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

DISSERTATION

Autoimmune PAR2 activation in vascular events

zur Erlangung des akademischen Grades
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von

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Abbreviations

AECA	Anti-endothelial cell antibodies
ADP	Adenosine diphosphate
Ang II	Angiotensin II
AR	Adrenergic receptor
AT_1R	Angiotensin II type 1 receptor
B2M	Beta-2 microglobulin
bp	Base pair
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
Conc.	Concentration
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cells
ECL	Extracellular loop
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FDGlu	Fluorescein Di-β-D-Glucopyranosid
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HIF1 α	Hypoxia-induced factor 1 alpha
HIF2 α	Hypoxia-induced factor 2 alpha

HMEC-1	Human microvascular endothelial cells-1
HUVEC	Human umbilical vein endothelial cells
ICL	Intracellular loop
IgG	Immunoglobulin class G
IL-6	Interleukin 6
IL-8	Interleukin 8
JNK	c-Jun kinase
kD	Kilo Dalton
LB	Lysogeny broth
M	Molar
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
mL	Milliliter
mRNA	Messenger RNA
NFAT	Nuclear factor of activated T-cells
nm	Nanometer
PAR1	Protease-activated receptor 1
PAR2	Protease-activated receptor 2
PAR2-IgG	IgG targeting PAR2
PAR2 AP	PAR2-activating peptide
PAR3	Protease-activated receptor 3
PAR4	Protease-activated receptor 4
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PLB	Passive Lysis Buffer
P/S	Penicillin/Streptomycin

PTX	Pertussis toxin
qRT-PCR	quantitative Real Time-PCR
RhoA	Rho guanine nucleotide exchange factors
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
SD	Standard deviation
SEM	Standard error of the mean
SRE	Serum response element
SRF	Serum response factor
SSc	Systemic sclerosis
ssDNA	single-stranded DNA
STAT3	Signal transducer and activator of transcription 3
TGF-β	Tissue growth factor beta
TNF-α	Tumor necrosis factor alpha
TSH	Thyrotropin, thyroid-stimulating hormone
TSHR	Thyroid-stimulating hormone receptor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular endothelial growth factor receptor
α1-AR	α1-adrenergic receptor
β1-AR	β1-adrenergic receptor
β2-AR	β2-adrenergic receptor
µg	Microgram
µL	Microliter

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Abstract

Background: Antibodies targeting endothelial G protein-coupled receptors (GPCRs), such as Protease-activated receptor 2 (PAR2), bridge antibody-mediated rejection through endothelial pathology. Previous results from Prof. Dragun´s lab show that antibodies targeting PAR2 (KTx-IgG) can regulate vascular formation in kidney transplant recipients, indicating that PAR2 antibodies might be involved in transplant rejections. However, the structural basis of antibodies interacting with PAR2 in endothelial cells and the specific signaling pathways involved are still unknown.

Methods: The three extracellular loops (ECL) of PAR2 were mutated respectively to study their involvement in trypsin and KTx-IgG-induced PAR2 activation and downstream signaling. Two systems were adopted for the functional analyses. The MMY system is based on engineered yeasts, in which the activation of G-proteins can be observed by monitoring the yeast growth. Another system applied in human endothelial cells (HMEC-1) is the luciferase reporter system, which makes the autoantibody-induced transcription detectable.

Results: KTx-IgG partially activated PAR2. Trypsin and KTx-IgG activated $G_{\alpha 12}$, but not $G_{\alpha q}$ in the MMY system. In contrast, in HMEC-1 both $G_{\alpha q}$ and $G_{\alpha 12}$ were activated by trypsin and KTx-IgG. Mutation of ECL1 eliminated the trypsin-induced $G_{\alpha q}$ activation and lowered the KTx-IgG-induced $G_{\alpha q}$ activation. ECL2 was also shown to be also important in trypsin-induced $G_{\alpha q}$ activation. However, mutation of ECL2 enhanced the KTx-IgG-induced $G_{\alpha 12}$ and ERK activation. ECL3 mutation attenuated trypsin-induced $G_{\alpha q}$ activation and the baseline activation of $G_{\alpha 12}$, but had only small effects on KTx-IgG-induced PAR2 activation. PAR2-activating peptide and KTx-IgG also activated G_i in the yeast system. Inhibition of G_i downregulated trypsin-induced $G_{\alpha q}$ activation.

Conclusion: The study explored autoantibodies and natural agonist-induced PAR2 downstream signaling and the extracellular binding loops involved in the MMY system and mammalian endothelial cells. The results provide a rationale for further development of drugs targeting specific ECLs of PAR2 or PAR2 downstream signaling to improve the prognosis and life expectancy of kidney transplantation patients.

Abstrakt

Hintergrund: Autoantikörper, die endotheliale G Protein gekoppelte Rezeptoren (GPCRs), wie den Protease aktivierten Rezeptor 2 (PAR2) aktivieren, können über endotheliale Störungen zur Transplantatrejektion führen. Frühere Ergebnisse der AG Prof. Dragun zeigten, dass Autoantikörper, die PAR2 (NTx-IgG) aktivieren, die Gefäßbildung in Nierentransplantat-empfängern regulieren und damit an einer Transplantatrejektion beteiligt sein könnten. Jedoch war es bisher völlig unbekannt, welche strukturellen Interaktionen zwischen den Autoantikörpern und PAR2 und welche spezifischen Signalwege dabei in den Endothelzellen eine Rolle spielen.

Methoden: Um die durch den natürlichen Liganden von PAR2 Trypsin und die NTx-IgG induzierte Aktivierung von PAR2, sowie nachfolgende Signalwege zu untersuchen, wurden die drei extrazellulären Loops (ECL) von PAR2 mutiert. Zur Bestimmung der funktionellen Bedeutung dieser Mutationen wurden zwei unterschiedliche Assays eingesetzt. Zum einen wurde das MMY-Hefesystem verwendet, das auf gentechnisch veränderten Hefen beruht und bei dem die Aktivierung eines G-Proteins direkt anhand des Wachstums der Hefen gemessen werden kann. Die zweite Methode, die in humanen Endothelzellen (HMEC-1) etabliert wurde, ist das Luziferase Reporter System, womit eine autoantikörperinduzierte Transkription detektiert werden kann.

Ergebnisse: NTx-IgG aktivierte PAR2. Im MMY-Hefesystem kam es durch Trypsin und NTx-IgG zu einer Aktivierung von $G_{\alpha 12}$, nicht jedoch von $G_{\alpha q}$. Im Gegensatz dazu wurden in HMEC-1 sowohl Gαq, als auch Gα12 durch Trypsin und NTx-IgG aktiviert. Die Mutation des ECL1 verhinderte die Gαq-Aktivierung durch Trypsin und reduzierte diejenige durch NTx-IgG. Darüberhinaus zeigte sich, dass für die Gαq-Aktivierung durch Trypsin auch der ECL2 wichtig ist. Jedoch führte die ECL2 Mutation zu einer verstärkten Aktivierung von Gα12 and ERK bei der Zellstimulation durch NTx-IgG. Die Mutation des ECL3 verminderte sowohl die trypsininduzierte Gαq-Aktivierung als auch die basale Aktivierung von Gα12, hatte aber nur einen geringen Einfluss auf die NTx-IgG induzierte PAR2 Aktivierung. Weiterhin konnte gezeigt werden, dass das PAR2 aktivierende Peptid und NTx-IgG im Hefesystem G_i aktivieren. Die Hemmung von G_i reduzierte die Gαq-Aktivierung durch Trypsin.

Zusammenfassung: In dieser Studie wurden sowohl die Aktivierung von Signalwegen als auch die Bindung an extrazelluläre Loops von PAR2 durch Autoantikörper und natürliche Aktivatoren

im MMY Hefesystem und in humanen Endothelzellen untersucht. Die Ergebnisse dieser Arbeit liefern erste Erkenntnisse für potenzielle drug targets, die spezifisch gegen die ECLs von PAR2 und PAR2 abhängige Signalwege gerichtet sind. Dies könnte die Prognose und Lebenserwartung für nierentransplantierte Patienten verbessern.

1 Introduction

1.1 Clinical background

Kidney transplantation is the best available therapy for chronic kidney failure patients. However, organ rejection still remains a clinical / critical problem in transplantation [1]. Within different pathogenic mechanisms of rejection, antibody (Ab)- mediated rejection (ABMR) is the leading cause of renal allograft loss [2]. It has been illustrated that non-HLA Abs targeting allograft endothelial targets, such as angiotensin II type I receptor (AT_1R) and endothelin-1 type A receptor (ET_AR), play essential roles in ABMR [3, 4]. These anti-endothelium Abs are involved in allograft vascular pathology through mediating injuries to the endothelial barrier, and they disturb the cardiovascular system of the recipients [5]. Protease-activated receptor 2 (PAR2), as a typical G protein-coupled receptor (GPCR), is expressed on human endothelium and acts as a target for autoantibodies which participate in microvascular damage [6]. Antibodies targeting PAR2 are capable of regulating vascular functions in kidney transplantation, yet their titers have been found to be lower in kidney transplant recipients than in healthy individuals [7]. PAR2 Abs might be involved in kidney transplant rejection through regulating endothelial physiological and pathophysiological functions. However, the autoimmune activating mechanisms of PAR2 in this process remain largely unknown.

1.2 Protease-activated receptor family and PAR2

GPCRs comprise a family of receptors which are localized in cellular and endosomal membranes. With almost 1000 members, GPCRs constitute the biggest protein superfamily[8]. The identical structural element of GPCRs is the seven trans-membrane domains which transduce signals through GTP-binding proteins (Fig.1). Drugs targeting GPCRs make up more than 40% of all marketed drugs and GPCR-based drugs have made great contribution to medical development. The most famous of these drugs are antihistamines, angiotensin receptor inhibitors, beta adrenergic blockers, dopamine agonists, neuroleptics, opioids, and triptanes [9].

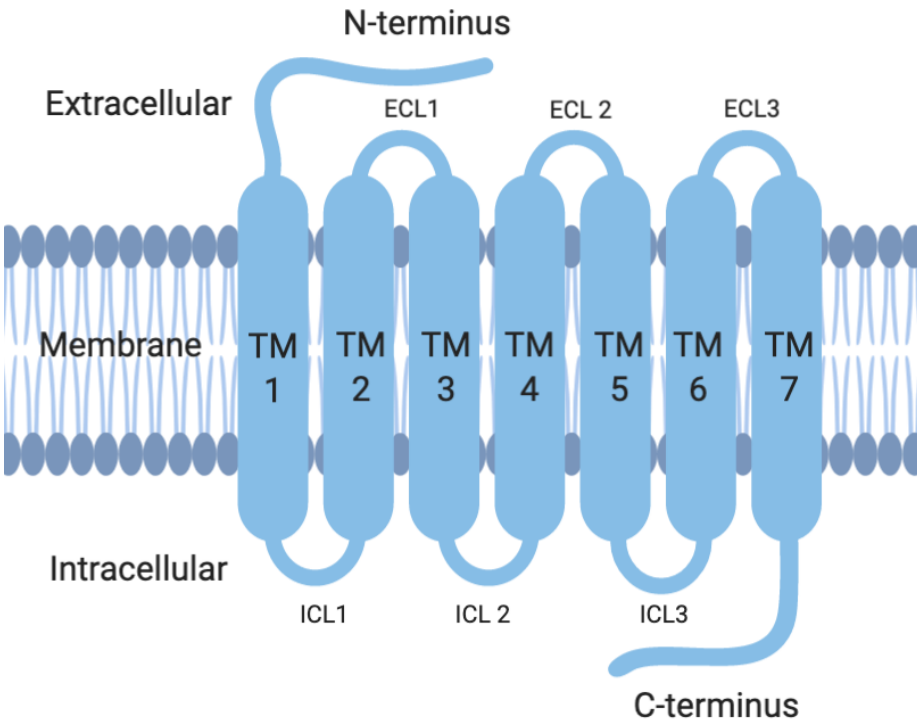

Figure 1. Schematic diagram of a GPCR. ECL, extracellular loop, TM, transmembrane domain, ICL, intracellular loop.

Since the recognition in the 1960s of hormone-like effects of proteases , many proteases have been studied [10]. Among these proteases, thrombin, which functions mainly in the coagulation system, drew attention. In addition to cleaving fibrinogen, thrombin also acts as an agonist of some receptors through a unique proteolytic mechanism. These receptors were named thrombin-activated receptors [11]. Since then, other proteases have also been found to be capable of activating these receptors. Thus, thrombin-activated receptors are now referred to as protease-activated receptors (PARs) [12].

PARs belong to the GPCR protein family, and all members of the PAR family share the cleavage activating mechanism by proteases. In detail, those proteases cleave PARs at a specific site on the N-terminus to expose a new N-terminus, which then functions as a tethered ligand and binds intramolecularly to activate the receptor (Fig.2). There are four members of the PAR family: PAR1, PAR2, PAR3 and PAR4. All of them are activated by thrombin except for PAR2. Like the other GPCRs, PARs exhibit an extracellular N-terminus, an intracellular C-terminus, seven transmembrane helices connected by three extracellular and three intracellular loops, and a small helix within the intracellular C-terminus. PAR1 and PAR3 also contain a negatively charged hirudin-like region on the N-terminus that binds to the exosite I of thrombin. PAR4, which does not

contain a hirudin-like region, interacts with the active site of thrombin [13].

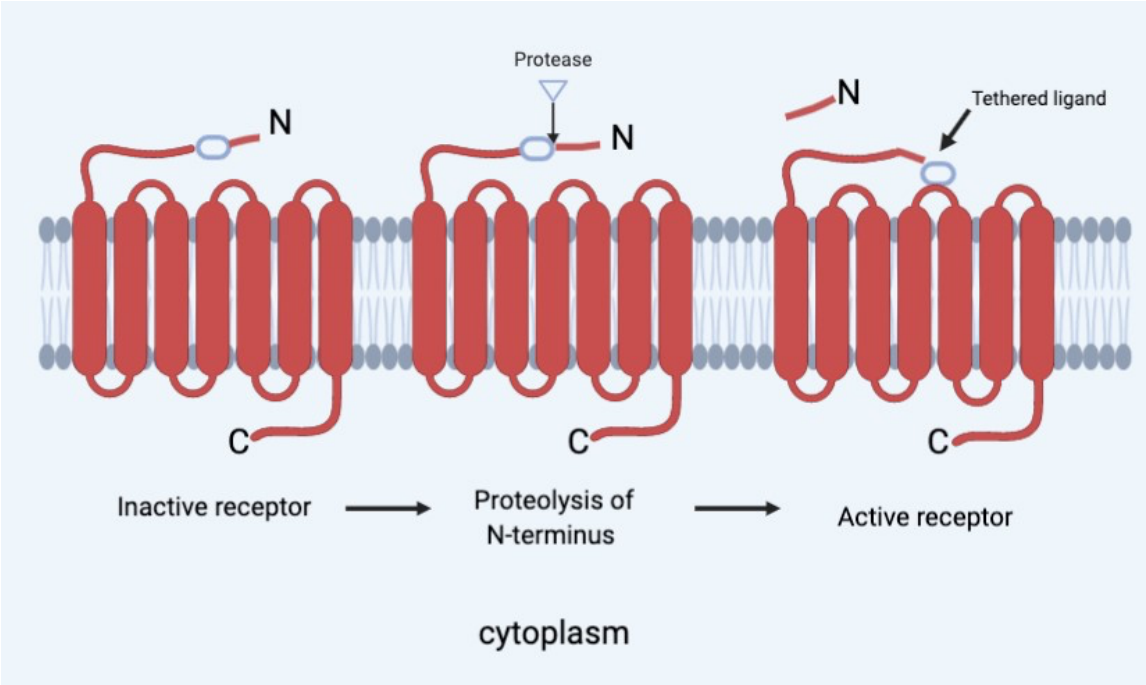

Figure 2. Activation process of the PAR family (aquired and modified from [14]).

1.2.1 PAR2

Protease-activated receptor 2 (PAR2), also known as coagulation factor II receptor-like 1 (FeRL1) or G protein 11, is the second member of the PAR family (Fig.3). It is encoded by the *G2RL1* gene. Unlike other members of the PAR family, PAR2 is not activated by thrombin, but cleaved by trypsin, tryptase, factor Xa/VIIa, matriptase, granzyme A, kallikreins (KLK 2/3/5/6/14), matrix metalloproteinase-1(MMP-1), cathepsin S, elastase, acrosin, transmembrane protease serine 2 (TMPRSS2), chitinase, bacterial gingpains and testisin [15]. Canonically, PAR2 is cleaved by trypsin, tryptase or some other proteases at position R^{36}/S^{37}, which reveals the tethered ligand SLIGKV(human) or SLIGRL(mouse) [13].

