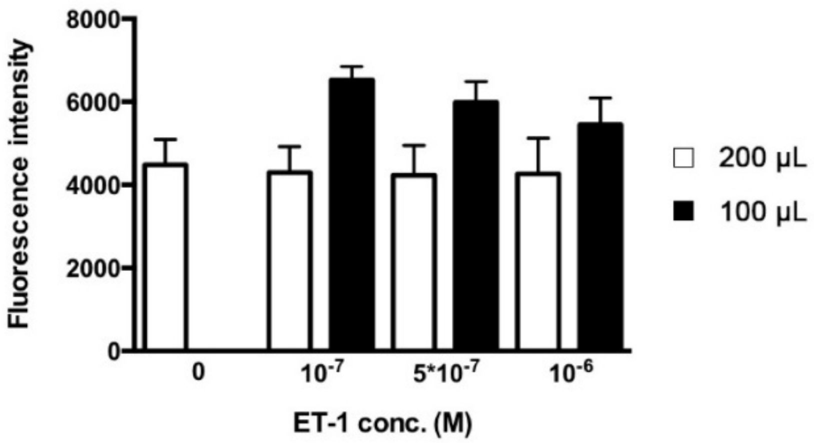

Figure 13. Yeast growth after ET-1 stimulation. Two methods of inoculation were compared. Yeast growth is reflected by the fluorescence intensity. Ten wells of a single colony were measured and the data are presented as mean ± SD.

4.2.2 ET_AR activation by ET_AR-IgG and involvement of the extracellular loops

MMY14 strain expressing $G_{q/11}$ was transformed with p426GPD ET_AR WT or mutant. In this model, it has already been shown that ET-1 activates ET_AR dose-dependently [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy, http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000100230]. Therefore, the focus was on the IgG-mediated activation of the receptor. Yeasts were stimulated with increasing concentrations of ET_AR-IgG (0, 0.5, 1 and 1.5 mg/mL) isolated from systemic sclerosis (SSc) patients tested positive for ET_AR-IgG in ELISA. After 16 hours of incubation, fluorescence intensity was measured.

4.2.2.1 ET_AR-IgG activate the wild-type receptor

When the yeast cells were stimulated with ET_AR-IgG after transformation with the wild-type receptor, the yeast growth increased significantly when comparing stimulated yeasts to non-stimulated yeasts (Figure 14). Therefore, IgG isolated from patients was able to induce activation of the receptor.

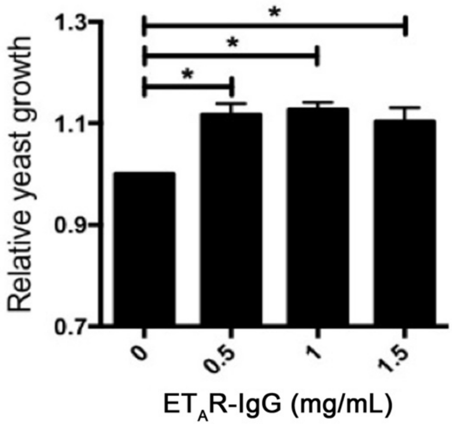

Figure 14. MMY 14 ET$_A$R wild-type yeast growth in GPCR activation model stimulated with ET$_A$R-IgG. Yeast growth is depicted as relative to yeast growth without stimulation. Data are from six experiments and presented as mean ± SEM, * $p < 0.05$.

4.2.2.2 Involvement of the extracellular loops in ET$_A$R activation

4.2.2.2.1 The second extracellular loop is not involved in ET$_A$R activation

A thesis done in the laboratory had already shown that the second extracellular loop was not necessary for the receptor activation [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy, http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000100230]. Hence, the focus of this study was on the first and third loops.

4.2.2.2.2 The first extracellular loop is involved in ET$_A$R activation

Yeast expressing the receptor mutated in the first ECL showed a trend to a higher growth rate even without antibodies stimulation. Increasing the concentrations of the stimuli did not bring any further activation in yeasts expressing the mutant receptor (Figure 15), suggesting that there could be a constitutive activation of the receptor due to the modification of the first ECL structure.

