# Significance of actins in the investigation of the pathomechanism of tissue remodeling

PhD Thesis

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## 1. Introduction

Tissue remodeling is a reversible or irreversible structural and functional alteration of a well-differentiated tissue, induced by any type of injuries. The processof tissue remodeling is the same for all tissues. Injured cells and activated immune cells produce cytokines, chemokines and growth factors, leading to the activation of myofibroblasts, which are key players of tissue remodeling.

Myofibroblasts play a central role in tissue repair due to their increased proliferation rate, motility and ability to produce extracellular matrix (ECM), which serves as structural basis for the renewal of functional cell layers. The main marker of myofibroblasts is the α smooth muscle actin (α-SMA), which is the structural element of their stress fiber system and is responsible for their contraction, thus ensuring cell migration and wound contraction.

The complete regeneration of injured tissue requires a delicate balance of ECM deposition and demolition, cell division and apoptosis. Otherwise, the structural alteration of tissue, and thereby its decreased function become permanent. This lattercan be observed in various chronic inflammatory diseases, including the different diseases of kidney or gastrointestinal tract, as permanent activation of myofibroblast leads to excessive accumulation of ECM and formation of scar tissue. Despite the obvious medical demand, there is no effective therapy against abnormal tissue remodeling or fibrosis. Therefore, our research group aimed to investigate the pathomechanism of tissue remodeling and exploring novel molecular pathways, which facilitates the identification of potential new therapeutic targets.

Actin family consists of six different isoforms, including α-SMA and ß-actin, which have special importance in the research related to tissue remodeling. Indeed, α-SMA is one of the main markers depicting the

presence and activity of myofibroblasts, and ß-actin is widely used as internal control in many molecular biological measurements. Although actin isoforms are encoded by different genes, the similarity between them is significant. The homology in the amino acid or nucleotide sequences of the different actin isoforms is over 90%, making it a real challenge to selectively measure their expression.
In the present study we aimed to develop isoform-specific real-time PCR methods to selectively measure the expression of mouse, human or rat α-SMA and ß-actin. Moreover, using fibrotic kidney samples we unequivocally demonstrated the inaccuracy of the real time PCRs caused by the used of non-specific α-SMA or ß-actin primer pairs.

Interleukin 24 (IL-24) is a member of IL-20 cytokine subfamily, and is mainly produced by immune cells. IL-24 acts on IL-20RA/IL-20RB and IL-22RA/IL-20RB receptor heterodimers. The most well-known function of IL-24 is the regulation of innate immune responses and repair processes at epithelial surfaces. Moreover, its possible role in wound healing and chronic inflammation has been suggested. Our research group reported increased expression of IL-24 in biological samples of patients with coeliac disease (CD), inflammatory bowel diseases and chronic kidney disease. In this study, we performed *in vitro* and *in vivo* experiments to investigate the exact biological role of IL-24.

Parkinson's disease 7 (PARK7) is a multifunctional, cytoprotective, antioxidant molecule, which is primarily investigated in connection with neurodegenerative diseases, but its role is probable also in other diseases associated with oxidative damage. Indeed, our research group reported elevated expression of PARK7 in the duodenal mucosa of therapy naive children with CD, and genome wide association studies identified PARK7 polymorphisms as predisposing factor in CD and ulcerative

colitis. In the present study, we aimed to investigate the possible role of PARK7 in the pathomechanism of CD, with particular attention to oxidative damage of intestinal epithelial cells.

## 2. Objectives

In the present dissertation the cellular and molecular mechanism of tissue remodeling was investigated with the help of actin-related molecular biological measurements. Our objectives were the following:

- To investigate the specificity of various PCR primers used to evaluate the expression of α-SMA or ß-actin in tissue remodeling-related literature.
- To investigate the effect of non-specific actin PCR primers on gene expression measurements performed on tissue samples derived from *in vivo* experiment.
- To design, optimize and validate mouse, human and rat α-SMA and ß-actin PCR primers.
- To investigate the role of IL-24 in the pathomechanism of renal and intestinal remodeling with a special focus on myofibroblast activation.
- To investigate the role of PARK7 in the pathomechanism of CD-related tissue remodeling with a special focus on oxidative damage of intestinal mucosa.