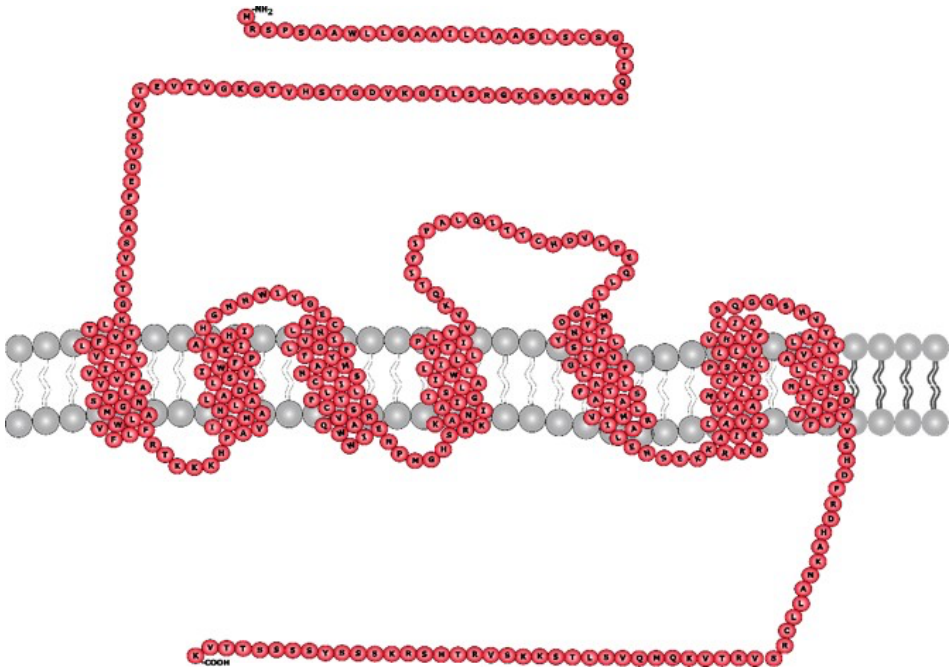

Figure 3. Schematic diagram of PAR2.

1.2.2 Physiological and pathological functions of PAR2

PAR2 is widely expressed through the human body in different systems and organs, including the lung, gastrointestinal tract, skin, kidney, pancreas, liver, heart, smooth muscle cells, fibroblasts, nerves, and immune and inflammatory cells (T-cells, monocytes, macrophages, neutrophils, mast cells, and eosinophils). It is expressed on numerous cell types, especially epithelial and endothelial cells. Thus, it has a role in many physiological processes associated with respiratory and gastrointestinal functions, tissue metabolism, immunity, and neuronal signaling [12, 13]. In the cardiovascular system, PAR2 is widely expressed on endothelial and smooth muscle cells of arteries and veins, and regulates vascular tone and proliferation [16]. In the gastrointestinal tract, PAR2 is also prominently expressed, especially in the small intestine, colon, liver and pancreas, mediating fluid and electrolyte secretion, and it controls gastrointestinal motility and exocrine secretion [17]. Furthermore, PAR2 is involved in transmission of nociceptive messages in the nervous system [18] and in many other physiological processes [19].

Moreover, PAR2 activation and up-regulation have been associated with a number of pathophysiologic conditions, such as metabolic dysfunction, arthritis and multiple sclerosis. PAR2 also plays important roles in modulating inflammatory, gastrointestinal, cardiovascular, respiratory,

and metabolic diseases, and in asthma and cancers [20]. A study by Huesa C. et al. showed that PAR2 deficiency regulated pain, cartilage damage and bone pathology in experimental osteoarthritis [21]. In other studies, PAR2 was also found to be involved in interstitial fibrosis in IgA nephropathy[22]. Systemic PAR2 deletion in ApoE(-/-) mice significantly decreased the expression of inflammatory molecules in the aorta in an atherosclerosis model, indicating the important role of PAR2 in vascular inflammation [6]. In human endothelial cells, PAR2 was activated by autoantibodies from kidney transplantation patient plasma and induced an anti-angiogenic effect, which linked PAR2 to endothelial non-human leukocyte antigens-induced vascular events [7]. Besides, PAR2 plays important role in cancer progression in multiple systems. In breast cancer experimental models, inhibition of tumor growth and angiogenesis was observed after blocking the signaling function of TF but not its coagulation activity, and after inhibition of PAR2 [23]. A similar phenotype was observed in glioblastoma [24]. The PAR2 involved inflammation also plays a role in cancer, especially in nervous system [25].

1.2.3 Classical downstream signaling pathways of PAR2

After activation, PAR2 signals through G proteins $G_{\alpha q}$, G_i , $G_{\alpha 12}$ or β-arrestin 1 and 2 to induce downstream signaling (Fig.4) [26]. $G_{\alpha q}$ is the most commonly activated G-protein, which induces production of inositol triphosphate and diacylglycerol, resulting in downstream activation of protein kinase C and mobilization of calcium ions. In contrast, the G_i pathway inhibits cAMP via inhibition of adenylyl cyclase while also activating the mitogen-activated protein kinase (MAPK) pathways. The $G_{\alpha 12}$ pathway activates the c-Jun N-terminus kinases (JNK) and RhoA thereby promoting cell migration, differentiation and growth, ERK phosphorylation, and subsequent receptor internalization and degradation. Finally, it recruits β-arrestins (Fig.4) [27].

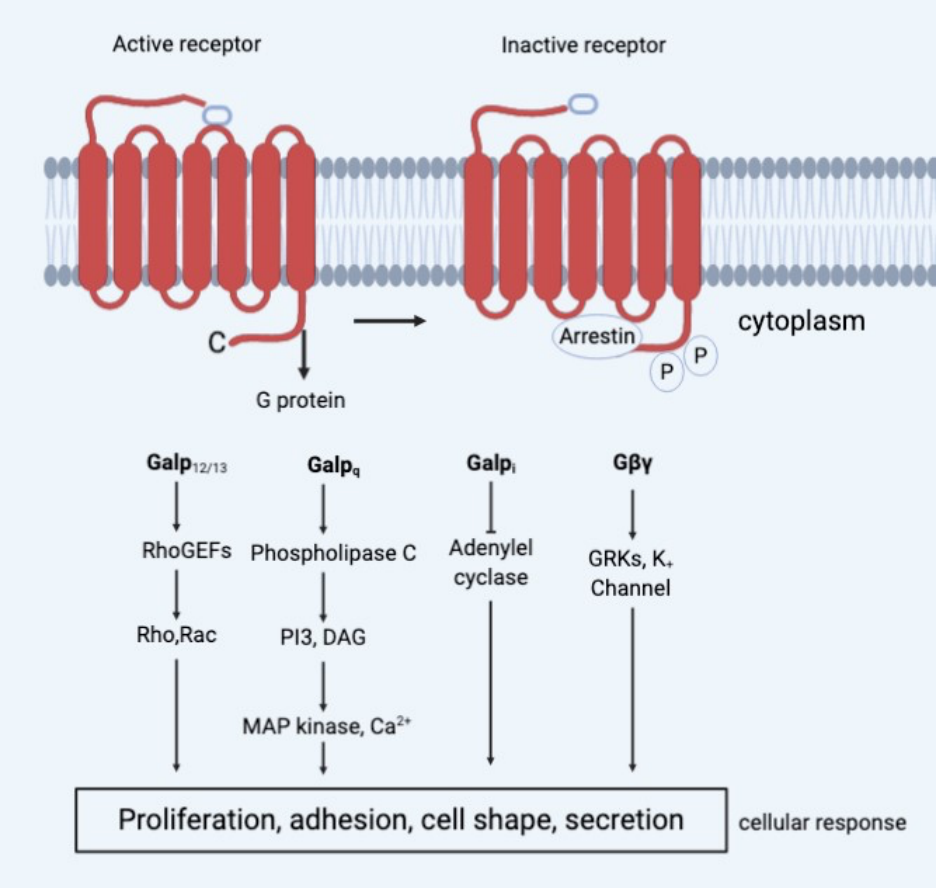

Figure 4. Activation mechanism and classical downstream signaling pathways of PAR2. Protein RhoGEFs, Rho, Rac. MAP kinase mitogen-activated protein kinase, DAG diacylglycerol, PI3 inositol (1,4,5)-trisphosphate, GRKs G-protein-coupled receptor kinases. Rho means Ras homologous family, GEF means guanine nucleotide exchange factor, Rac is a subfamily of the Rho family of GTPase, PI3 stands for phosphatidylinositol-3, DAG stands for diacylglycerol, GRKs stands for G protein-coupled receptor kinases (aquired and modified from [14]).

1.2.4 Biased signaling of PAR2

Apart from the classical agonists, non-canonical agonists such as neutrophil elastase, proteinase-3, and cathepsin G (Fig.5) cleave PAR2 at distinct sites and induce biased signaling [28]. Some of them only induce parts of the pathways. Elastase does not induce Ca^{2+} signals, but triggers PAR2-ERK phosphorylation. It does not trigger β-arrestin recruitment nor receptor-dependent internalization after activating PAR2. Cathepsin G or proteinase-3 does not stimulate PAR2-dependent Ca^{2+} signals or activate ERK phosphorylation or receptor internalization [29]. Some agonists cannot be as efficient as natural ligands. The synthetic peptides of PAR2-tethered ligand, SLIGRL-NH2 (human) and SLIGKV-NH2 (mouse), show lower potency than canonical agonists

[28, 30, 31]. The potency of small molecules, such as AC-98170, is even lower [32]. Small molecule can even activate parts of the PAR2 pathways while suppressing other parts of the pathway. For instance, GB88 is a competitive antagonist for both trypsin and AP-induced Ca2+ signaling, but it can still selectively activate other PAR2-dependent pathways at the same time [15].

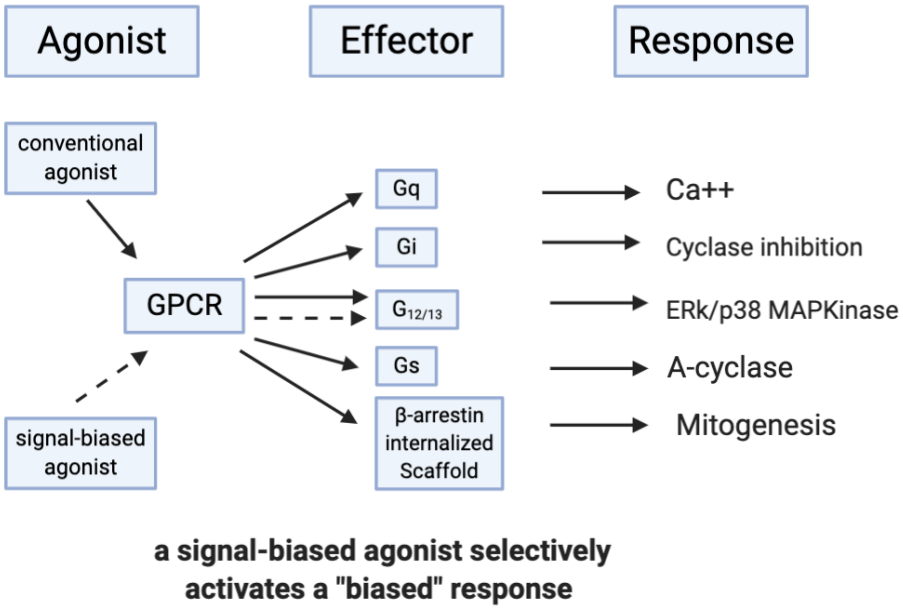

Figure 5. Scheme of PAR2-signaling (aquired and modified from [33]).

1.3 Autoantibodies against GPCRs in kidney graft rejection

1.3.1 Kidney transplantation

Since 1933, when the first kidney transplantation surgery was performed by a Soviet Union surgeon [34], many attempts have been made to improve the prognosis of patients with end-stage renal disease (ESRD). However, the first successful transplantation was not performed until 1950, when a 44-year-old woman with polycystic kidney disease received a kidney graft, and lived another five years with the new organ. In 1964, with rejection prevention therapy, the success rate of transplantation increased dramatically. This therapy then became routine practice after transplantation [35]. Nowadays, kidney transplantation has become the most beneficial treatment for patients who need kidney replacement therapy. Compared to dialysis, it gives ESRD patients a better prognosis and longer life expectancy [36, 37]. However, allograft loss due to rejection still

remains an issue after transplantation. Moreover, complications such as cardiovascular disease and cancer rank in first and second place as the most common causes of mortality and morbidity in kidney transplant recipients [38].

1.3.2 Kidney graft rejection

Rejection has always been a major drawback in transplantation. Once a kidney allograft is transplanted to a recipient, it acts as an alloantigen. This allograft induces an immune response in the recipient which is directed against itself. This whole process is called allograft rejection and if not controlled, it will destroy the graft [1].

Depending on the histopathology and immunological characteristics, renal transplantation rejections can be broadly classified into five categories: hyperacute rejection; acute rejection, divided into antibody-mediated rejection and acute T-cell mediated rejection; chronic rejection; and a mixture of acute rejection superimposed on chronic rejection [39].

The etiology of rejection is complicated and not fully clarified. However, risk factors include: prior sensitization-high panel reactive antibodies; type of transplants, grafts from deceased donors have higher rejection risks than a living donation; advanced age of the donor; prolonged cold ischemia time; HLA (human leukocyte antigen) mismatch; positive B cell crossmatch; and ABO incompatibility [39, 40]. Apart from the classical risk factors, recent studies have illustrated that HLA mismatches between donors and recipients, as well as antibodies targeting non-HLA antigens, such as AT_1R antibodies, participating in acute rejection and long-term kidney allograft survival [4, 41].

1.3.3 GPCR-Abs in kidney graft rejection

1.3.3.1 Autoantibodies against GPCRs

Autoantibodies are able to bind to GPCRs [42]. These antibodies targeting GPCRs activate specific cellular pathways which contribute to various diseases, including organ graft rejection, graft versus host disease, systemic sclerosis, pre-eclampsia, chronic fatigue syndrome, cardiovascular diseases, Alzheimer's disease, and cancer [43-50].

Pathophysiologic GPCR-Abs were initially described in 1956 in Grave's disease. These antibodies are directed against the thyroid-stimulating hormone (TSH) receptor and stimulate the proliferation of the thyroid gland and the absorption of iodine [51]. Later, the pathophysiologic role of anti-beta adrenergic antibodies was found in Chagas disease, idiopathic dilated cardiomyopathy and allergic asthma, successively [52-54].

1.3.3.2 AT_1R-Abs and ET_AR-Abs in kidney transplantation

Many non-HLA antigens have been recently discovered, such as the bioactive C-terminal fragment of perlecan (LG3), Fms-like tyrosine kinase-3 (FLT3) and intercellular adhesion molecule 4 (ICAM4) [55]. Among all the endothelial antigens, two GPCRs, angiotensin type 1 receptor (AT_1R) and endothelin-1 type A receptor (ET_AR), have been studied most frequently [56, 57]. AT_1R is a typical GPCR and mediates mostly the physiologic and pathophysiologic effects of angiotensin II (Ang II) by promoting vasoconstriction, inflammation, proliferation, and fibrosis. Similar actions of endothelin-1 are exerted via ET_AR. [56, 57] On quiescent kidney allograft endothelium, low expression levels of both AT_1R and ET_AR can be detected [3, 58, 59]. However, in activated endothelium, the expression of both receptors is increased, showing their potential to be endothelial antigens [4].

Different from HLA -Abs that need to interact with integrins in order to induce signal transduction, AT_1R-Abs and ET_AR-Abs can interact directly with the receptors and increase downstream GPCR signaling [5]. After activating the receptors, AT_1R-Abs and ET_AR-Abs initiate a common pathologic process, starting with vascular hyperreactivity and microvascular inflammation culminating finally in vascular remodeling, obliterative vasculopathy and progressive tissue fibrosis. Although the two receptors are involved in many different pathologic processes, they induce downstream signaling in a disease-specific manner [60]. For instance, vasoconstrictive mechanisms induced by AT_1R-Abs and ET_AR-Abs in systemic sclerosis and in renal transplantation are G protein-dependent, while AT_1R-Abs induced adrenocortical pathway is G protein-independent [61, 62]. These two GPCRs participate in ABMR mainly through regulating vascular pathology.

1.3.3.3 Antibodies targeting PAR2 in kidney transplantation

PAR2 is expressed on endothelium and involved in the regulation of different vascular functions

and dysfunctions, such as vasodilation and vascular inflammation [6, 63]. Antibodies targeting PAR2 have been proven to regulate angiogenesis in renal transplantation [7]. However, whether PAR2-Abs participate in further pathomechanisms in the transplant, such as endothelial injury, is still largely unknown.

2 Hypothesis and objectives

2.1 Hypothesis

Antibodies against endothelial G protein-coupled receptors (GPCRs) bridge antibody-mediated rejection with vasculopathy. Protease-activated receptor 2 (PAR2) is a GPCR expressed on endothelium. It is involved in vascular physiological and pathophysiological processes, such as vasodilation [63] and vascular inflammation [6]. The levels of antibodies targeting PAR2 seem to be lower in kidney transplant recipients than in healthy people, and these antibodies might be involved in transplant rejection through disturbing the physiological function of the endothelium [7]. However, the structural basis of the antibodies interacting with PAR2 in endothelium is still unknown. We hypothesized that antibodies activating PAR2 by interacting with one of the three extracellular loops induce the activation of the $G_{\alpha q}$, G_i and $G_{\alpha 12}$ pathways differently from PAR2 natural (peptide) agonists, which initiate PAR2 signaling through the second extracellular loop.

2.2 Objectives

In order to test the hypothesis, it was necessary to study each extracellular loop separately. Thus, the objectives of this work were:

1. To test the binding of antibodies with PAR2 extracellular loops (ECLs):

The three ECLs were mutated to alanine stretches respectively, and subcloned into suitable vectors to enable tests in endothelium or yeast.