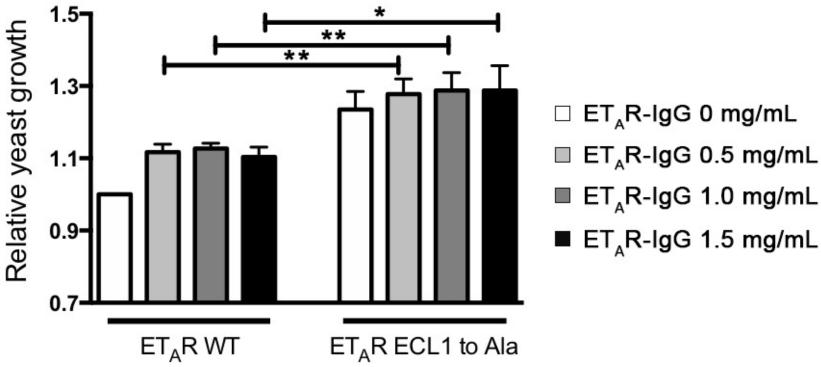

Figure 15. Yeast growth in GPCR activation model stimulated with ET_AR-IgG. Yeast growth is depicted relative to yeast growth without stimulation. Data are from six (WT) and four (ECL1) experiments and presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$.

4.2.2.2.3 Mutating ECL3 results in ET_AR constitutive activation

As for the third ECL, mutation to Alanine induced a significantly stronger yeast growth already in absence of immune stimulation (Figure 16), which means that there is a constitutive activation of the mutated receptor.

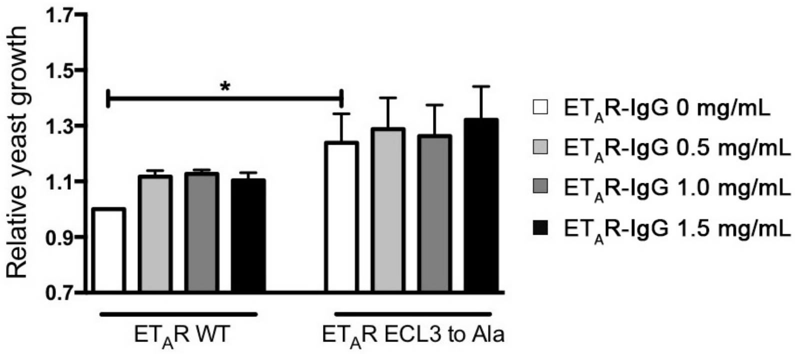

Figure 16. Yeast growth in GPCR activation model stimulated with ET_AR-IgG. Yeast growth is depicted relative to yeast growth without stimulation. Data are from six (WT) and seven (ECL3) experiments and presented as mean ± SEM, * $p < 0.05$.

4.3 Extracellular loops of ET_AR trigger G-protein activation

To investigate whether structural changes affecting extracellular loops of ET_AR induce the same effects in a more complex environment as in the MMY model, activation of G-proteins upon stimulation of ET_AR was monitored. Human Endothelin-1 type A receptor binds to two G-proteins, $G_{q/11}$ and $G_{12/13}$, upon activation by its peptide agonist, ET-1 [9]. In order to characterize the intracellular pathways activated after receptor stimulation with IgG and to determine involvement of specific extracellular loops, the mutants used in the yeast assay were subcloned in pcDNA3 to allow their expression in mammalian cells.

$G_{q/11}$ and $G_{12/13}$ activation was monitored in luciferase reporter assays by measuring the binding of the transcription factors NFAT (Nuclear factor of activated T-cells) and SRF (Serum response factor), respectively to DNA. Human microvascular endothelial cells (HMEC-1) were transiently transfected to express either wild-type or mutated ET_AR and a NFAT or SRF luciferase reporter plasmid. Cells were stimulated with the peptide agonist of the receptor, ET-1, or ET_AR-IgG.

4.3.1 G-protein activity is increased upon ET_AR WT activation

In the NFAT luciferase reporter assay, cells transfected with wild-type ET_AR showed a significant increase in NFAT activity in response to both ET-1 and ET_AR-IgG stimulation, antibodies inducing a slightly higher increase than the peptide agonist (Figure 17A). This result was concordant with those previously obtained in yeasts.