## 3. Methods

### 3.1. *In vivo* and *ex vivo* experiments

In our experiments 6-8 weeks old, 20-24 g male C57BL/6J (WT) and *Il20rb* -/- mice with C57BL/6J background were used.

#### 3.1.1. Unilateral ureteral obstruction (UUO)

UUO model was used to investigate the process of renal fibrosis on WT and *Il20rb* -/- mice. The left ureter of mice was isolated and ligated using non-absorbable surgical suture. Sham-operated mice were used as control. Experiment was terminated 7 or 14 days after the initiation of UUO.

#### 3.1.2. Dextran sulphate sodium (DSS)-induced colitis

DSS-induced colitis model was used to investigate the process of tissue remodeling in WT and *Il20rb* -/- mice. Animals were treated with 2.5% DSS dissolved in the drinking water for 7 days, then watered with normal water for another 12 days. Control animals received normal drinking water only. Clinical parameters, including body weight and disease activity was monitored daily. Experiment was terminated 19 days after induction of colitis.

#### 3.1.3. Intracolonic injection

Local treatment of colonic mucosa was used to investigate the direct effect of IL-24 on the remodeling of colonic tissue in WT mice. After standard midline laparotomy the bowel was isolated and 0.1 µg IL-24 in 50 µl PBS was injected into the mucosa. Control mice were treated with PBS only. Experiment was terminated 24 hours after the injection.

### 3.1.4. *Ex vivo* mucosal permeability measurement

Effect of PARK7 on intestinal permeability was investigated *ex vivo*, using intestinal sacs prepared from duodenum of WT mice treated with a PARK7 binding compound, Comp23. Intestinal sacs were filled with 0.1% evans blue diluted in medium with or without $H_2O_2$ supplementation and placed into PBS containing tubes. Permeation of evans blue was determined by measuring the absorbance in every 20 minutes.

## 3.2. Duodenal biopsies

Role of PARK7 in the pathomechanism of CD and the oxidative stress induced damage of mucosa was investigated using immunofluorescence staining in duodenal biopsies derived from pediatric therapy naive CD patients and controls. CD was diagnosed based on the criteria of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. Controls were referred with chronic abdominal pain, growth retardation and diarrhoea and an upper gastrointestinal endoscopy was part of their diagnostic procedure.

## 3.3. *In vitro* experiments

The effect of recombinant IL-24 on myofibroblast was investigated using primary myofibroblasts isolated from duodenal mucosa of control children.

The effect of PARK7 on the oxidative stress was investigated using $H_2O_2$ treated duodenal epithelial cells (FHs74Int) in the presence or absence of PARK7-binding Comp23.

## 3.4. Viability measurements

The viability of FHs74Int cells after various treatments was investigated using MTT cell viability, LDH cytotoxicity and Annexin V apoptosis assays.

### 3.5. Reactive oxygen species (ROS) assay

Intracellular accumulation of ROS in H2O2 treated FHs74Int cells was determined using redox-sensitive 2',7'-Dichlorofluorescein diacetate (DCFDA) fluorescent dye.

### 3.6. Immunofluorescence staining

Presence and localisation of PARK7, α-SMA, zonula occludens and actin was investigated by immunofluorescence staining on tissue samples, FHs74Int cells and primer myofibroblasts. Nuclei were counterstained with DAPI. Samples were analyzed with an Olympus IX81 fluorescence microscope.

α-SMA stress fiber orientation of primer myofibroblasts and the ratio of FHs74Int cells with cytoskeletal damage was graphically analyzed using ImageJ software.

### 3.7. mRNS expression measurement

Gene expression measurement were performed after RNA isolation, complementer DNA synthesis and real-time PCR. Relative mRNA expression was determined by comparison with internal control and presented as the ratio of control group.

### 3.8. Protein analysis

The protein level of α-SMA was measured by Western blot in renal and intestinal tissue samples of mice. The relative protein level of α-SMA was determined by comparison with GAPDH as internal control and presented as the ratio of control group.

### 3.9. Histological staining

The extent of fibrotic tissue in renal tissue samples of mice was measured by Masson's trichrome and picro-SiriusRed staining. After digitalization of slides the ratio of positive-stained area was graphically analyzed using ImageJ software.