2. To study kidney transplantation antibodies induced PAR2 intracellular signals in the endothelium system:

PAR2 and its mutants were transfected into human microvascular endothelial cells (HMEC-1) and tested for involved downstream signals.

3. To investigate kidney transplantation antibodies induced PAR2 signals without disturbance from other GPCR signals:

Wild-type and mutated PAR2 were transformed to MMY yeast strains and GPCR activation assays were performed.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals and solutions

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	Promega
10X 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	Sigma Aldrich
dNTP mix	Thermo Fisher Scientific
Dulbecco's Modified Eagle Medium 4.5g/LGlucose	BioWest
Dulbecco's Modified Eagle Medium 1.5 g/L Glucose	BioWest
Dulbecco’s Phosphate-buffered saline	Biochrom

Chemicals and solutions	Manufacturer
Dextrose (D(+)-Glucose)	Applichem
EDTA	Carl Roth
Ethanol 99.8%	Carl Roth
Ethanol 96% (methyl ethyl ketone denaturated)	Herbeta Arzneimittel
FastStart Universal SYBR Green Master	Roche
Fetal calf serum (FCS)	Gibco
Fluorescein-D-glucopyranoside	Invitrogen
Gelatin	Sigma
Glycerin	Carl Roth
hEGF	Sigma Aldrich
Hydrocortisone	Sigma Aldrich
L-Glutamine	PAA
L-arginine	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

Chemicals and solutions	Manufacturer
L-histidine	Applichem
Lithium acetate	Applichem
MCDB-131	c.c. pro GmbH
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	PAA
Pertussis toxin	Enzo
Q5® High-Fidelity DNA Polymerase	New England BioLabs
RNase inhibitor	Thermo Fisher Scientific
single-stranded DNA	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	Sigma Aldrich

3.1.1 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
FLUOstar OPTIMA Microplate Reader	BMG LABTECH
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
T Professional BASIC XL 96 Thermocycler	Biometra
UV-transilluminator Gene Flash	SYNGENE

3.1.2 Kits

Kits	**Manufacturer**
Direct-zol™ RNA MiniPrep	Zymo Research
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific
HiTrap Protein G HP	GE Healthcare
In-Fusion ® HD Cloning Kit	Takara
Luciferase Assay System	Promega
NucleoBond®Xtra Midi/Maxi EF	Macherey-Nagel
NucleoBond®Xtra Mini	Macherey-Nagel

PeqGOLD MicroSpin Cycle pure Kit	PEQLAB
Q5® Site-Directed Mutagenesis Kit	New England BioLabs
Xfect Transfection Reagent	Takara

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

Plasmid	**Manufacturer**
p426 GPD	GlaxoSmithKline
pcDNA3.1(+)	Thermo Fisher Scientific

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 offered by Dr. H.D. Orzechowski

Yeast strains	**Manufacturer**
MMY 14	GlaxoSmithKline
MMY 19	GlaxoSmithKline
MMY 23	GlaxoSmithKline
MMY 24	GlaxoSmithKline

Enzymes and provided buffer	**Manufacturer**

*Hind*III-HF	New England BioLabs
*Xho*I	New England BioLabs
CutSmart Buffer	New England BioLabs

3.1.4 Agonist

Agonist	**Manufacturer**
PAR2-activating peptide (SLIGRL-NH2)	Abcam

3.1.5 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	
Binding buffer, pH 7.0	Na_2HPO_4	20 mM
	In H_2O	
	Filtered (0.45 µm)	
BU salts 10X, pH 7.0	$Na_2HP0_4·7H2O$	7% m/v
	NaH_2PO_4	3% m/v
	In H_2O	
	Sterilized by autoclaving	

Buffer	Reagent	Final conc.
DNA sample loading buffer 6x	Glycerol	30% v/v
	Xylene cyanole	0.25% v/v
	Bromphenolblue	0.25% m/v
	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

Buffer	Reagent	Final conc.
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
	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	

3.1.6 Media

Cell culture media

Complete medium	Conc.

L-glutamine	10 mM
hEGF	10 ng/mL
Hydrocortisone	10 nM
FCS	5% v/v
Penicillin/Streptomycin	100 U/mL/100 µg/mL
	in MCDB-131

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

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	

Yeast media

WHAUL powder	Mass
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
Adjust pH to 7.0	
Sterilize by autoclaving	
Supplemented with	
YNB 10X	10%v/v
40% glucose	5%v/v

WHAUL-His agar, pH 7.0	**Conc.**
WHAUL powder	1.1 g
Bacto agar	20 g
	Milli-Q water 850 mL
Adjust pH to 7.0	
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

3.1.7 Primers

Primers for mutagenesis experiments were designed with the NEBaseChanger™ software online. All primers were provided by Biolegio. Primers used to mutate PAR-2 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'**
PAR2 ECL1 Ala (from nucleotides 412 to 447)	F: GCCTATCACATACATGCCGCCGCCGCGGCTGCTGCGGCAG R: GGCTGCTGCGGCAGCTGCTGCTGCTGTGCTTATTGGCTTTT
PAR2 ECL2Ala (from nucleotides 634 to 705)	F:GTCACCATCCCTTTGTATGTCGTGAAGCAGACCATCTTCATTCCTGCCCTGA R: CTGAACATCACGACCTGTCATGATGTTTTGCCTGAGCAGC TCTTGGTGGG
PAR2 ECL3 Ala (from nucleotides 952 to 969)	F: TTCTGATTAAGAGCCAGGGCCAGAGCCAT R: GCCATGTCTATGCCCTGTACATTGTA

Table 1. Primers for mutagenesis.

Subcloning primer	**5'- to -3'**
PAR2 pcDNA3	F: AGGGAGACCCAAGCTTATGGAAACCCTTTGCCTCA R: TAGATGCATGCTCGAGTCAGTTCATGCTGTCCTTAT

Table 2. Primers for subcloning from Flag-plasmid to pcDNA3-plasmid.

Sequencing primers	**5'- to -3'**
p426GPD from nucleotides 4025 to 4046)	Forward: TTGACCCACGCATGTATCTATC

pcDNA CMV (from nucleotides 769 to 789)	Forward: CGCAAATGGGCGGTAGGCGTG

Table 3. Primers for sequencing.

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

Table 4. Primers for quantitative Real Time-PCR.

3.2 Methods

3.2.1 Subcloning of PAR2

3.2.1.1 Total RNA isolation from HMEC-1 cells

HMEC-1 cells were cultured to 90% confluence in a 6-well plate. RNA extraction was performed using Qiazol reagent (Qiagen) following manufacturer's instructions. RNA pellets were dissolved in DEPC water and concentrations were determined with a NanoDrop® spectrophotometer.

3.2.1.2 Reverse transcription

1µg of total RNA was reverse transcribed to cDNA by M-MuLV Reverse transcriptase in the system described in Table 5, and the reaction conditions are shown in Table 6.

Content	**Volume/Quantity**	**Final Conc.**
10X M-MuLV Reverse Transcriptase Buffer	5µL	1X
dNTP mix 10 mM	8µL	1.6mM
RNase inhibitor 40U/ul	1µL	0.8U/µL

M-MuLV Reverse Transcriptase 200,000U/ml	0.25μL	1U/μL
Oligo	1μL	1μM
RNA	500ng	10ng/mL
Total	50μL	

Table 5. Reverse-transcription reaction.

Temperature (°C)	**Duration (min)**
25	10
40	60
90	5
4	--

Table 6. Reverse-transcription reaction conditions.

3.2.1.3 PCR amplification of target fragments

PAR2 fragments has previously been amplified in the laboratory and described in a thesis [7] performed in the laboratory. PAR2 was amplified using the CloneAmp HiFi PCR Premix with HMEC-1 cDNA as a template. Agarose gel electrophoresis was performed to confirm that a single DNA fragment with the expected size had been obtained.

3.2.2 Site-directed mutagenesis PCR

The wild-type PAR2 plasmids (pcDNA 3.1-PAR2 WT and p426 GPD-PAR2 WT) obtained previously [7] were used as templates to generate mutated constructs for mammalian cell and yeast experiments. Briefly, the components in Table 7 were mixed according to the Q5 site-directed mutagenesis kit instructions and the reaction followed the cycling conditions shown in Table 8 and Table 9.

Component	**25**µL RXN	**Final conc./Quantity**
Q5 Hot Start High-Fidelity 2X Master Mix	12.5µL	1X
10 µM Forward Primer	1.25µL	0.5 µM
10 µM Reverse Primer	1.25µL	0.5 µM
Template DNA	1µL	1-25 ng
Nuclease-free water	9.0µL	

Table 7. Exponential amplification (PCR) components.

STEP	**TEMP (°C)**	**TIME (sec)**
Initial Denaturation	98	30
25 Cycles	98	10
	50-72 (annealing temp was explored with gradient PCR and shown in Table 8)	30
	72	180
Final extension	72	120
Hold	4	--

Table 8. Thermocycling conditions for PCR.

Construct	**Annealing**
PAR2 ECL1 Ala	67.6°C 30 s
PAR2 ECL2 Ala	60°C 30 s
PAR2 ECL3 Ala	70°C 30 s

Table 9. Annealing conditions for the different mutagenesis primers in mutagenesis experiments.

3.2.3 KLD reaction

Next, KLD (Kinase, Ligase & Dpni) treatment was performed (Table 10).

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 10. KLD components.

3.2.4 Bacteria transformation

The KLD product was then transformed in chemically competent NEB 5-alpha Competent *E.coli* (Table 11).

Bacteria	**Provider**	**Genotype**
NEB 5-alpha Competent *E.coli*	NEB	*fhuA2, Δ(argF-lacZ) U169, phoA, glnV44, Φ80Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*

Table 11. Profile of NEB 5-alpha Competent *E.coli*.

Briefly, a tube of NEB 5-alpha Competent *E. coli* cells (Table 11) was thawed on ice. 5µL of the KLD product was added to the tube of thawed cells and mixed by flicking. The mixture was placed on ice for 30 minutes before being heat-shocked at 42°C for 30 seconds. Then the mixture was placed on ice for five minutes. 950 µL of room temperature SOC was pipetted into the mixture and incubated at 37°C for 60 minutes under shaking. The cells were mixed thoroughly by flicking the tube and inverting, and 50 to 100 uL were spread onto a 10cm LB agar plate containing 100 µg/mL

of ampicillin for selection and were incubated overnight at 37°C.

3.2.4.1 Mini-prep

The next step was to purify the plasmid DNA. The day after bacterial transformation, individual isolated colonies were picked from the LB plates and plasmid DNA isolation was performed with the GeneJet kit (Thermo Fisher) according to the manufacturer's instructions. The concentrations were determined with a NanoDrop® spectrophotometer.

3.2.4.2 Verification

These plasmids were then sent for sequencing by LGC Genomics GmbH to verify the sequence of the plasmids with the primers described above (Table 3). The resulting sequences were analyzed using the NCBI standard nucleotide BLAST tool to confirm the absence of mutation.

3.2.4.3 Midi-prep and Bacterial Glycerol Stocks

To obtain a larger amount of plasmid, a midi-prep was performed with the NucleoBond® Xtra Midi endotoxin-free kit, following the high-copy protocol.

3.2.5 IgG isolation from kidney transplant patients

Autoantibody levels against PAR2 from plasma of kidney transplant patients (KTx) and healthy individuals were measured by CellTrend GmbH using a solid-phase ELISA (ethical approval for serum samples: EA2/068/07).

Isolation of immunoglobulin G (IgG) was performed with HiTrap Protein G columns (GE Healthcare). Plasma was thawed overnight at 4 °C and was centrifuged at 5000 rpm for 15 minutes. Supernatant was then filtered with 0.45 µm PVDF syringe filters to remove the remaining cell debris. Filtered liquid was diluted with equal volume of binding buffer and ready to be loaded to columns.

The isolation started by equilibrating the HiTrap Protein G column with binding buffer (50mL). Next, samples were loaded on the column. Flow-through was loaded three times to increase the

efficiency of IgG binding. The column was then washed with binding buffer and captured IgG was eluted with elution buffer before being neutralized with neutralization buffer.

Isolated IgG was then dialyzed at 4 °C against DMEM low glucose for cell experiments, or WHAUL medium for yeast assay, and stirred constantly. The final concentration of IgG was determined by Labor Berlin.

3.2.6 PAR2-IgG levels detection

The PAR-2 autoantibody levels were determined in healthy controls (healthy con) and kidney transplantation patients (KTx Pat) by ELISA. 197 healthy controls were involved and their mean serum PAR2-IgG level was 17.68 units with a median value of 15.24 units. However, the PAR2-IgG level of the KTx Pats was much lower, with a mean value of 4.5 U/mL (Fig. 6) [7].

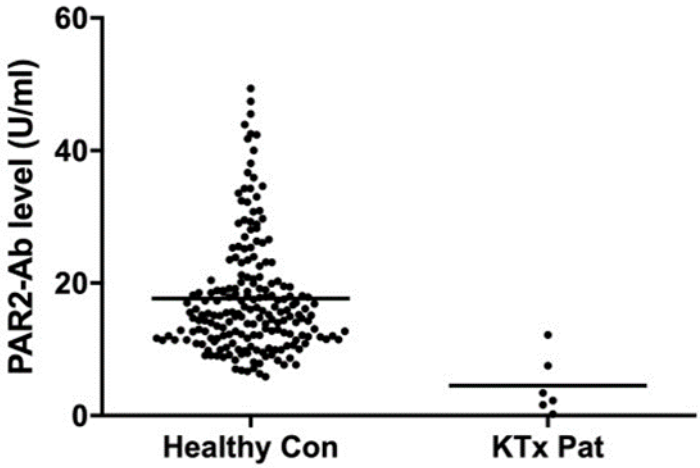

Figure 6. Measurement of the titers of PAR2-IgG in healthy individuals and kidney transplantation patients. The 197 serum samples from healthy individuals were provided by Celltrend Company. Kidney transplantation patients were patients who had received at least one kidney transplant before sample collection. PAR2-IgG levels were detected using Celltrend PAR2 autoantibodies ELISA kit [7].

3.2.7 PAR2 activation assay in yeasts

The GPCR activation assay in yeast is based on the functional coupling of heterologous G proteins to the pheromone response pathway of budding yeast *Saccharomyces cerevisiae*. It has been established as an experimental system for the detection of GPCR activation [64]. The MMY yeast strains were been modified to express chimeric G proteins in which the five C-terminal residues of

Gpa1p were replaced by the corresponding sequence of the human Gα subunits (Table12). In these strains, the heterologous G protein-coupled receptor are functionally coupled to the pheromone-response pathway of the budding yeast *Saccharomyces cerevisiae*[64]. Further modifications were introduced to this system to analyze mammalian heterologous GPCR [64].

Strain	**Genotype**
MMY14	*MAT***a** *his3 leu2 trp1 ura3 can1 gpa1*Δ*::ADE2 far1*Δ*::ura3 sst2*Δ*::ura3*Δ *fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2*Δ*::G418^R*
MMY19	MMY11 *TRP1::Gpa1/Gq$^{(5)}$*
MMY23	MMY11 *TRP1::Gpa1/Gi$^{1(5)}$*
MMY24	MMY11 *TRP1::Gpa1/Gi$^{3(5)}$*

Table 12. Profiles of yeast strains.

3.2.7.1 Yeast culture

A yeast strain was spread on a YPD plate. After two days of incubation at 30°C, a single colony was picked and pre-cultured in 3mL of YPD media overnight. The yeasts were then expanded by inoculating 1.5mL of the pre-culture into 100mL of YPD media and cultivated under shaking at 30°C for three hours.

3.2.7.2 Yeast transformation

Transformation was performed using the lithium acetate (LiAc)/ single-stranded DNA (ssDNA)/ polyethylene glycol (PEG) method and PAR-2 plasmid was transformed into the yeast strains. Finally, yeast cells were plated on WHAUL-His plates and incubated for three days.

3.2.7.3 Working stock

Colonies of transformed yeast strains were reinoculated on a new WHAUL-His plate as a working stock which could be stored in 4°C fridge for a few months.

3.2.7.4 mRNA level quantification

Yeasts were picked from each colony with toothpicks and pre-cultured in 3mL of YPD media at

30°C under shaking. The next day, total RNA was isolated from these pre-cultures with the Direct-zol™ RNA MiniPrep kit (Company) according to the manufacturer's protocol. The RNA concentration was then measured by NanoDrop® spectrophotometer. Reverse Transcription was performed to obtain cDNA, as previously described, related components are shown in Table 13. Quantitative Real Time-PCR (qRT-PCR) was performed with Applied Biosystems® 7500 qRT-PCR System according to the manufacturer's protocol (Table 14). The amount of fluorescence released during an amplification cycle is proportional to the relative quantity of mRNA initially present.

Content	**Volume**	**Final Conc.**
10X M-MuLV Reverse transcriptase Buffer	5µL	1X
dNTP mix 10 mM	8µL	1.6mM
RNase inhibitor 40U/ul	1µL	0.8U/µL
M-MuLV Reverse transcriptase 200,000U/ml	0.25µL	1U/µL
Oligo	1µL	1µM
RNA	500ng	10ng/mL
Total	50µL	

Table13. Reverse transcription reaction.

Content	**Volume**	**Final Conc.**
SYBR Green	4µL	--
Primer se 10 µM	0.5µL	0.39µM
Primer ase 10 µM	0.5µL	0.39µM
ddH2O	7µL	--
cDNA	1µL	--
Total	13µL	

Table 14. qRT-PCR reaction.