Furthermore, cells transfected with wild-type ET_AR showed that ET-1 and ET_AR-IgG both significantly increased SRF activity. IgG-mediated activation of the receptor induced a significantly stronger activation of $G_{12/13}$ than ET-1 (Figure 17B). In conclusion, antibodies stimulating ET_AR trigger a higher activation of both $G_{q/11}$ and $G_{12/13}$ than does the peptide agonist.

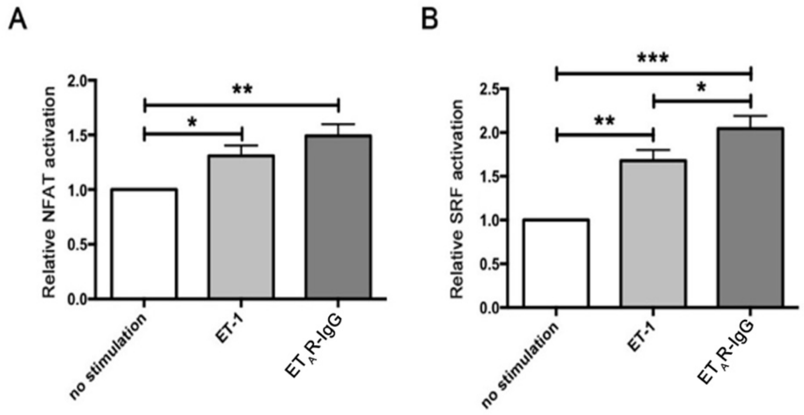

Figure 17. Relative NFAT activity (A) and SRF activity (B) in response to ET-1 and ET_AR-IgG stimulation in HMEC cells transfected with ET_AR wild-type. NFAT or SRF activity is depicted relative to NFAT or SRF activity in absence of stimulation, respectively. Data are from nine (NFAT) and eleven (SRF) experiments and presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

4.3.2 The second extracellular loop of ET_AR is required for ET-1 but not for IgG-induced G-protein activation

In the yeast GPCR activation assay, replacing the second extracellular loop of ET_AR by ECL2 of AT_1R did not affect endogenous or IgG-mediated activation of the receptor [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy, http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000100230]. In order to verify these results in mammalian cells, this mutant was used in both NFAT and SRF luciferase reporter assay.

When the cells were transfected with pcDNA3 ET_AR with ECL2 of AT_1R, stimulation with ET_AR-IgG led to significantly increased NFAT activity in comparison to non-stimulated cells (Figure 18A). Stimulation with ET-1 also showed a slight increase of NFAT activity, but it was not significant. This confirms the results observed in yeast. However, it should be noted that the $G_{q/11}$ activation upon antibodies stimulation was stronger in the mutant cells than in the wild-type cells, something that was not seen in yeasts.

In SRF luciferase reporter assay, cells that were transfected with pcDNA3 ET_AR with ECL2 of

AT_1R and stimulated with ET-1 showed SRF activity at non-stimulated level, revealing that intact ECL2 structure is essential for ET_AR activation via its peptide agonist. Conversely, cells transfected with ET_AR mutant and stimulated with ET_AR-IgG showed a higher activity of SRF in comparison to both non-stimulated wild-type and mutant ET_AR transfected cells, even if significance was only found with wild type (Figure 18B). Hence, ECL2 does not seem necessary for antibody-triggered ET_AR intracellular signaling.

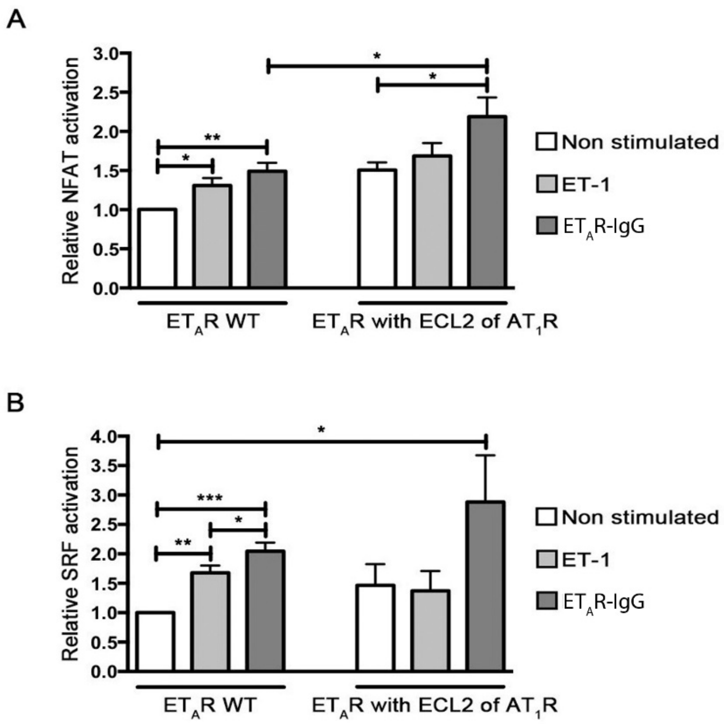

Figure 18. Relative NFAT activity (A) and SRF activity (B) in response to ET-1 and ET_AR-IgG stimulation in HMEC cells transfected with ET_AR wild type and ET_AR with AT_1R ECL2. NFAT or SRF activity is depicted relative to NFAT or SRF activity in absence of stimulation, respectively. Data are from eleven (WT) and seven (ECL2) experiments and presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

4.3.3 The first extracellular loop of ET_AR is required for $G_{12/13}$ activation

Cells transfected with ET_AR ECL1 Ala mutant and stimulated with ET_AR-IgG showed a significantly higher activity of NFAT in comparison to non-stimulated wild type ET_AR cells. However, no constitutive activation of the receptor was observed (Figure 19A). Hence, mutation of the receptor's first extracellular loop does not influence the activation of $G_{q/11}$ mediated by antibodies.

When the cells were transfected with ET_AR ECL1 Ala and stimulated with ET-1, SRF activity stayed at non-stimulated level and was significantly decreased in comparison to the stimulation of the wild-type receptors, revealing that normal structure of ECL1 is essential for ET_AR-mediated $G_{12/13}$ activation via its peptide agonist. Conversely, cells transfected with ET_AR ECL1 Ala and stimulated with ET_AR-IgG showed a significantly higher activity of SRF in comparison to non-stimulated wild type ET_AR and mutant transfected cells (figure 19B). Therefore, ECL1 is not necessary for antibody-triggered ET_AR intracellular signaling.

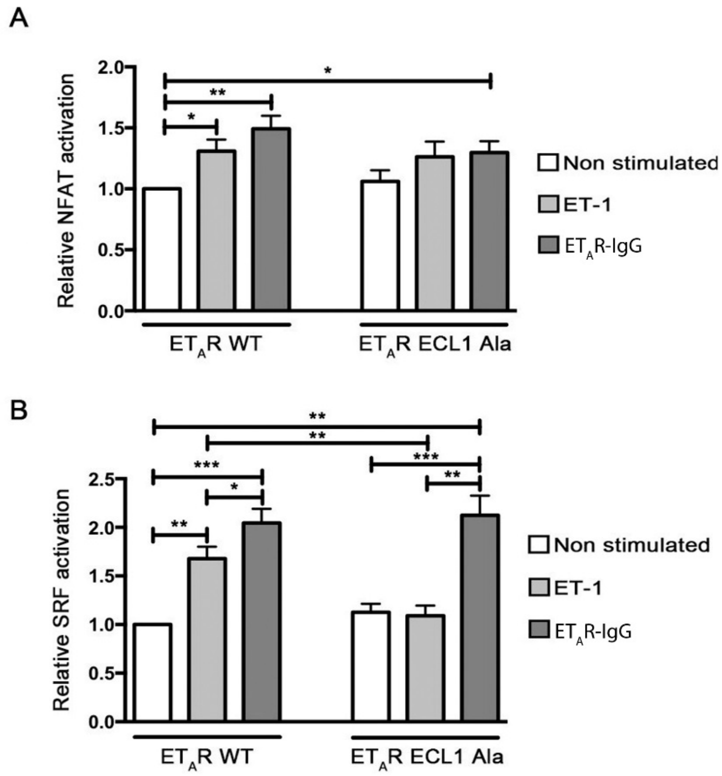

Figure 19. Relative NFAT activity (A) and SRF activity (B) in response to ET-1 and ET_AR-IgG stimulation in HMEC cells transfected with ET_AR wild type and ET_AR ECL1 Ala. NFAT or SRF activity is depicted relative to NFAT or SRF activity in absence of stimulation, respectively. Data are from eleven (WT) and eight (ECL1) experiments and presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