3.2.7.5 Yeast G protein activation assay

Yeast colonies from working stock were picked and transferred individually into WHAUL medium containing histidine and incubated overnight at 30°C under shaking. These cultures were then added to the FDGlu assay medium containing different stimulants. Plates were protected from light and incubated 20 hours at 30°C under shaking. After incubation, fluorescent measurement was performed with FLUOstar OPTIMA Microplate Reader at an excitation of 485 nm and emission of 535 nm.

3.2.8 Luciferase assay

HMEC-1 cells were grown in 24-well plates with complete growth medium in 37 °C, 5% CO2 incubator. After reaching 90% confluency, cells were transfected with luciferase reporter plasmids with the Xfect transfection kit. Transfected cells were then grown in 0.5% FCS medium and incubated for 4 hours before changing back to complete growth medium. After overnight incubation, cells were stimulated with trypsin or KTx-IgG. 6 hours after stimulation, cells were washed three times with 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.

3.2.9 G_i α subunit inhibition

Bordetella pertussis toxin (PTX) was applied one hour prior to other stimulations at a concentration of 5ng/µL.

3.2.10 Statistical analysis

Data are expressed as mean ± SEM, *n* stands for the number of independent experiments. Comparison between two groups were performed by student t-test. While one-way-ANOVA with tukey's post-hoc test was used when comparisons were made among three or more groups.

Differences were considered significant when p value was smaller than 0.05. GraphPad Prism software version 5.01 was used for the calculations.

4 Results

4.1 Levels of PAR2-IgG in healthy individuals and renal transplant patients

As mentioned in the Method part, PAR2-IgG levels were detected in the laboratory before this study. In this work, healthy individuals showed a much higher PAR2-IgG level than kidney transplant patients, whose mean value was 4.5 U/mL (Fig. 6)[7].

4.2 Activation of PAR2 pathways in HMEC-1 cells and yeast

PAR2 is widely expressed in mammalian endothelium and plays a role in vascular disease. [6] The expression and activation of PAR2 has been detected in primary human endothelial cells, as well as in the endothelial cell line HMEC-1 [7] [13]. In this study, PAR2-activating mechanisms and downstream pathways were explored in yeast and HMEC-1 cells.

4.2.1 Activation of PAR2 pathways in yeasts

The MMY yeast assay system is a G protein-activating assay system. It is based on the functional coupling of a human GPCR with the pheromone response pathway of budding yeast *Saccharomyces cerevisiae.* Modifications were made to make it possible for this yeast system to detect specific G proteins activation individually [64]. As most GPCRs interact inefficiently with *Gpa1* and mammalian G_{α} subunits have low affinity for the yeast $G_{\beta\gamma}$, a series of *Gpa1*–G_{α} chimeras has been developed to incorporate receptor-binding properties of mammalian subunits into a *Gpa1* subunit that interacts with the yeast signaling machinery. The endogenous receptor (*Ste2*) was removed by gene disruption to prevent sequestering G-proteins into inactive complexes. Readouts of growth (*His3*) or color (*LacZ*) were achieved by coupling expression of the reporter genes to the pheromone response element (PRE) of the *Fus1* promoter [65].

4.2.1.1 Optimization of the GPCR activation assay

To investigate the G protein activation induced by PAR2 natural agonist trypsin and KTx-IgG, PAR2 was transformed into the strains MMY14 ($G_{\alpha q}$) and MMY19 ($G_{\alpha 12}$).

The following experiment was performed to optimize plasmid concentrations to achieve a better

PAR2 expression level for further experiments. An amount of 0, 0.5, 1 or 3 µg of p426GPD PAR2 WT plasmid was transformed into MMY14 and MMY 19 yeast strains individually. RNA was extracted for measurement of the PAR2 mRNA level. For MMY14, transformation with 0.5µg plasmid increased the PAR2 transcription level (around 15000-fold), and higher effects could also be seen in the 1µg and 3 µg transformed yeasts (Fig. 7A). For MMY19, 3 µg increased the PAR2 transcription level by 280-fold (Fig.7B).Thus, 3µg was chosen for both yeast strains.

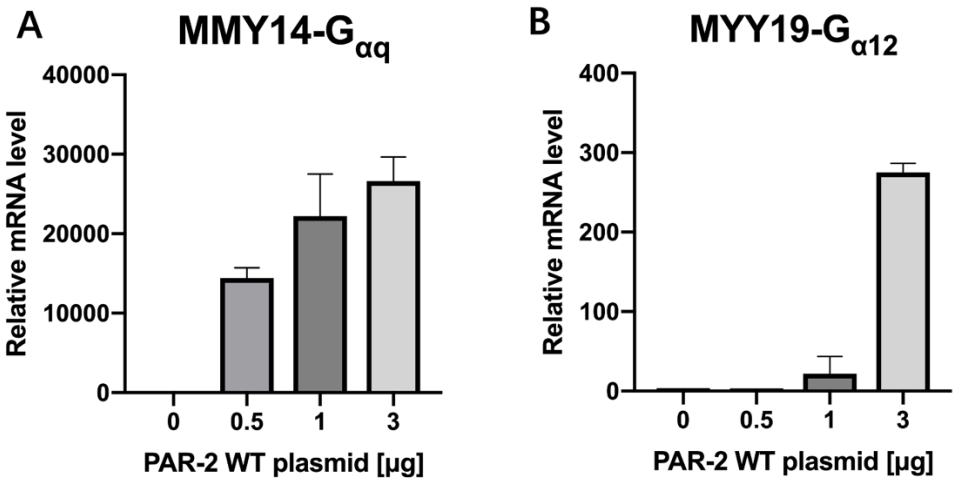

Figure 7. Wild-type PAR2 (PAR2 WT) transcription levels in two yeast strains. MMY14 ($G_{\alpha q}$) (A) and MMY19 ($G_{\alpha 12}$) (B) yeasts were transformed with different amounts of plasmid and incubated for three days. Values are from three colonies and are presented as mean ± SEM.

4.2.1.2 Trypsin selectively activates PAR2 in yeasts

To explore the G alpha subunits involved in trypsin-induced PAR2 activation in yeasts, MMY14 and 19 strains transformed with PAR2 WT were treated with different concentrations of trypsin and incubated for 20 hours. As shown in Figure 8A, $G_{\alpha q}$ was not activated by trypsin. However, $G_{\alpha 12}$ was significantly activated with 1nM trypsin ($p < 0.0001$) (Fig. 8B). In conclusion, $G_{\alpha 12}$ is involved in the trypsin-induced PAR2 activation.

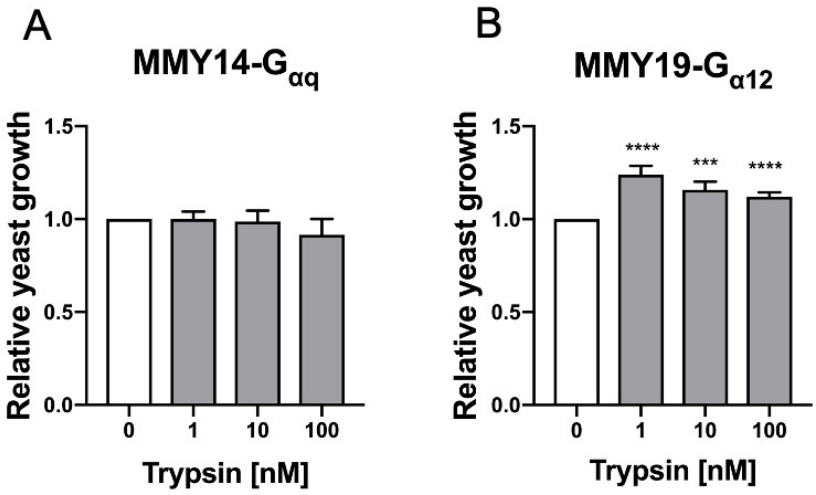

Figure 8. Trypsin selectively activates PAR2 in yeasts. Yeast strains MMY14 (A)or MMY19 (B) were stimulated with different concentrations of trypsin and incubated for 20 hours. Data are presented as mean ± SEM. *** or**** compares to the unstimulated group, *** $p < 0.001$, **** $p < 0.0001$, n=9.

4.2.1.3 KTx-IgGs activate PAR2 in yeasts

Further, the effect of kidney transplantation IgGs (KTx-IgG) activating PAR2 was explored in MMY14 and MMY19 strains. These IgGs were isolated from the plasma of seven kidney transplantation patients, with a mean PAR2-IgG level of 4.5 U/mL. MMY14 or MMY19 transformed with PAR2 WT was stimulated with 0, 0.5, 1or 1.5 mg/mL of KTx-IgG for 20 hours. The fluorescence intensity was then detected to assess yeast growth. In MMY14, only a concentration of 1.5 mg/mL of KTx-IgGs significantly activated $G_{\alpha q}$ (*$p<0.05$) (Fig.9A). In MMY19, KTx-IgGs activated $G_{\alpha 12}$ (Fig.9B). Thus, KTx-IgG activate both $G_{\alpha q}$ and $G_{\alpha 12}$, but $G_{\alpha 12}$ was more sensitive to KTx-IgG.

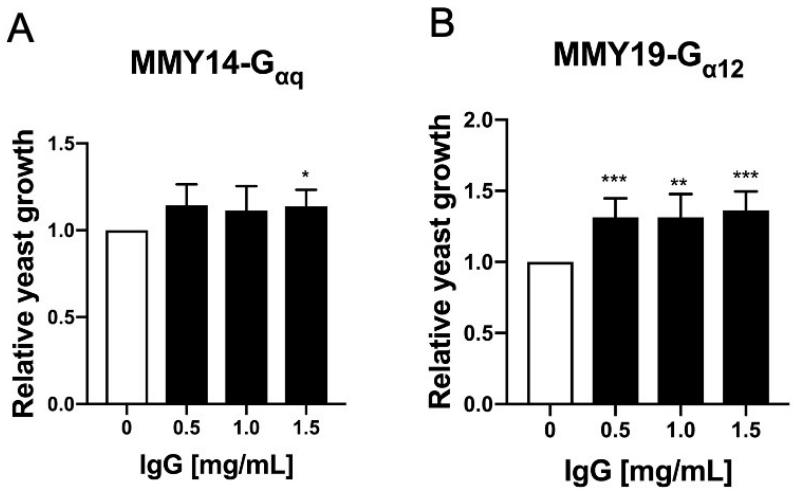

Figure 9. KTx-IgGs activate PAR2 in yeasts. Yeast strains MMY 14(A) or MMY 19(B) were stimulated with different concentrations and incubated for 20 hours. Data are presented as mean ± SEM. *, ** or *** compare to the unstimulated group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=18.

In conclusion, the results of the yeast experiments showed that KTx-IgG activated both $G_{\alpha q}$ and $G_{\alpha 12}$, whereas trypsin only activated $G_{\alpha 12}$.

4.2.2 Function of PAR2 extracellular loops in downstream signaling in HMEC-1 cells

Consistent with all GPCRs, PAR2 has three extracellular loops which are potentially accessible for binding of extracellular ligands. However, the detailed binding mechanisms between PAR2 and its agonists still need to be understood. Moreover, the specific pathways involved in each activation model are not clarified.

Wild-type PAR2 was manipulated by mutating its three extracellular loops to alanine to generate three mutations, PAR2 ECL1-Ala, PAR2 ECL2-Ala and PAR2 ECL3-Ala in order to investigate the functions of the individual extracellular loops in downstream signaling. Along with wild-type PAR2, these mutants were inserted in pcDNA 3.1, and overexpressed in HMEC-1 cells. Luciferase reporters SRF ($G_{\alpha 12}$), SRE (ERK) or NFAT ($G_{\alpha q}$) were used in HMEC-1 to monitor the activation of the different G alpha subunits and related downstream signaling.

4.2.2.1 Optimization of trypsin concentration in HMEC-1

To optimize the concentration of trypsin in further experiments, different concentrations of trypsin (1nM, 10nM and 100nM) were used to stimulate PAR2 WT expressing HMEC-1 cells (Fig.10). Luciferase reporter assays for NFAT, SRF and SRE were performed to detect the activation of PAR2 signaling pathways mediated by $G_{\alpha q}$, $G_{\alpha 12}$ and ERK, respectively. Figure 10A shows that $G_{\alpha q}$ is activated by trypsin with the highest effect observed with 100nM, so that this concentration was chosen for later experiments. $G_{\alpha 12}$ was more strongly activated than $G_{\alpha q}$, so that 10nM was chosen to keep the effects comparable (Fig. 10B). Finally, for the ERK pathway, significant activation was already seen with 1nM and 10nM, so that 10nM was kept for further experiments (Fig. 10C). These concentrations were applied for all experiments.

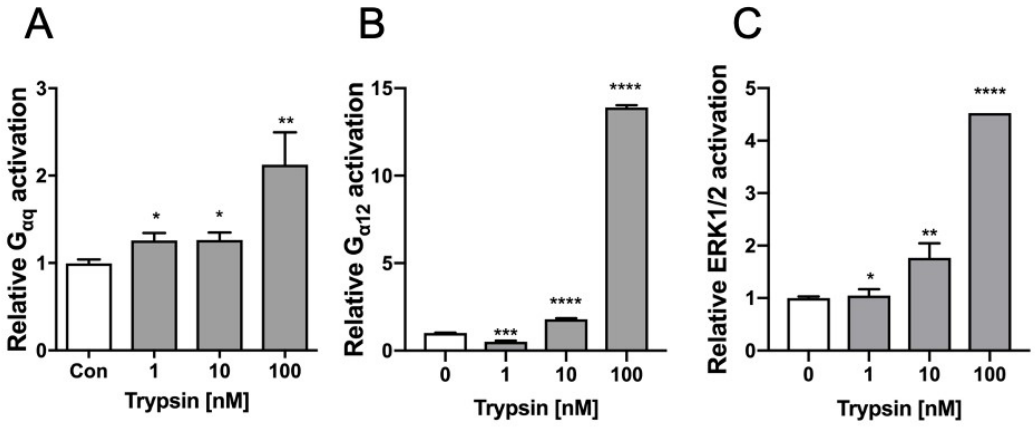

Figure 10. Trypsin activation of $G_{\alpha q}$ (A), $G_{\alpha 12}$ (B) and ERK (C). HMEC-1 were transfected with PAR2 WT (100ng per well in 24-well plates) and reporter plasmids (NFAT 200ng, SRF 100ng or SRE 100ng per well in 24-well plates); luciferase activity was detected six hours later. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=12.

4.2.2.2 Involvement of PAR2 extracellular loops in $G_{\alpha q}$ activation in HMEC-1

Trypsin-induced $G_{\alpha q}$ activation was detected by an NFAT luciferase reporter assay in HMEC-1 transfected with PAR2 WT or one of the ECL mutants, PAR2 ECL1-Ala, PAR2 ECL2-Ala, or PAR2 ECL3-Ala.

After stimulation with 100nM trypsin, PAR2 WT induced $G_{\alpha q}$ significant activation ($p < 0.0001$).

Conversely, $G_{\alpha q}$ was not activated in any of the three mutations after stimulation (Fig.11).

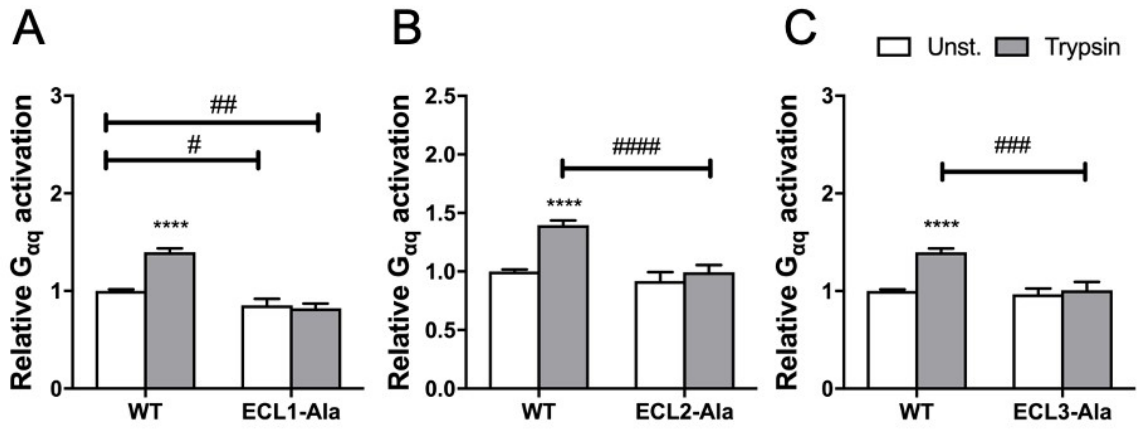

Figure 11. Trypsin-induced $G_{\alpha q}$ activation in wild-type or mutated PAR2-expressing HMEC-1 cells.

NFAT activity is depicted relative to NFAT activity in absence of stimulation. * , **, *** or **** compares to the unstimulated group with the same PAR2 genotype, while #, ##, ###, or #### compares between genotypes. Data is presented as mean ± SEM, * or # $p < 0.05$, ** or ## $p < 0.01$, *** or ### $p < 0.001$,**** or ####$p < 0.0001$, n=21.