4.3.4 Mutation of the third extracellular loop results in constitutive binding of G-proteins

When the cells were transfected with ET_AR ECL3 Ala, a significant increase of NFAT activity in comparison to the wild-type ET_AR transfected cells could already be seen in the absence of stimulation (figure 20A). This revealed a constitutive binding of $G_{q/11}$ to the receptor which was consistent with the results obtained in yeast experiments.

As in the NFAT study, when the cells were transfected with ET_AR ECL3 Ala, a significant increase of SRF activity could be seen in the absence of stimulation, revealing a constitutive binding of $G_{12/13}$ to the mutated receptor (figure 20B).

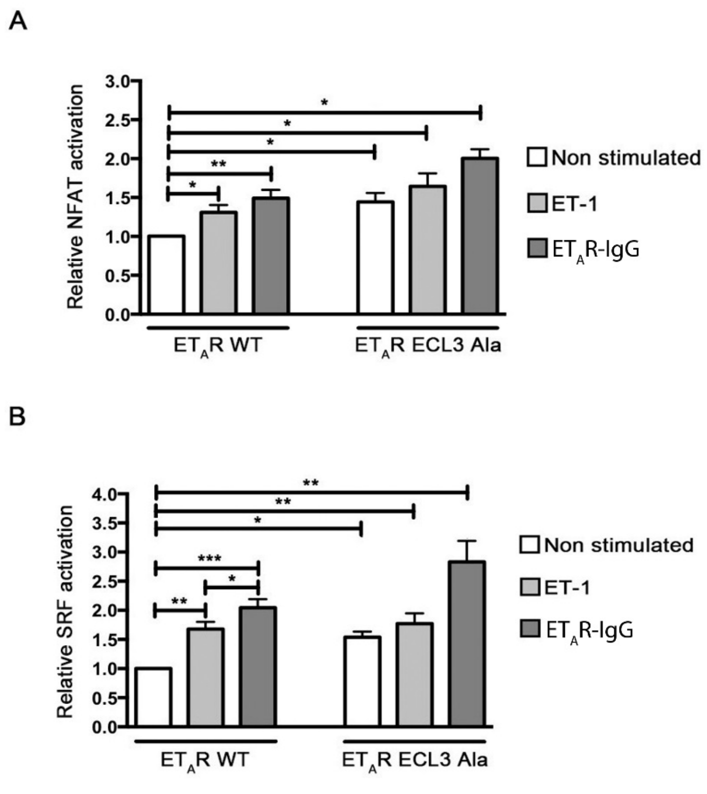

Figure 20. Relative NFAT activity (A) and SRF activity (B) in response to ET-1 and ET_AR-IgG stimulation in HMEC cells transfected with ET_AR wild type and ET_AR ECL3 Ala. NFAT or SRF activity is depicted relative to NFAT or SRF activity in absence of stimulation, respectively. Data are from eleven (WT) and eight (ECL3) experiments and presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

5 Discussion

In this work, involvement of the Endothelin-1 type A receptor (ET_AR) extracellular loops in the receptor activation and resulting intracellular signaling was investigated. IgG binding to ET_AR triggered a stronger activation of $G_{q/11}$ and $G_{12/13}$ signaling pathways as the natural peptide agonist ET-1. GPCR activation assay in yeasts using mutations of the extracellular loops of the receptor demonstrated that disturbing the third ECL structure might constitutively activate the receptor. Similar results were obtained in mammalian cells, where mutating ECL3 resulted in the constitutive binding of $G_{q/11}$ and $G_{12/13}$ to ET_AR. Furthermore, changes in ECL1 structure had only a small impact on ET_AR-mediated $G_{q/11}$ pathway activation. On the other hand, without preservation of ECL1 structure, ET_AR-triggered $G_{12/13}$ activation after stimulation with ET-1 was lost. Replacement of the second extracellular loop of the receptor by ECL2 of AT_1R confirmed that this domain is only involved in ET-1 induced $G_{12/13}$ pathway activation. These results shed light on the influence of the extracellular loops on ET_AR activation and show that antibodies bind to the receptor in a completely different way than the peptide agonist.

Yeast GPCR activation assays, applications and models

In this project, a yeast model was used to assess GPCR activation. The yeast mating pathway is often used to study GPCR activation and signal transduction. The yeast's growth depends on activation of one single GPCR which can be replaced by a mammalian GPCR, which then activates a preserved pathway [52], providing an eukaryotic assay system with no other endogenous responses [51]. To activate the mating pathway, GPCRs must be coupled to a heterotrimeric G-protein consisting of G_α (Gpa1), G_β and G_γ. Compatibility between the receptor and the G-proteins depends on the C-terminus of the G_α subunit. It has been shown that the chimeric G_α subunit consisting of five C-terminal amino acids of a human G-protein fused with the yeast G-protein alters the specificity of G_α without disturbing the signal transduction [49]. Yeast G_β and G_γ are homologous to mammalian G_β and G_γ subunits, so that yeast $G_{\beta\gamma}$ may have close structural and functional similarities with mammalian $G_{\beta\gamma}$ subunits [53]. When an agonist binds to a receptor, the

$G_{\beta\gamma}$ subunit transduces signals to the mitogen-activated protein kinase (MAPK) cascade, resulting in cell cycle arrest and gene expression, as *FUS1* for mating. The MMY system used in this study is based on the *Saccharomyces cerevisiae* strain that uses *FUS1* as a pheromone-responsive promoter and the *HIS3* gene as a reporter. *HIS3* encoding the key enzyme that produces histidine, an essential amino acid for yeast growth, is located behind the *FUS1* promoter. As a result, only the cells, in which the human GPCR is activated, can grow in histidine-free selective medium. Experiments have already been performed in the MMY system to study different GPCRs. The role of the second and third extracellular loops of the adenosine A_1 receptor was studied using the MMY24 strain, which expresses chimeric Gpa1/$G_{\alpha i3}$. By Means of Alanine scan, the authors demonstrated a strong regulatory role for the ECL2 and the importance of many residues of ECL2 and ECL3 for adenosine A_1 receptor activation [54]. Furthermore, adenosine A_{2B} receptor was investigated in the MMY24 strain. Random mutagenesis was introduced in transmembrane domains four and five. The screening identified 22 single and double mutant receptors showing constitutive activity and decreased agonist potency [55]. Many features of the MMY model enable rapid analysis of structural and functional aspects of GPCRs. However, this is not the only existing yeast model to study GPCRs. One of the difficulties in using the yeast system to analyze the signaling of human GPCR is that the coupling between a yeast G-protein and a human GPCR can be ineffective and results in poor signal transduction. Hence, Fukuda *et al.* described a novel strategy for increasing the sensitivity of agonist-mediated signal transduction of human GPCR expressing yeast [56]. Stimulation of ligand induces the expression of both GFP, a reporter gene, and G_β gene. Overexpressed G_β competes with endogenous G_β present in the $G_{\alpha\beta\gamma}$ heterotrimer for G_α binding, wherein the free $G_{\beta\gamma}$ complex can further activate the MAPK cascade and amplify the signal. In parallel, Li *et al.* described a novel yeast genetic screen that only selects for mutant receptors containing inactivation point mutations that is capable of identifying single-point mutations that abolish the receptor function [57]. The yeast strain contains the pheromone-sensitive *FUS2-CAN1* reporter gene encoding the cytotoxic arginine-canavanine permease. Yeasts expressing a functional receptor in the presence of an agonist will not grow, while yeasts expressing a mutant containing inactivating point mutation in a medium containing both carbachol and canavanine will grow.