KTx-IgG-induced $G_{\alpha q}$ activation was observed with an NFAT luciferase reporter assay in HMEC-1 transfected with PAR2 WT or one of the mutants PAR2 ECL1-Ala, PAR2 ECL2-Ala, or PAR2 ECL3-Ala. IgGs isolated from the plasma of seven kidney transplantation patients were used to stimulate HMEC-1 at a concentration of 1mg/mL which had been confirmed in a previous doctoral work to be the optimal concentration [7]. In contrast to trypsin, KTx-IgGs did not activate $G_{\alpha q}$ in HMEC-1 expressing PAR2 WT. However, mutation of PAR2 ECL1, but none of the other extracellular loops, induced $G_{\alpha q}$ activation after KTx-IgG stimulation (Fig.12).

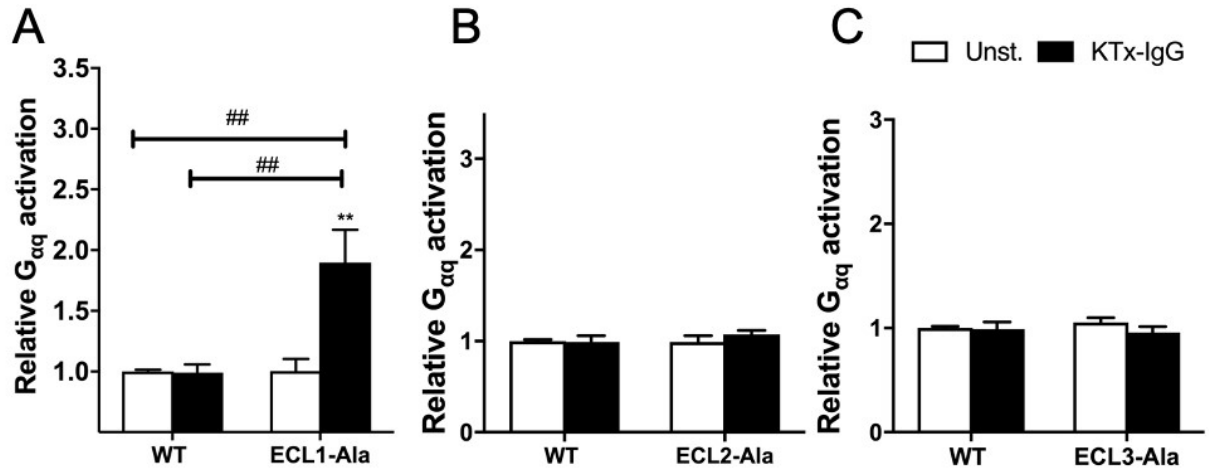

Figure 12. KTx-IgG-activated $G_{\alpha q}$ in wild-type or mutated PAR2-transfected HMEC-1 cells.

NFAT activity is depicted relative to NFAT activity in absence of stimulation.** compares to the unstimulated group with the same PAR2 genotype, ## compares between genotypes. Data is presented as mean ±SEM, ** or ## $p < 0.01$, n=15.

The results show that trypsin activation depends on the three extracellular loops. Conversely, $G_{\alpha q}$ is normally not activated by KTx-IgG, but gets activated when the first extracellular loop is mutated. Thus, ECL1 is involved in KTx-IgG regulation of $G_{\alpha q}$.

4.2.2.3 Involvement of PAR2 extracellular loops in $G_{\alpha 12}$ activation in HMEC-1

Trypsin-induced $G_{\alpha 12}$ activation was studied using an SRF luciferase reporter assay in HMEC-1 transfected with PAR2 WT or one of the mutants, PAR2 ECL1-Ala, PAR2 ECL2-Ala, or PAR2 ECL3-Ala.

After stimulation with 10nM trypsin, WT PAR2 significantly activated $G_{\alpha 12}$ ($p < 0.001$) (Fig. 13). $G_{\alpha 12}$ was also significantly activated in PAR2 mutants, PAR2 ECL1-Ala and ECL2-Ala albeit at lower levels (Fig. 13 A and B). However, PAR2 ECL3-Ala triggered $G_{\alpha 12}$ even without stimulation (Fig. 13 C), which indicated a constitutive activation.

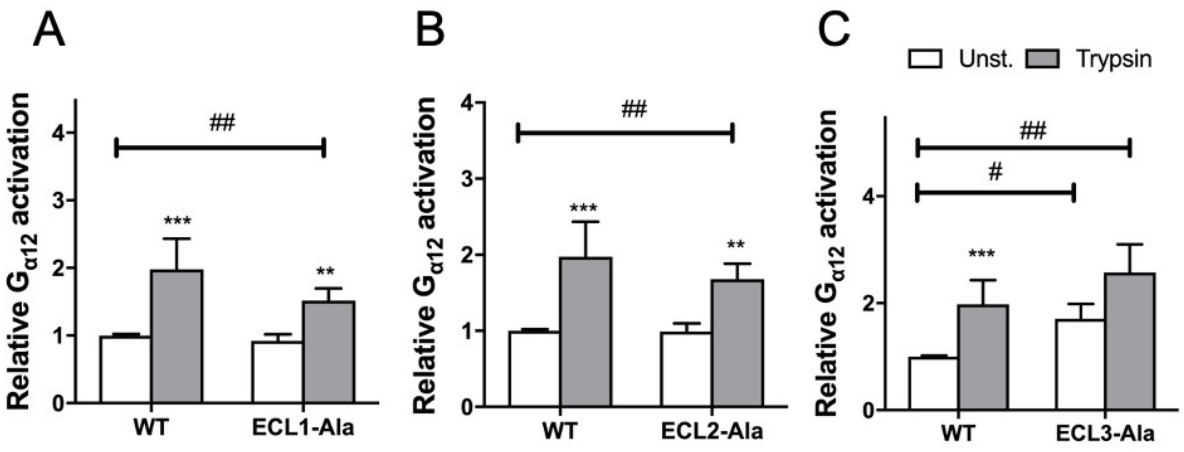

Figure 13. Trypsin-induced $G_{\alpha 12}$ activation in wild-type or mutated PAR2-expressing HMEC-1 cells.

SRF activity is depicted relative to SRF activity in absence of stimulation. * , ** or *** compares to the unstimulated group with the same PAR2 genotype,# or ## compares between genotypes. Data is presented as mean ±SEM, # $p < 0.05$, ** or ## $p < 0.01$, *** $p < 0.001$, n=21.

An SRF luciferase reporter assay was used to detect KTx-IgG-induced $G_{\alpha 12}$ activation in HMEC-1 transfected with plasmids of PAR2 WT, PAR2 ECL1-Ala, PAR2 ECL2-Ala or PAR2 ECL3-Ala. $G_{\alpha 12}$ was activated by PAR2 WT (Fig. 14). Mutation of the first or second extracellular loops induced a significantly higher activation of $G_{\alpha 12}$ than with WT PAR2 (Fig. 14 A and B). However, PAR2 ECL3-Ala induced $G_{\alpha 12}$ activation similar to that with PAR2 WT (Fig. 14).

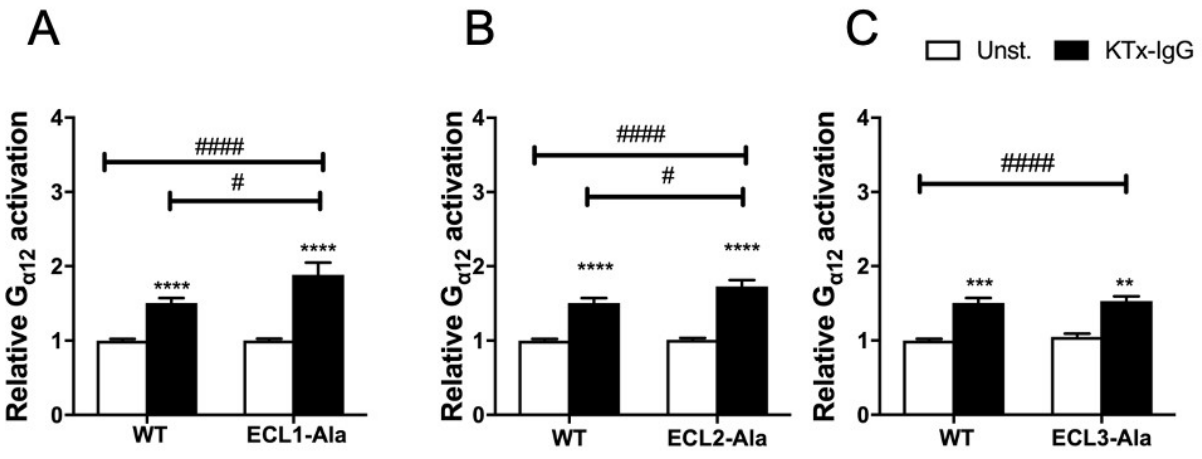

Figure 14. KTx-IgG- induced $G_{\alpha 12}$ activation in wild0type or mutated PAR2-expressing HMEC-1 cells.

SRF activity is depicted relative to SRF activity in absence of stimulation. **, *** or **** compares to the unstimulated group with the same PAR2 genotype, # or #### compares between genotypes. Data is presented as mean ± SEM, # $p < 0.05$, **$p < 0.01$, *** $p < 0.001$,**** or ####$p < 0.0001$,n=15.

In this section, results show that trypsin and KTx-IgG activate $G_{\alpha 12}$ in HMEC-1 regardless of the replacement of the three ECLs. Mutation of ECL3 even increased the baseline level of $G_{\alpha 12}$ activation when stimulated with trypsin, which suggests a constitutive activation. When stimulated with KTx-IgG, there is an increased activation when mutating ECL1 or 2. In conclusion, changing the structure of ECL1 or 2 facilitates the activation of PAR2 by KTx-IgG perhaps because the epitope where the IgG bind becomes more accessible.

4.2.2.4 Involvement of PAR2 extracellular loops in ERK1/2 pathway activation in HMEC-1

Trypsin-induced ERK1/2 pathway activation in HMEC-1 transfected with PAR2 or one of its three mutants was assessed by an SRE luciferase reporter assay. In HMEC-1 transfected with PAR2 WT, 10nM trypsin significantly activated ERK ($p < 0.01$). Mutating ECLs of PAR2 changed ERK1/2 baseline activity. PAR2 ECL1-Ala showed a trend to increase the baseline activity of the ERK1/2 pathway (Fig.15 A), while ECL2-Ala and ECL3-Ala showed decreases (Fig.15 B and C). The ERK1/2 pathway was activated in all three mutants, but at a lower level than in PAR2 WT (Fig.15).

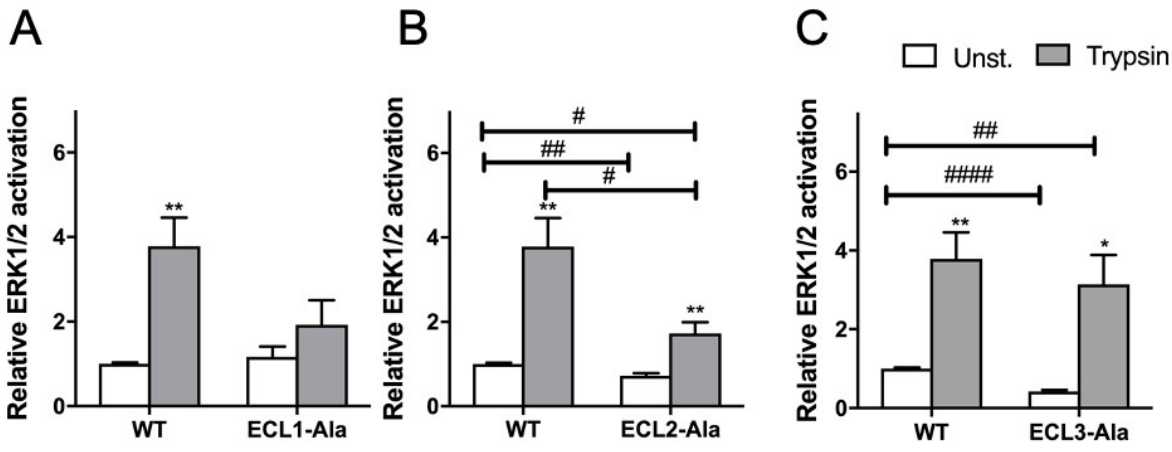

Figure 15. Trypsin-induced ERK1/2 pathway activation in wild-type or mutated PAR-expressing HMEC-1 cells. SRE activity is depicted relative to SRE activity in absence of stimulation. * or** compares to the unstimulated group with the same PAR2 genotype, #, ##, or #### compares between genotypes. Data is presented as mean ±SEM, * or # $p < 0.05$, ** or ## $p < 0.01$, ####$p < 0.000,1$, n=21.

In HMEC-1 transfected with wild-type PAR2 or one of its three mutations, an SRE luciferase reporter assay was used to detect KTx-IgG-induced ERK1/2 pathway activation. After KTx-IgG stimulation, the ERK1/2 pathway was significantly activated in HMEC-1 expressing PAR2 WT (Fig. 16). Mutated PAR2 also activated the ERK1/2 pathway. In PAR2 ECL1-Ala and ECL3-Ala, the ERK1/2 pathway was activated at almost the same level as with PAR2 WT (Fig. 16 A and C), while PAR2 ECL2-Ala induced a higher activation (Fig.16 B).

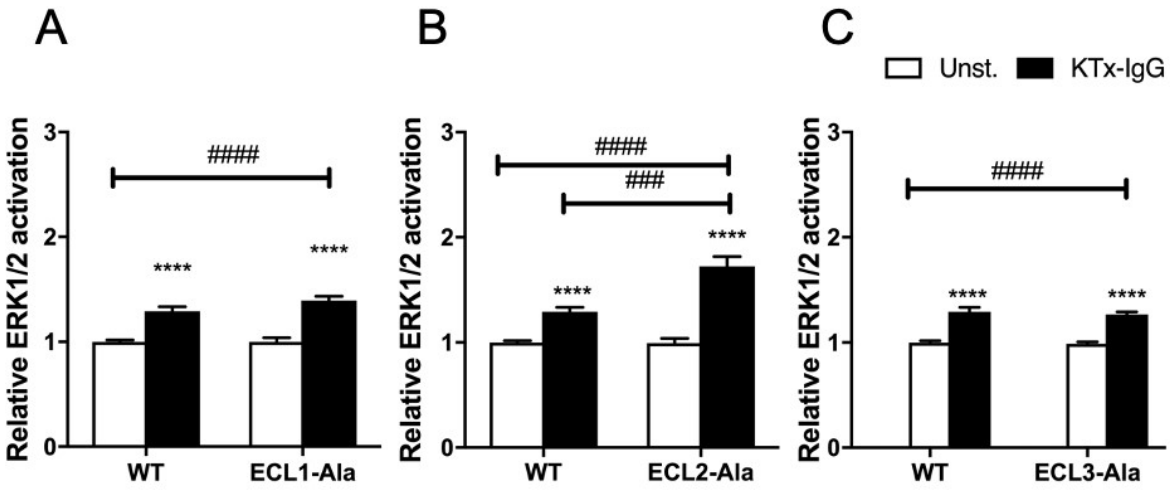

Figure 16. KTx-IgG-induced ERK1/2 pathway activation in wild-type or mutated PAR2-expressing HMEC-1 cells. SRE activity is depicted relative to SRE activity in absence of stimulation. **** compares to the unstimulated group with the same PAR2 genotype, ### or #### compares between genotypes. Data is presented as mean ± SEM, ### $p < 0.001$,**** or ####$p < 0.0001$, n=15.

This indicates that 10 nM trypsin or KTx-IgGs were able to activate the ERK1/2 pathway in PAR2 WT and ECL mutants. Mutations decreased the activation induced by trypsin, and PAR2 ECL2-Ala increased the activation induced by KTx-IgGs. Moreover, all three mutations changed the baseline activation of ERK when stimulated with trypsin. Taken together, we demonstrate here that mutating the extracellular loop impairs the activation of the ERK1/2 pathway by trypsin. Concerning the IgG, mutating ECL2 increased ERK1/2 activation, meaning that KTx-IgGs bind more efficiently to PAR2 without an intact ECL2 and improve ERK1/2 activation.

4.2.3 G_i participates in PAR2 activation in yeast and HMEC-1

$G_{\alpha q}$ is one of the PAR2-coupled G-proteins. Results presented in 4.2.2.2 showed that KTx-IgG did not activate $G_{\alpha q}$ in HMEC-1, which conflicted with the results in yeast experiments. Since NFAT might also be regulated by G alpha subunit i (G_i), G_i was studied in the yeast system and mammalian cells [66]. The yeast strain MMY24, in which human GPCR couples with G_i, was used. In HMEC-1, a G_i inhibitor, pertussis toxin (PTX), was used to explore the involvement of G_i in PAR2-mediated NFAT activation.

4.2.3.1 PAR2 AP and KTx-IgG activate G_i in yeast system

The effect of different PAR2 modulators on G_i was tested in the yeast system. PAR2-activating peptide (AP) was able to activate G_i (Fig. 17 A), while trypsin showed almost no effect (Fig. 17 B). A variation between different KTx-IgGs was observed: some of the IgGs (Pat.2, Fig. 17C) were able to activate G_i, albeit not significantly, while others (Pat.3 and Pat.4, Fig. 17 E and F) could not. To understand these differences, PAR2 levels from the serum samples of kidney transplantation patients were measured (Fig. 17 G). KTx-IgGs with higher PAR2-IgG levels were more likely to activate G_i.