Constitutively active mutants (CAMs) of GPCRs

Using the MMY yeast model, binding of ET_AR-IgG to the extracellular domains of ET_AR was observed. Interestingly, mutation of ECL3 led to a constitutive activation of ET_AR. Constitutive receptor activation is defined as the spontaneous formation of a signaling conformation of a receptor, resulting in the production of a second messenger without agonist binding [58]. ECL3 is usually a short loop, so it can constrain TM6 and TM7, limiting their movement and maintaining the receptor's ground state [59]. Substitution in ECL3 relieves this limitation and may affect the conversion of ground state into active state [59]. The ECL3 of β_2-adrenergic receptor (β_2AR) replaced by the ECL3 of the $\alpha_{1a}AR$, has a higher binding affinity to agonists and a higher agonist-independent basic adenylyl cyclase activity compared to the wild type β_2AR, with no influence on the affinity to antagonist [60]. In contrast, replacement of the ECL3 of the TSHR by the ECL3 of the β_2AR did not affect the basal signaling, but decreased the potency and extent of the TSH-stimulated cAMP response [61]. Other constitutive mutations have been described in GPCRs. The mutation of $Asn295^{7.46}$ to Ala was involved in the constitutive activation of AT_1R, probably due to the disruption of two hydrogen bonds between $Asn295^{7.46}$ and $Asn111^{3.35}$, which stabilize the inactive conformation [62]. Asn111 in TM3 helix plays a central role in AT_1R activation through direct interaction with the Tyr^4 side chain of Ang II [63].

Role of ECL1 and ECL2 in other GPCRs

Further new insights from thesis work are that ECL1 and ECL2 of ET_AR are necessary for ET-1-induced intracellular signaling activation. Crystal structure of Endothelin-1 type B receptor was recently published [11]. As ET_AR and ET_BR vary a lot in their amino acid sequences, it is difficult to draw conclusions from this structure. However, some studies showed the importance of these two ECLs in ET-1 binding to the ET_A receptor. Substitution of ECL1 of ET_AR with the ET_B counterparts significantly reduced the antagonist binding of BQ-123, indicating that the ECL1 is important for binding [64]. Mutations of Gly97, Lys140, Lys159, Gln165 and Phe315, each located in TM 1,2,3,3 and 6, result in decreased radio-ligand binding to receptor, which has similar levels expressed in cells [13]. In contrast to its involvement in ET-1 binding, mutational studies indicated that ECL2 is not involved in the binding of IgG to ET_AR. This is different to other studies on GPCRs and antibodies. Magnusson *et al.* demonstrated that the specific target of stimulatory autoantibodies in patients with dilative cardiomyopathy was the second extracellular loop of the β_1AR [65]. Chiale *et al.* confirmed the finding that the autoantibodies bind to the second extracellular loop of the β_1AR, and they identified agonistic autoantibodies to β_2AR [66]. In addition, Unal *et al.* demonstrated that the conformational kinetics of the ECL2 of AT_1R produce an epitope for AT_1R autoantibodies [67]. This allows the antibodies to bind and stabilize the active state of AT_1R. Epitope mapping studies in human AT_1R revealed that autoantibodies in patients with preeclampsia bind to epitope "AFHYESQ", the autoantibodies in patients with malignant hypertension and refractory vascular allograft rejection recognize and bind to are epitopes "ENTNIT" and "AFHYESQ" [68].

Putative pathogenic mechanisms for $G_{q/11}$ and $G_{12/13}$ activation

Interestingly, antibodies binding to the ET_AR elicited a much stronger $G_{q/11}$ and $G_{12/13}$ activation compared to the binding of the peptide agonist ET-1. This could be responsible for many aspects of pathophysiology of systemic sclerosis. SSc is characterized by abnormal vascular alterations, endothelial cell injury considered as a crucial initiating event leading to vascular remodeling. It has been shown that dysregulation of apoptosis is associated with this process [69]. Apoptosis is a highly conserved cellular mechanism characterized by cell shrinkage, chromatin condensation and nuclear fragmentation. It occurs under many physiological and pathophysiological conditions [70]. Increased endothelial apoptosis has been shown to represent the earliest microvascular abnormality in SSc animal models, suggesting that endothelial cell apoptosis is the initial trigger [71] [72]. Endothelial cell apoptosis has been observed under various pathogenic conditions *in vivo* and can be induced *in vitro* by anti-endothelial cell antibodies in systemic sclerosis [73] [74]. Apoptosis in HeLa cell can be induced by overexpression of active $G_{q/11}$ or stimulation of M_1 muscarinic acetylcholine receptor [75]. One study confirmed the importance of $G_{q/11}$ and PKC in