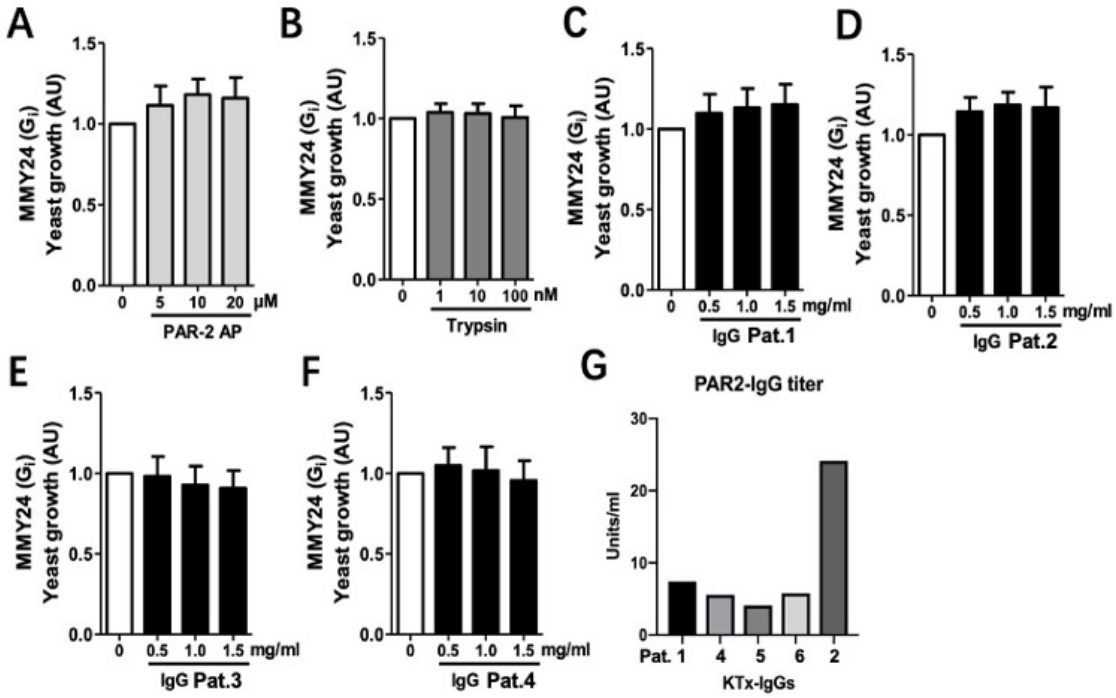

Figure 17. G_i activation in PAR2-expressing yeasts and PAR2-IgG levels. Yeast strain MMY24 was transformed with PAR2 WT and stimulated with PAR2 AP (A), trypsin (B) , KTx-IgGs (C, D, E, and F) and incubated for 20 hours, PAR2-IgG levels were shown in G. Pat. patient.

4.2.3.2 G_i inhibition of PAR2 in HMEC-1

NFAT activity in PAR2 WT expressing HMEC-1 was detected with or without G_i inhibitor. Trypsin induced a higher NFAT activation than KTx-IgGs (Fig. 18). PTX decreased NFAT activation induced by trypsin. However, PTX had almost no effect on the activation of NFAT by KTx-IgGs (Fig.18).

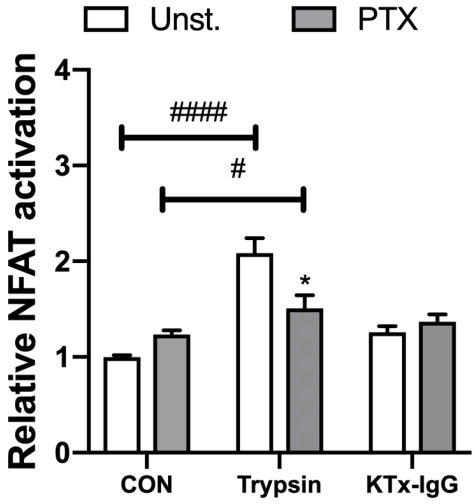

Figure 18. NFAT activity with or without G_i inhibitor in HMEC-1 expressing PAR2 WT.

NFAT activity is depicted relative to NFAT activity in absence of stimulation. HMEC-1 cells expressing PAR2 WT were incubated with or without 5ng/ul PTX before being stimulated with or without trypsin (100nM) or KTx-IgGs (1mg/mL) for six hours. *compares to trypsin-stimulated group without PTX treatment, #or #### compares to control group. Data is presented as Mean ± SEM, * or # $p < 0.05$, #### $p < 0.0001$, n=15.

These results indicate that PAR2 AP, but not trypsin, was able to activate G_i in yeasts. KTx-IgGs with higher PAR2-IgG levels were more likely to activate G_i. In HMEC-1, G_i inhibition decreased the activation of NFAT induced by trypsin, but not by KTx-IgGs. This result indicates that Gi is also involved in trypsin-induced NFAT activation in PAR2.

In summary, trypsin was able to activate all three intracellular pathways, $G_{\alpha q}$, $G_{\alpha 12}$ and ERK. All extracellular loops were involved in the $G_{\alpha q}$ and ERK pathways. On the other hand, KTx-IgG activated $G_{\alpha 12}$ and ERK, but not $G_{\alpha q}$. ECL1 was involved in $G_{\alpha q}$ and $G_{\alpha 12}$ activation, while ECL2 was only involved in $G_{\alpha 12}$ activation, and ECL3 participated in ERK activation. Moreover, mutation of ECL3 even increased the baseline activity of $G_{\alpha 12}$.

5 Discussion

Kidney transplantation is the optimal therapy for patients who need renal replacement treatment. However, allograft rejection is an obstacle and increases graft loss after transplantation [1]. The pathophysiology of rejection is mainly an inflammation induced by the immune response in the recipient against the kidney allograft [67]. In this immune response, antigens such as HLA antigens and non-HLA antigens, play important roles as targets [68]. The immune response differs, depending on the type of rejection. In hyperacute rejection, the pre-existing circulating antibodies in the recipient directed against the foreign antigens (usually the ABO blood group and HLA) cause rejection in minutes or hours after transplantation. With acute T-cell mediated rejection, T cells are activated by antigen-presenting cells recognizing foreign antigens and infiltrate the allograft causing damage. In antibody-mediated rejection, antibodies against donor antigens (HLA or non-HLA) lead to damage through activation of complement-dependent or -independent pathways which recruit NK cells and other immune cells to attack the allograft[1].

The treatment plan depends on multiple factors, including the type of rejection, the severity of the histological lesions, the chronicity score, and the recipient comorbidity [35]. For hyperacute rejection, prevention, such as determining ABO-compatibility and pre-transplant crossmatch is key. For antibody-mediated rejection (ABMR), plasma exchange is needed to remove the high-level antibodies. Moreover, high-dose intravenous immunoglobulin and Bortezomib, which is a proteasome inhibitor, are also used. In addition, Rituximab, which is a chimeric monoclonal antibody against the CD20 antigen and induces B cell lysis via complement-dependent cytotoxicity and antibody-mediated cellular cytotoxicity, is also used [69]. For T-cell mediated rejection, immune suppressants are used. In chronic rejections, ABMR is the main cause and can be addressed as described above [70, 71].

Antibodies targeting G protein-coupled receptors (GPCR-Abs), constituting the majority of the non-HLA antibodies, participate in ABMR and contribute to acute or chronic graft loss. The corresponding antigens are mainly expressed in the endothelium and binding of the antibodies results in activation of the targeted receptor, contributing to or inducing vasculopathy [2]. PAR2

belongs to the GPCR family, is located in the endothelium and has been associated with vascular events in previous studies [6]. Lower titer PAR2-Abs of kidney transplantation patients have previously been detected compared to healthy individuals [7].

In this work, the structural basis of PAR2-IgG binding to PAR2 and the activation of downstream intracellular pathways were studied in human endothelial cells and yeasts. The results showed that PAR2 coupled with different G proteins and induced specific pathways when activated by its natural ligand trypsin or IgG isolated from patients after kidney transplantation. In the yeast GPCR-activating system, KTx-IgGs triggered activation of both the $G_{\alpha 12}$ and $G_{\alpha q}$ pathways, while trypsin selectively activated $G_{\alpha 12}$. In HMEC-1, trypsin stimulating PAR2-WT activated the $G_{\alpha q}$, $G_{\alpha 12}$ and ERK pathways, while KTx-IgGs only activated $G_{\alpha 12}$. Furthermore, 3 ECL mutants of the receptor were investigated. ECL2 mutation induced KTx-IgGs-dependent $G_{\alpha q}$ activation and a higher baseline activation of the ERK pathway. Moreover, ECL3 mutation induced a constitutive activation of $G_{\alpha 12}$. Further, all 3 mutants eliminated trypsin-induced $G_{\alpha q}$ activation and decreased the ERK activation induced by trypsin. In the G_i yeast activation assay, PAR2-activating peptide and KTx-IgGs with higher PAR2-IgG titers induced G_i activation. In HMEC-1, G_i inhibition attenuated the activation of NFAT induced by trypsin, but not by KTx-IgGs.

5.1 GPCR activation assays

In this study, the function and downstream pathways of PAR2 G-proteins were measured in a yeast GPCR activation assay. As described in the Methods section, the yeast strains were engineered to increase their sensitivity and specificity in detecting GPCR signals [64]. Many studies support the power of this system. A. P. Ijzerman et.al [72] described a few constitutively active and gain of function mutations for the human adenosine A_{2B} receptor. Furthermore, a second-site mutation was found to restore the function to a mutant deficient M3 muscarinic receptor [73]. Glucose and sucrose were reported to act as agonists and mannose as antagonist ligands of Gpr1 [74]. An attempt has also been made to improve the yeast systems. A refined yeast-based GPCR biosensor employing a high-sensitivity strain that incorporated both a G protein alpha subunit engineered receptor and a fluorescent reporter (ZsGreen) was reported [75]. The efficiency of this new system was tested in

the HTR1A receptor with site-directed mutations. However, some GPCRs were reported to fail to couple to the yeast pheromone response system. In some cases, the yeast cell wall can provide a barrier for some ligands and prevent access to GPCR at the cell surface [64, 65, 76].

Apart from the yeast GPCR activating assay system used in this study, other methods are available to make up for the drawbacks of yeast system. Multiple models have been developed to assess GPCR signaling pathways in mammalian cells, such as guanine nucleotide-binding assays, cAMP assays, and intracellular calcium assays [77]. These models are based on different mechanisms and have advantages in detecting specific pathways in different systems. GPCR-mediated guanine nucleotide exchange is monitored by measuring [^{35}S]GTPγS binding to plasma membranes prepared from cells expressing GPCRs of interest [78]. This method is attractive because guanine nucleotide exchange is a proximal event to receptor activation and not easily disturbed by other cellular processes. Unfortunately, this assay is generally limited to G_i-coupled receptors [78]. Yet, focus of this study was the investigation of $G_{\alpha q}$ and $G_{\alpha 12}$ in addition to G_i.

cAMP assays detect cAMP in whole cells or adenylyl cyclase activity in membranes to sense the activation of upstream GPCRs. Screening G_s-coupled receptors is generally straightforward, whereas screening G_i-coupled receptors in cAMP assays can be considerably more difficult [79, 80]. Intracellular calcium assay for GPCR signaling detection is based on the concept that activation of GPCRs can lead to increases in calcium in cells. Measuring intracellular calcium has advantages due to the availability of calcium-sensitive fluorescent dyes and automated real- time charge-coupled device-based fluorescence plate readers, which make this method sensitive and amenable to automation [81, 82]. In the present study, the yeast assay system was adopted since yeasts express a single human GPCR, so there is no interference from other receptors There is only a single pathway regulated by this GPCR and these yeasts also only express one G protein. The yeasts are thus a tightly regulated model which enables preliminary studies before a more complex environment is introduced.

5.2 PAR2-mediated signaling pathways activation is ligand-dependent

Trypsin is the natural agonist of PAR2; it cleaves PAR2 in its N-terminus, revealing the tethered ligand, which then interacts with the second extracellular loop to activate multiple G-protein-dependent and -independent pathways [13].The results of this study show that trypsin stimulated PAR2-WT-triggered $G_{\alpha q}$, $G_{\alpha 12}$ and ERK pathway activation in HMEC-1, which is in agreement with previous findings in other cell types [33]. Trypsin has shown potential in regulating endothelial functions, especially vasorelaxation [83]. Moreover, PAR2 has also been found to be involved in endothelial physiology and pathophysiology through activating the $G_{\alpha q}$, $G_{\alpha 12}$ and ERK pathways. A study in glioblastoma showed that PAR2 induced VEGF production , which is an important proangiogenic component, through the ERK pathway [84].$G_{\alpha q}$ also contributes critically to VEGF-A-dependent permeability control and angiogenic behavior in endothelium [85]. A previous doctoral work on PAR2 also showed that trypsin increased the promoter activity of VEGF [7]. In combination with those studies on trypsin and PAR2 downstream pathways, it suggests that trypsin has a potential in regulating endothelial functions, especially angiogenesis, through PAR2 in the HMEC-1 system. However, other physiological or pathophysiological functions of trypsin-induced PAR2 activation in HMEC-1 still need to be explored.

Results with KTx-IgG showed a different signaling pattern compared to trypsin. KTx-IgG binding to PAR2 activated the $G_{\alpha 12}$ and ERK pathways, but not the $G_{\alpha q}$ pathway. These data are in agreement with other studies. Unlike the conventional agonists, GPCR-Abs usually bind to other sites of the receptor, triggering different activations [2]. Moreover, compared to the natural agonist Ang II, AT_1R-Abs induced prolonged stimulating activity of AT_1R in endothelium of kidney transplantation recipients [2, 5]. Antibodies binding to ET_AR triggered a stronger signal than the natural agonist ET-1 in systemic sclerosis [86]. As shown in the Results section, PAR2 Ab titers are higher in healthy individuals than in kidney transplant patients. A previous doctoral work also found that PAR-2-IgG exerted both agonistic and proangiogenic activities in endothelial cells in kidney transplantation patients[7]. KTx-IgG inducing specific signals might be involved in the vascular events leading to kidney graft rejection. For instance, in a previous doctoral work performed in the laboratory, the ERK and P38 pathways have been identified as being involved in the upregulation

of the VEGF (vascular endothelial growth factor) level in endothelial cells when stimulated with PAR2 autoantibodies and sd participating in the antiangiogenic effect [7].

5.3 Involvement of extracellular loops in PAR2 signaling

In this work, the role of the three extracellular loops in the activation of PAR2 was studied. In the past decades, the less conserved extracellular loops have received increasing interest, particularly after the publication of several GPCR crystal structures that clearly showed the extracellular loops to be involved in ligand binding [87, 88].

Previous research regarding the ECL1 role in GPCR is limited. Nevertheless, it has been shown that mutation to alanine of six conserved amino acids in ECL1 of neurotensin receptor 1 resulted in an agonist-specific loss of maximal binding capacity, leading to the remodeling of this receptor [89]. In the case of the human cannabinoid receptor 1, ECL1 mutation exhibited a loss of affinity for its agonist CP 55940, revealing that ECL1 has a crucial role for CP 55940-induced receptor activation [90]. Moreover, a single glycine in ECL1 was found to be the critical determinant for the pharmacological specificity of dopamine D2 and D3 receptors. In this study, ECL1 also demonstrated an important role, predominantly in $G_{\alpha q}$ activation

ECL2 is the longest loop of the three, not only in PAR2, but also in many other GPCRs. Futhermore, it was found to be crucial for the activation of many GPCRs. Previous studies from Prof. Dragun's laboratory regarding AT_1R and ET_AR also clarified the importance of ECL2 in the activation of the receptors [91]. In 2017, the crystal structure of PAR2 in complex with two distinct antagonists and a blocking antibody was reported, and ECL2 was found to be involved in these three interactions [92]. Decades ago, the importance of ECL2 in PAR2 activation was reported by Hollenberg M. D. et al., who showed that site-directed mutagenesis of ECL2 decreased the potency of trypsin and synthesized ligands by various extents [93]. Another study also identified ECL2 to be the primary determinant of agonist specificity [94]. In this study, ECL2 was involved in $G_{\alpha q}$, $G_{\alpha 12}$ and ERK pathway activation after stimulation with trypsin or ECL2 agonist peptide. Moreover, the results in this study show that replacement of ECL2 increased the sensitivity of the $G_{\alpha 12}$ and ERK1/2 pathways to KTx-IgG stimulation. These results show both the multiple and important role of ECL2 in PAR2 activation.

The results of this study additionally shows an important role of ECL3 at least for the downstream signaling of PAR2 by trypsin and for an inhibitory function on the basal receptor activation status. Mutation of ECL3 eliminated the activation of $G_{\alpha q}$ and enhanced the activation of ERK induced by trypsin. The change of ECL3 also induced a $G_{\alpha 12}$ activation without stimulation. Nevertheless, ECL3 is less involved in KTx-IgG PAR2 activation. ECL3 is the shortest extracellular loop and the least studied. Apart from simply passive linking between transmembrane domains, it also participates in agonist recognition [94, 95]. Substitutions of ECL3 in the FSH receptor increased FSH-induced receptor internalization, while another mutation exert impaired ERK1/2 phosphorylation [96]. Moreover, the noradrenaline transporter ECL3 contributed to substrate and inhibitor selectivity [97].