Angiotensin II-induced myocyte apoptosis [76]. The MAP kinase pathway is also involved in the regulation of apoptosis. Two related MAPK kinases, apoptosis signal-regulation kinase 1 (ASK1) and MAPK kinase kinase 1 (MEKK1), stimulate c-Jun kinase (JNK) activity and induce apoptosis. In COS-7 cells, $G_{12/13}$ stimulated the JNK pathway in an ASK-1 and MEKK1-dependent manner during apoptosis [77]. ET-1 may also promote the proliferation of vascular smooth muscle cells and fibroblasts, causing vascular wall thickening [78] [79]. AT_1R-IgG and ET_AR-IgG specifically bind to their receptors on endothelial cells, induce phosphorylation of ERK1/2 and increase the gene expression of TGF-β, indicating their potential involvement in fibrosis [35]. This process could involve $G_{12/13}$. The $G_{12/13}$ subfamily is of great significance in mediating signaling pathways such as Rho/Rho-associated protein kinase (ROCK)-dependent formation of actin stress fibers [80] and in vascular smooth muscle cell contraction [81], suggesting that the $G_{12/13}$ subfamily may play a considerable role in various physiological effects ET-1 induced such as cell growth, migration, transformation and gene expression.

The results generated in this work show the involvement of the first and second extracellular loops of Endothelin-1 type A receptor in ET-1-induced receptor activation. On the contrary, binding of ET_AR-IgG does not rely on the presence of intact ECL1 to 3 and activates $G_{q/11}$ and $G_{12/13}$ more strongly than ET-1. Further studies are required to determine if other extracellular domains of ET_AR, like the N-terminus, are involved in the binding of the antibodies to the receptor. These results constitute the first step towards a better understanding of ET_AR immune activation and towards designing new drugs able to impair it specifically.

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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publications

Publication 1: **Wu Sumin**, Wang Yucheng, Zhen Jie, Wang Ying, Gu Ying. Antibacterial Activity of Ru Photosensitizer: An in vitro Study on Pseudomonas Aeruginosa Strains. Chinese Journal of Laser Medicine & Surgery. 2014.

Publication 2: **Wu Sumin**，Wang Ying，Gu Ying. Photodynamic Antibacterial Activation Pseudomonas Aeruginosa in Planktonic and Biofilm Culture in vitro. Chinese Journal of Laser Medicine & Surgery. 2014.

Publication 3: **Wu Sumin**，Wang Ying，Gu Ying. Photodynamic inactivation of Pseudomonas aeruginosa by Ru complex. Chinese Journal of Microecology. 2013.

Publication 4: Wang Y, Zhou Q, Wang Y, Ren J, Zhao H, **Wu S**, Yang J, Luo Y, Wang X, Gu Y, In Vitro Photodynamic Inactivation Effects of Ru(Ⅱ) Complexes on Clinical Methicillin-resistant *Staphylococcus aureus* Planktonic and Biofilm Cultures. Photochemistry and Photobiology, 2015.

Publication 5: Yucheng Wang, Ying Wang, **Sumin Wu** et al, *In vitro* sensitivity of Candida spp. to hematoporphyrin monomethyl ether-mediated photodynamic inactivation, *Proc. SPIE* 9268, Optics in Health Care and Biomedical Optics Ⅵ, 92680L, 2014.

Publication 6: Zhao H, Yin R, Wang Y, Lee YH, Luo T, Zhang J, Qiu H, Ambrose S, Wang L, Ren J, Yao J, Chen D, Wang Y, Liang Z, Zhen J, **Wu S**, Ye Z, Zeng J, Huang N, Gu Y, Modulating mitochondrial morphology enhances antitumor effect of 5-ALA-mediated photodynamic therapy both in vitro and in vivo. J Photochem Photobiol B, 2017.

Affidavit

“I, Sumin, Wu certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Structural and functional basis of Endothelin-1 type A receptor (ET_AR) activation] I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

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