5.4 PAR2 and G_i

As described in the Results section, PTX, an inhibitor of G_i, decreased the activation of NFAT induced by trypsin in endothelial cells. However, PTX was not able to reduce KTx-IgG-induced NFAT activation, which indicated a lesser involvement of G_i in this process.

G_i and G_o proteins are both substrates for pertussis toxin produced by *Bordetella pertussis*, the infectious agent in whooping cough [98]. Pertussis toxin is an ADP-ribosylase enzyme that adds an ADP-ribose moiety on one particular cysteine residue in G_i and G_o proteins, preventing their coupling to and activation by GPCRs, thus turning off G_i and G_o cell signaling pathways [99, 100]. PAR2 is believed to couple to G_i [15]. It has been reported that regulators of G-protein-mediated signaling, RGS16 and RGS18, inhibit PAR2/G_i-mediated signaling [101]. In contrast, the study of McCoy et al. showed opposite results in COS-7 cells, that PAR1 but not PAR2 coupled to G_i [102]. This evidence indicates that PAR2 might couple to G_i in some specific cells, and G_i was only activated by certain agonists in special environments.

Further, the G_i yeast assay system was used to test the participation of G_i in PAR2 activation. In MMY24, trypsin was not able to induce G_i activation, while PAR2 AP could activate this G protein. PAR2 AP activates the receptor by binding directly on the ECL2, while trypsin activates PAR2 by cleaving the receptor on its N- terminus to expose the agonist which binds on the ECL2 [19]. The

difference in activating mechanism might contribute to the different results of PAR2 AP and trypsin. Moreover, there was a variation between different KTx-IgGs: some of them could activate G_i, while some could not. In spite of the differences between modulators and their biased signaling, these results suggest that G_i is able to be activated by PAR2 in the yeast system.

G_i alpha subunit, namely G protein i (G_i), is one of the heterotrimeric G protein alpha subunits. Like other G alpha subunits, G_i is also capable of activating intracellular signaling pathways in response to activation of cell surface G protein-coupled receptors (GPCRs) [103]. G_i proteins primarily inhibit the cAMP-dependent pathways by inhibiting adenylyl cyclase activity, decreasing the production of cAMP from ATP, which, in turn, results in decreased activity of cAMP-dependent protein kinase [104]. Therefore, additional measurements of cAMP generation might have strengthened the functional G_i coupling of PAR2.

6 Future Prospects

PAR2 plays a crucial role in regulating multiple cell functions in vascular inflammation. The present study illustrated that PAR2 autoantibodies modulate PAR2 activation and downstream signaling most probably by binding to specific ECLs These antibodies also activate specific G proteins which are partly different from those induced by the natural ligand trypsin and which might regulate specific cellular functions such as cellular metabolism, proliferation angiogenesis and wound healing processes involved in the pathophysiology of graft failures. Specific identified targets should be addressed in further studies for drug development to treat antibody-mediated rejection.

7 References

1. Nankivell, B.J. and S.I. Alexander, *Rejection of the kidney allograft.* N Engl J Med, 2010. **363**(15): p. 1451-62.
2. Dragun, D., R. Catar, and A. Philippe, *Non-HLA antibodies in solid organ transplantation: recent concepts and clinical relevance.* Current opinion in organ transplantation, 2013. **18**(4): p. 430-435.
3. Banasik, M., M. Boratyńska, K. Kościelska-Kasprzak, M. Krajewska, O. Mazanowska, D. Kamińska, D. Bartoszek, M. Zabińska, M. Myszka, B. Nowakowska, A. Hałoń, T. Dawiskiba, P. Chudoba, and M. Klinger, *The impact of non-HLA antibodies directed against endothelin-1 type A receptors (ETAR) on early renal transplant outcomes.* Transplant immunology, 2014. **30**(1): p. 24-29.
4. Dragun, D., D.N. Muller, J.H. Brasen, L. Fritsche, M. Nieminen-Kelha, R. Dechend, U. Kintscher, B. Rudolph, J. Hoebeke, D. Eckert, I. Mazak, R. Plehm, C. Schonemann, T. Unger, K. Budde, H.H. Neumayer, F.C. Luft, and G. Wallukat, *Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection.* N Engl J Med, 2005. **352**(6): p. 558-69.
5. Dragun, D., A. Philippe, R. Catar, and B. Hegner, *Autoimmune mediated G-protein receptor activation in cardiovascular and renal pathologies.* Thrombosis and haemostasis, 2009. **101**(4): p. 643-648.
6. Hara, T., P.T. Phuong, D. Fukuda, K. Yamaguchi, C. Murata, S. Nishimoto, S. Yagi, K. Kusunose, H. Yamada, T. Soeki, T. Wakatsuki, I. Imoto, M. Shimabukuro, and M. Sata, *Protease-Activated Receptor-2 Plays a Critical Role in Vascular Inflammation and Atherosclerosis in Apolipoprotein E-Deficient Mice.* Circulation, 2018. **138**(16): p. 1706-1719.
7. Chen, L., *Funktionelle Charakterisierung der endothelvermittelten autoimmunen PAR-2 Aktivierung*, in *Charité - Universitätsmedizin Berlin*. 2020, (Accessed 2020-06-17T08:41:41Z, at https://refubium.fu-berlin.de/handle/fub188/27311).
8. De Francesco, E.M., F. Sotgia, R.B. Clarke, M.P. Lisanti, and M. Maggiolini, *G Protein-Coupled Receptors at the Crossroad between Physiologic and Pathologic Angiogenesis: Old Paradigms and Emerging Concepts.* Int J Mol Sci, 2017. **18**(12).
9. Hauser, A.S., M.M. Attwood, M. Rask-Andersen, H.B. Schiöth, and D.E. Gloriam, *Trends in GPCR drug discovery: new agents, targets and indications.* Nature Reviews Drug Discovery, 2017. **16**(12): p. 829-842.
10. Rieser, P., *The insulin-like action of pepsin and pepsinogen.* Acta Endocrinol (Copenh), 1967. **54**(2): p. 375-9.
11. Vu, T.K., D.T. Hung, V.I. Wheaton, and S.R. Coughlin, *Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation.* Cell, 1991. **64**(6): p. 1057-68.
12. Ramachandran, R., F. Noorbakhsh, K. Defea, and M.D. Hollenberg, *Targeting proteinase-activated receptors: therapeutic potential and challenges.* Nat Rev Drug Discov, 2012. **11**(1): p. 69-86.
13. Macfarlane, S.R., M.J. Seatter, T. Kanke, G.D. Hunter, and R. Plevin, *Proteinase-*

activated receptors. Pharmacol Rev, 2001. **53**(2): p. 245-82.
14. Wojtukiewicz, M.Z., D. Hempel, E. Sierko, S.C. Tucker, and K.V. Honn, *Protease-activated receptors (PARs)—biology and role in cancer invasion and metastasis.* Cancer and Metastasis Reviews, 2015. **34**(4): p. 775-796.
15. Yau, M.K., L. Liu, and D.P. Fairlie, *Toward drugs for protease-activated receptor 2 (PAR2).* J Med Chem, 2013. **56**(19): p. 7477-97.
16. Bucci, M., F. Roviezzo, and G. Cirino, *Protease-activated receptor-2 (PAR2) in cardiovascular system.* Vascular Pharmacology, 2005. **43**(4): p. 247-253.
17. Sekiguchi, F., K. Takaoka, and A. Kawabata, *Proteinase-activated receptors in the gastrointestinal system: a functional linkage to prostanoids.* Inflammopharmacology, 2007. **15**(6): p. 246-251.
18. Noorbakhsh, F., N. Vergnolle, M.D. Hollenberg, and C. Power, *Proteinase-activated receptors in the nervous system.* Nature Reviews Neuroscience, 2003. **4**(12): p. 981-990.
19. Coelho, A.M., V. Ossovskaya, and N.W. Bunnett, *Proteinase-activated receptor-2: physiological and pathophysiological roles.* Curr Med Chem Cardiovasc Hematol Agents, 2003. **1**(1): p. 61-72.
20. Liu, H., F. Liu, Y. Peng, Y. Liu, L. Li, X. Tu, M. Cheng, X. Xu, X. Chen, G. Ling, and L. Sun, *Role of mast cells, stem cell factor and protease-activated receptor-2 in tubulointerstitial lesions in IgA nephropathy.* Inflammation research 2010. **59**(7): p. 551-559.
21. Huesa, C., A.C. Ortiz, L. Dunning, L. McGavin, L. Bennett, K. McIntosh, A. Crilly, M. Kurowska-Stolarska, R. Plevin, R.J. van 't Hof, A.D. Rowan, I.B. McInnes, C.S. Goodyear, J.C. Lockhart, and W.R. Ferrell, *Proteinase-activated receptor 2 modulates OA-related pain, cartilage and bone pathology.* Annals of the rheumatic diseases, 2016. **75**(11): p. 1989-1997.
22. Grandaliano, G., P. Pontrelli, G. Cerullo, R. Monno, E. Ranieri, M. Ursi, A. Loverre, L. Gesualdo, and F.P. Schena, *Protease-activated receptor-2 expression in IgA nephropathy: a potential role in the pathogenesis of interstitial fibrosis.* Journal of the American Society of Nephrology : JASN, 2003. **14**(8): p. 2072-2083.
23. Schaffner, F., H.H. Versteeg, A. Schillert, N. Yokota, L.C. Petersen, B.M. Mueller, and W. Ruf, *Cooperation of tissue factor cytoplasmic domain and PAR2 signaling in breast cancer development.* Blood, 2010. **116**(26): p. 6106-13.
24. Dutra-Oliveira, A., R.Q. Monteiro, and A. Mariano-Oliveira, *Protease-activated receptor-2 (PAR2) mediates VEGF production through the ERK1/2 pathway in human glioblastoma cell lines.* Biochem Biophys Res Commun, 2012. **421**(2): p. 221-7.
25. Shimamoto, R., T. Sawada, Y. Uchima, M. Inoue, K. Kimura, Y. Yamashita, N. Yamada, T. Nishihara, M. Ohira, and K. Hirakawa, *A role for protease-activated receptor-2 in pancreatic cancer cell proliferation.* Int J Oncol, 2004. **24**(6): p. 1401-6.
26. Hilger, D., M. Masureel, and B.K. Kobilka, *Structure and dynamics of GPCR signaling complexes.* Nat Struct Mol Biol, 2018. **25**(1): p. 4-12.
27. Kimple, A.J., D.E. Bosch, P.M. Giguere, and D.P. Siderovski, *Regulators of G-protein signaling and their Galpha substrates: promises and challenges in their use as drug discovery targets.* Pharmacol Rev, 2011. **63**(3): p. 728-49.
28. Dulon, S., D. Leduc, G.S. Cottrell, J. D'Alayer, K.K. Hansen, N.W. Bunnett, M.D.

Hollenberg, D. Pidard, and M. Chignard, *Pseudomonas aeruginosa elastase disables proteinase-activated receptor 2 in respiratory epithelial cells.* Am J Respir Cell Mol Biol, 2005. **32**(5): p. 411-9.
29. Ramachandran, R., K. Mihara, H. Chung, B. Renaux, C.S. Lau, D.A. Muruve, K.A. DeFea, M. Bouvier, and M.D. Hollenberg, *Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2 (PAR2).* J Biol Chem, 2011. **286**(28): p. 24638-48.
30. Gardell, L.R., J.N. Ma, J.G. Seitzberg, A.E. Knapp, H.H. Schiffer, A. Tabatabaei, C.N. Davis, M. Owens, B. Clemons, K.K. Wong, B. Lund, N.R. Nash, Y. Gao, J. Lameh, K. Schmelzer, R. Olsson, and E.S. Burstein, *Identification and characterization of novel small-molecule protease-activated receptor 2 agonists.* J Pharmacol Exp Ther, 2008. **327**(3): p. 799-808.
31. Tanaka, Y., F. Sekiguchi, H. Hong, and A. Kawabata, *PAR2 triggers IL-8 release via MEK/ERK and PI3-kinase/Akt pathways in GI epithelial cells.* Biochem Biophys Res Commun, 2008. **377**(2): p. 622-626.
32. Seitzberg, J.G., A.E. Knapp, B.W. Lund, S. Mandrup Bertozzi, E.A. Currier, J.N. Ma, V. Sherbukhin, E.S. Burstein, and R. Olsson, *Discovery of potent and selective small-molecule PAR-2 agonists.* J Med Chem, 2008. **51**(18): p. 5490-3.
33. MD, H., D. Mihara K Fau - Polley, J.Y. Polley D Fau - Suen, A. Suen Jy Fau - Han, D.P. Han A Fau - Fairlie, R. Fairlie Dp Fau - Ramachandran, and R. R, *Biased signalling and proteinase-activated receptors (PARs): targeting inflammatory disease.* Br J Pharmacol, 2014. **171**(5): p. 1180-94.
34. Matevossian, E., H. Kern, N. Huser, D. Doll, Y. Snopok, J. Nahrig, J. Altomonte, I. Sinicina, H. Friess, and S. Thorban, *Surgeon Yurii Voronoy (1895-1961) - a pioneer in the history of clinical transplantation: in memoriam at the 75th anniversary of the first human kidney transplantation.* Transpl Int, 2009. **22**(12): p. 1132-9.
35. Halloran, P.F., *Immunosuppressive Drugs for Kidney Transplantation.* New England Journal of Medicine, 2004. **351**(26): p. 2715-2729.
36. McDonald, S.P. and G.R. Russ, *Survival of recipients of cadaveric kidney transplants compared with those receiving dialysis treatment in Australia and New Zealand, 1991-2001.* Nephrol Dial Transplant, 2002. **17**(12): p. 2212-9.
37. Naesens, M., D.R. Kuypers, K. De Vusser, P. Evenepoel, K. Claes, B. Bammens, B. Meijers, B. Sprangers, J. Pirenne, D. Monbaliu, I. Jochmans, and E. Lerut, *The histology of kidney transplant failure: a long-term follow-up study.* Transplantation, 2014. **98**(4): p. 427-35.
38. Haberal, M., F. Boyvat, A. Akdur, M. Kirnap, U. Ozcelik, and F. Yarbug Karakayali, *Surgical Complications After Kidney Transplantation.* Exp Clin Transplant, 2016. **14**(6): p. 587-595.
39. Joosten, S.A., Y.W. Sijpkens, C. van Kooten, and L.C. Paul, *Chronic renal allograft rejection: pathophysiologic considerations.* Kidney Int, 2005. **68**(1): p. 1-13.
40. Pallardo Mateu, L.M., A. Sancho Calabuig, L. Capdevila Plaza, and A. Franco Esteve, *Acute rejection and late renal transplant failure: risk factors and prognosis.* Nephrol Dial Transplant, 2004. **19 Suppl 3**: p. iii38-42.
41. Reindl-Schwaighofer, R., A. Heinzel, G.A. Gualdoni, L. Mesnard, F.H.J. Claas, and R. Oberbauer, *Novel insights into non-HLA alloimmunity in kidney transplantation.*

Transplant international : official journal of the European Society for Organ Transplantation, 2020. **33**(1): p. 5-17.
42. Meyer, C. and H. Heidecke, *Antibodies Against GPCR.* Front Biosci (Landmark Ed), 2018. **23**: p. 2177-2194.
43. Fu, M.L., H. Herlitz, G. Wallukat, E. Hilme, T. Hedner, J. Hoebeke, and A. Hjalmarson, *Functional autoimmune epitope on alpha 1-adrenergic receptors in patients with malignant hypertension.* Lancet, 1994. **344**(8938): p. 1660-3.
44. Wallukat, G., V. Homuth, T. Fischer, C. Lindschau, B. Horstkamp, A. Jupner, E. Baur, E. Nissen, K. Vetter, D. Neichel, J.W. Dudenhausen, H. Haller, and F.C. Luft, *Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor.* J Clin Invest, 1999. **103**(7): p. 945-52.
45. Duffner, U., B. Lu, G.C. Hildebrandt, T. Teshima, D.L. Williams, P. Reddy, R. Ordemann, S.G. Clouthier, K. Lowler, C. Liu, C. Gerard, K.R. Cooke, and J.L. Ferrara, *Role of CXCR3-induced donor T-cell migration in acute GVHD.* Exp Hematol, 2003. **31**(10): p. 897-902.
46. Fox, R.I., *Sjogren's syndrome.* Lancet, 2005. **366**(9482): p. 321-31.
47. Kohr, D., P. Singh, M. Tschernatsch, M. Kaps, E. Pouokam, M. Diener, W. Kummer, F. Birklein, A. Vincent, A. Goebel, G. Wallukat, and F. Blaes, *Autoimmunity against the beta2 adrenergic receptor and muscarinic-2 receptor in complex regional pain syndrome.* Pain, 2011. **152**(12): p. 2690-700.
48. Li, H., D.C. Kem, S. Reim, M. Khan, M. Vanderlinde-Wood, C. Zillner, D. Collier, C. Liles, M.A. Hill, M.W. Cunningham, C.E. Aston, and X. Yu, *Agonistic autoantibodies as vasodilators in orthostatic hypotension: a new mechanism.* Hypertension, 2012. **59**(2): p. 402-8.
49. Hiemann, N.E., R. Meyer, E. Wellnhofer, C. Schoenemann, H. Heidecke, N. Lachmann, R. Hetzer, and D. Dragun, *Non-HLA antibodies targeting vascular receptors enhance alloimmune response and microvasculopathy after heart transplantation.* Transplantation, 2012. **94**(9): p. 919-24.
50. Riemekasten, G., A. Philippe, M. Näther, T. Slowinski, D.N. Müller, H. Heidecke, M. Matucci-Cerinic, L. Czirják, I. Lukitsch, M. Becker, A. Kill, J.M. van Laar, R. Catar, F.C. Luft, G.R. Burmester, B. Hegner, and D. Dragun, *Involvement of functional autoantibodies against vascular receptors in systemic sclerosis.* Ann Rheum Dis, 2011. **70**(3): p. 530-6.
51. Morshed, S.A., R. Ma, R. Latif, and T.F. Davies, *Biased signaling by thyroid-stimulating hormone receptor-specific antibodies determines thyrocyte survival in autoimmunity.* Sci Signal, 2018. **11**(514).
52. Venter, J.C., C.M. Fraser, and L.C. Harrison, *Autoantibodies to beta 2-adrenergic receptors: a possible cause of adrenergic hyporesponsiveness in allergic rhinitis and asthma.* Science, 1980. **207**(4437): p. 1361.
53. Fraser, C.M., J.C. Venter, and M. Kaliner, *Autonomic abnormalities and autoantibodies to beta-adrenergic receptors.* N Engl J Med, 1981. **305**(20): p. 1165-70.
54. Magnusson, Y., S. Marullo, S. Hoyer, F. Waagstein, B. Andersson, A. Vahlne, J.G. Guillet, A.D. Strosberg, A. Hjalmarson, and J. Hoebeke, *Mapping of a functional autoimmune epitope on the beta 1-adrenergic receptor in patients with idiopathic dilated*

cardiomyopathy. J Clin Invest, 1990. **86**(5): p. 1658-63.
55. Jackson, A.M., T.K. Sigdel, M. Delville, S.-C. Hsieh, H. Dai, S. Bagnasco, R.A. Montgomery, and M.M. Sarwal, *Endothelial cell antibodies associated with novel targets and increased rejection.* Journal of the American Society of Nephrology : JASN, 2015. **26**(5): p. 1161-1171.
56. Boesen, E.I., *Endothelin receptors, renal effects and blood pressure.* Current opinion in pharmacology, 2015. **21**: p. 25-34.
57. Rüster, C. and G. Wolf, *Angiotensin II as a morphogenic cytokine stimulating renal fibrogenesis.* Journal of the American Society of Nephrology : JASN, 2011. **22**(7): p. 1189-1199.
58. Cresci, B., S. Giannini, L. Pala, C. Mavilia, C. Manuelli, P. Cappugi, E. Maggi, and C.M. Rotella, *AT1 and AT2 receptors in human glomerular endothelial cells at different passages.* Microvascular research, 2003. **66**(1): p. 22-29.
59. Davenport, A.P., R.E. Kuc, S.L. Hoskins, F.E. Karet, and F. Fitzgerald, *[125I]-PD151242: a selective ligand for endothelin ETA receptors in human kidney which localizes to renal vasculature.* British journal of pharmacology, 1994. **113**(4): p. 1303-1310.
60. Wisler, J.W., K. Xiao, A.R.B. Thomsen, and R.J. Lefkowitz, *Recent developments in biased agonism.* Current opinion in cell biology, 2014. **27**: p. 18-24.
61. Kem, D.C., H. Li, C. Velarde-Miranda, C. Liles, M. Vanderlinde-Wood, A. Galloway, M. Khan, C. Zillner, A. Benbrook, V. Rao, C.E. Gomez-Sanchez, M.W. Cunningham, and X. Yu, *Autoimmune mechanisms activating the angiotensin AT1 receptor in 'primary' aldosteronism.* The Journal of clinical endocrinology and metabolism, 2014. **99**(5): p. 1790-1797.
62. Becker, M.O., A. Kill, M. Kutsche, J. Guenther, A. Rose, C. Tabeling, M. Witzenrath, A.A. Kühl, H. Heidecke, H.A. Ghofrani, H. Tiede, R.T. Schermuly, N. Nickel, M.M. Hoeper, I. Lukitsch, M. Gollasch, W.M. Kuebler, S. Bock, G.R. Burmester, D. Dragun, and G. Riemekasten, *Vascular receptor autoantibodies in pulmonary arterial hypertension associated with systemic sclerosis.* American journal of respiratory and critical care medicine, 2014. **190**(7): p. 808-817.
63. Hennessey, J.C., B.D. Stuyvers, and J.J. McGuire, *Small caliber arterial endothelial cells calcium signals elicited by PAR2 are preserved from endothelial dysfunction.* Pharmacology research & perspectives, 2015. **3**(2): p. e00112-e00112.
64. Dowell, S.J. and A.J. Brown, *Yeast assays for G protein-coupled receptors.* Methods Mol Biol, 2009. **552**: p. 213-29.
65. Ladds, G., A. Goddard, and J. Davey, *Functional analysis of heterologous GPCR signalling pathways in yeast.* Trends in Biotechnology, 2005. **23**(7): p. 367-373.
66. Boss, V., D.J. Talpade, and T.J. Murphy, *Induction of NFAT-mediated transcription by Gq-coupled receptors in lymphoid and non-lymphoid cells.* The Journal of biological chemistry, 1996. **271**(18): p. 10429-10432.
67. Cornell, L.D., R.N. Smith, and R.B. Colvin, *Kidney transplantation: mechanisms of rejection and acceptance.* Annu Rev Pathol, 2008. **3**: p. 189-220.
68. Zhang, Q. and E.F. Reed, *The importance of non-HLA antibodies in transplantation.* Nature reviews. Nephrology, 2016. **12**(8): p. 484-495.
69. Garces, J.C., S. Giusti, C. Staffeld-Coit, H. Bohorquez, A.J. Cohen, and G.E. Loss,

Antibody-Mediated Rejection: A Review. The Ochsner journal, 2017. **17**(1): p. 46-55.
70. Sharif, A., S. Shabir, S. Chand, P. Cockwell, S. Ball, and R. Borrows, *Meta-analysis of calcineurin-inhibitor-sparing regimens in kidney transplantation.* J Am Soc Nephrol, 2011. **22**(11): p. 2107-18.
71. Webster, A.C., S. Wu, K. Tallapragada, M.Y. Park, J.R. Chapman, and S.J. Carr, *Polyclonal and monoclonal antibodies for treating acute rejection episodes in kidney transplant recipients.* Cochrane Database Syst Rev, 2017. **7**: p. CD004756.
72. Beukers, M.W., J. van Oppenraaij, P.P.W. van der Hoorn, C.C. Blad, H.d. Dulk, J. Brouwer, and A.P. Ijzerman, *Random Mutagenesis of the Human Adenosine A2B Receptor Followed by Growth Selection in Yeast. Identification of Constitutively Active and Gain of Function Mutations.* Molecular Pharmacology, 2004. **65**(3): p. 702.
73. Li, B., N.M. Nowak, S.K. Kim, K.A. Jacobson, A. Bagheri, C. Schmidt, and J. Wess, *Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast: identification of second-site mutations that restore function to a coupling-deficient mutant M3 receptor.* J Biol Chem, 2005. **280**(7): p. 5664-75.
74. Gomar-Alba, M., M.Á. Morcillo-Parra, and M.l.d. Olmo, *Response of yeast cells to high glucose involves molecular and physiological differences when compared to other osmostress conditions.* FEMS Yeast Research, 2015. **15**(5).
75. Nakamura, Y., J. Ishii, and A. Kondo, *Applications of yeast-based signaling sensor for characterization of antagonist and analysis of site-directed mutants of the human serotonin 1A receptor.* Biotechnology and Bioengineering, 2015. **112**(9): p. 1906-1915.
76. Beukers, M.W. and A.P. Ijzerman, *Techniques: How to boost GPCR mutagenesis studies using yeast.* Trends in Pharmacological Sciences, 2005. **26**(10): p. 533-539.
77. Thomsen, W., J. Frazer, and D. Unett, *Functional assays for screening GPCR targets.* Curr Opin Biotechnol, 2005. **16**(6): p. 655-65.
78. Milligan, G., *Principles: extending the utility of [35S]GTP gamma S binding assays.* Trends Pharmacol Sci, 2003. **24**(2): p. 87-90.
79. Gabriel, D., M. Vernier, M.J. Pfeifer, B. Dasen, L. Tenaillon, and R. Bouhelal, *High throughput screening technologies for direct cyclic AMP measurement.* Assay Drug Dev Technol, 2003. **1**(2): p. 291-303.
80. Williams, C., *cAMP detection methods in HTS: selecting the best from the rest.* Nat Rev Drug Discov, 2004. **3**(2): p. 125-35.
81. Chambers, C., F. Smith, C. Williams, S. Marcos, Z.H. Liu, P. Hayter, G. Ciaramella, W. Keighley, P. Gribbon, and A. Sewing, *Measuring intracellular calcium fluxes in high throughput mode.* Comb Chem High Throughput Screen, 2003. **6**(4): p. 355-62.
82. Eglen, R.M., *Functional G protein-coupled receptor assays for primary and secondary screening.* Comb Chem High Throughput Screen, 2005. **8**(4): p. 311-8.
83. Nakayama, T., K. Hirano, J. Nishimura, S. Takahashi, and H. Kanaide, *Mechanism of trypsin-induced endothelium-dependent vasorelaxation in the porcine coronary artery.* British Journal of Pharmacology, 2001. **134**(4): p. 815-826.
84. Dutra-Oliveira, A., R.Q. Monteiro, and A. Mariano-Oliveira, *Protease-activated receptor-2 (PAR2) mediates VEGF production through the ERK1/2 pathway in human glioblastoma cell lines.* Biochemical and Biophysical Research Communications, 2012. **421**(2): p. 221-227.

85. Sivaraj, K.K., R. Li, J. Albarran-Juarez, S. Wang, D. Tischner, M. Grimm, J.M. Swiercz, S. Offermanns, and N. Wettschureck, *Endothelial Galphaq/11 is required for VEGF-induced vascular permeability and angiogenesis.* Cardiovasc Res, 2015. **108**(1): p. 171-80.
86. Wu, S., *Structural and functional basis of Endothelin-1 type A receptor (ETAR) activation*, in *Charité - Universitätsmedizin Berlin*. 2019, (Accessed 2019-03-04T12:00:30Z, at https://refubium.fu-berlin.de/handle/fub188/24036).
87. Nishimasu, H., S. Okudaira, K. Hama, E. Mihara, N. Dohmae, A. Inoue, R. Ishitani, J. Takagi, J. Aoki, and O. Nureki, *Crystal structure of autotaxin and insight into GPCR activation by lipid mediators.* Nature Structural & Molecular Biology, 2011. **18**(2): p. 205-212.
88. Rasmussen, S.G.F., H.-J. Choi, D.M. Rosenbaum, T.S. Kobilka, F.S. Thian, P.C. Edwards, M. Burghammer, V.R.P. Ratnala, R. Sanishvili, R.F. Fischetti, G.F.X. Schertler, W.I. Weis, and B.K. Kobilka, *Crystal structure of the human β2 adrenergic G-protein-coupled receptor.* Nature, 2007. **450**(7168): p. 383-387.
89. Härterich, S., S. Koschatzky, J. Einsiedel, and P. Gmeiner, *Novel insights into GPCR—Peptide interactions: Mutations in extracellular loop 1, ligand backbone methylations and molecular modeling of neurotensin receptor 1.* Bioorganic & Medicinal Chemistry, 2008. **16**(20): p. 9359-9368.
90. Murphy, J.W. and D.A. Kendall, *Integrity of extracellular loop 1 of the human cannabinoid receptor 1 is critical for high-affinity binding of the ligand CP 55,940 but not SR 141716A.* Biochemical Pharmacology, 2003. **65**(10): p. 1623-1631.
91. Zhu, N., *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*, in *Charité - Universitätsmedizin Berlin*. 2015, (Accessed 2015-12-07T12:11:19.838Z, at https://refubium.fu-berlin.de/handle/fub188/2530).
92. Cheng, R.K.Y., C. Fiez-Vandal, O. Schlenker, K. Edman, B. Aggeler, D.G. Brown, G.A. Brown, R.M. Cooke, C.E. Dumelin, A.S. Dore, S. Geschwindner, C. Grebner, N.O. Hermansson, A. Jazayeri, P. Johansson, L. Leong, R. Prihandoko, M. Rappas, H. Soutter, A. Snijder, L. Sundstrom, B. Tehan, P. Thornton, D. Troast, G. Wiggin, A. Zhukov, F.H. Marshall, and N. Dekker, *Structural insight into allosteric modulation of protease-activated receptor 2.* Nature, 2017. **545**(7652): p. 112-115.
93. Al-Ani, B., M. Saifeddine, A. Kawabata, and M.D. Hollenberg, *Proteinase activated receptor 2: Role of extracellular loop 2 for ligand-mediated activation.* Br J Pharmacol, 1999. **128**(5): p. 1105-13.
94. Lerner, D.J., M. Chen, T. Tram, and S.R. Coughlin, *Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function.* J Biol Chem, 1996. **271**(24): p. 13943-7.
95. Peeters, M.C., G.J.P. van Westen, Q. Li, and A.P. Ijzerman, *Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation.* Trends in Pharmacological Sciences, 2011. **32**(1): p. 35-42.
96. Banerjee, A.A. and S.D. Mahale, *Extracellular loop 3 substitutions K589N and A590S in FSH receptor increase FSH-induced receptor internalization and along with S588T substitution exhibit impaired ERK1/2 phosphorylation.* Arch Biochem Biophys, 2018.

659: p. 57-65.
97. Lynagh, T., T.S. Khamu, and L.J. Bryan-Lluka, *Extracellular loop 3 of the noradrenaline transporter contributes to substrate and inhibitor selectivity.* Naunyn Schmiedebergs Arch Pharmacol, 2014. **387**(1): p. 95-107.
98. Pittman, M., *Pertussis Toxin: The Cause of the Harmful Effects and Prolonged Immunity of Whooping Cough. A Hypothesis.* Reviews of Infectious Diseases, 1979. **1**(3): p. 401-412.
99. Teter, K., *Intracellular Trafficking and Translocation of Pertussis Toxin.* Toxins (Basel), 2019. **11**(8).
100. Mangmool, S. and H. Kurose, *G(i/o) protein-dependent and -independent actions of Pertussis Toxin (PTX).* Toxins (Basel), 2011. **3**(7): p. 884-99.
101. Kim, K., J. Lee, and S. Ghil, *The regulators of G protein signaling RGS16 and RGS18 inhibit protease-activated receptor 2/Gi/o signaling through distinct interactions with Gα in live cells.* FEBS Letters, 2018. **592**(18): p. 3126-3138.
102. McCoy, K.L., S.F. Traynelis, and J.R. Hepler, *PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology.* Mol Pharmacol, 2010. **77**(6): p. 1005-15.
103. Didsbury, J.R., Y.S. Ho, and R. Snyderman, *Human Gi protein alpha-subunit: deduction of amino acid structure from a cloned cDNA.* FEBS Lett, 1987. **211**(2): p. 160-4.
104. Momota, F., K. Hirano, M. Hirano, J. Nishimura, and H. Kanaide, *Involvement of Gi/o in the PAR-4-induced NO production in endothelial cells.* Biochemical and Biophysical Research Communications, 2006. **342**(2): p. 365-371.

Statutory Declaration

"I, Qing, Li, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic Autoimmune PAR2 activation in vascular events/Autoimmune PAR2 Aktivierung bei vaskulären Erkrankungen, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Curriculum Vitae

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

Publications

Publication 1: Xu X, Ning Y, Shang W, Li M, Ku M, **Li Q**, Li Y, Dai W, Shao J, Zeng R, Han M, He X, Yao Y, Lv Y, Liu X, Ge S, Xu G. Analysis of 4931 renal biopsy data in central China from 1994 to 2014. Ren Fail. 2016;38(7):1021-1030. doi:10.1080/0886022X.2016.1183443

Publication 2: Zhang Y, Wang J, Zhou QD, Zhang CH, **Li Q**, Huang S, Zhan J, Wang K, Liu YY, Xu G. Peroxisome proliferator-activated receptor-γ agonist pioglitazone fails to attenuate renal fibrosis caused by unilateral ureteral obstruction in mice. J Huazhong Univ Sci Technolog Med Sci. 2016;36(1):41-47. doi:10.1007/s11596-016-1539-1

Publication 3: Ku M, Guo S, Shang W, **Li Q**, Zeng R, Han M, Ge S, Xu G. Pregnancy Outcomes in Chinese Patients with Systemic Lupus Erythematosus (SLE): A Retrospective Study of 109 Pregnancies. PLoS One. 2016;11(7):e0159364. Published 2016 Jul 21. doi:10.1371/journal.pone.0159364

Publication 4: Catar RA, Chen L, Cuff SM, Kift-Morgan A, Eberl M, Kettritz R, Kamhieh-Milz J, Moll G, **Li Q**, Zhao H, Kawka E, Zickler D, Parekh G, Davis P, Fraser DJ, Dragun D, Eckardt KU, Jörres A, Witowski J. Control of neutrophil influx during peritonitis by transcriptional cross-regulation of chemokine CXCL1 by IL-17 and IFN-γ. J Pathol. 2020;251(2):175-186. doi:10.1002/path.5438

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