### 3.10. Statistical analysis

After testing normality with Kolmogorow-Smirnov test, data were analyzed using t-test or Mann-Whitney U-test for two groups and ANOVA or Kruskal-Wallis test for more than two groups. Multiple comparisons of row data derived from assay-like measurements were performed using multiple t-test and ordinary two-way ANOVA with Dunnett correction. To determine the correlation between the relative mRNA expressions, Pearson correlation analysis was performed. The ratio of cells with healthy or damaged cytoskeleton after various treatments were compared using Chi-square test. $p \leq 0.05$ was considered as statistically significant and was signed with various symbols, indicating the applied statistical test, as well. Results are presented as mean+SD.

# 4. Results

## 4.1. α-SMA and ß-actin specific PCR primers

### 4.1.1. Template specificity of self-designed mouse, human or rat α-SMA and ß-actin primers

We located our primers to the nucleotide sequences of the greatest possible difference among different actin genes, paying special attention to the 3′ end of our primers to maximize the chance of specific priming. Target specificity of primers were verified using artificial oligonucleotide templates. PCRs resulted in products with the corresponding templates only, we found no cross-reactions with the non-specific actins.

### 4.1.2. Template specificity of literary mouse α-SMA and ß-actin primers

Investigating the localisation of mouse α-SMA and ß-actin primers derived from papers published in different prestigious journals we found high homology between the sequence of actin isoforms at their annellation position. Indeed, we found that all primer pairs amplified both mouse α-SMA and ß-actin specific artificial DNA templates as well.

### 4.1.3. Demonstration of measurement inaccuracy caused by non-specific primers

To investigate the relevance of the non-specific primer binding, real-time PCRs were performed on kidney samples of mice underwent UUO and sham-operated controls using the different set of primer pairs. Relative α-SMA and ß-actin mRNA levels determined by self-designed and literary PCRs showed significant deviation. Our experiments showed, the cross-reactions of primers with the different actin isoforms may substantially alter the experimental results up to 50%.

## 4.2. Role of IL-24 in tissue remodeling

### 4.2.1. Effect of IL-24 on myofibroblasts

Primary myofibroblasts isolated from duodenal biopsies showed elongated morphology with parallel α-SMA stress fibers. After treatment with IL-24, we found circular fiber displacement and sheet-like shape of cells. Accordingly, IL-24 increased the expression of cytoskeletal components and cell morphology regulators.

### 4.2.2. Tissue remodeling in *Il20rb* -/- mice

Experiments on *Il20rb* -/- mice contribute to the better understanding of the role of IL-24 in tissue remodeling. We found increased level of α-SMA in the renal tissue of mice underwent on UUO, indicating myofibroblast accumulation. Expression of α-SMA and also the deposition of scar tissue increased in WT compared to *Il20rb* -/- mice on the 14$^{th}$ day.

Similarly, DSS treatment of mice resulted in increased expression of α-SMA and other fibrosis-related factors in their intestinal tissue. *Il20rb* -/- phenotype showed less extent ECM deposition in the colon of DSS treated mice. In contrary, local treatment of colon tissue with IL-24 was pro-fibrotic in WT mice.

## 4.3. Role of PARK7 in the oxidative damage of mucosa in coeliac disease

In this study duodenal epithelial cells (FHs74Int) and intestinal sacs exposed to oxidative stress were used to investigate the role of PARK7 in the maintenance of mucosal integrity. In these experiments we used Comp23, a PARK7-binding compound to enhance the antioxidant activity of PARK7.

### 4.3.1. Effect of Comp23 on intracellular ROS accumulation

As a short-time effect of oxidative stress we found strong ROS accumulation in the epithelial cells, which was decreased by Comp23 treatment. Investigating the molecular mechanism of its antioxidant effect our results showed that Comp23 treatment increased the expression of multitude of NRF2 dependent antioxidant response genes.

### 4.3.2. Effect of Comp23 on cell death

As a long-term effect, oxidative stress induced significant apoptosis in epithelial cells, which was decreased by Comp23 treatment. Investigating the molecular mechanism of its cytoprotective effect our results showed that Comp23 increase the expression of P53 and other cell-cycle regulators.

### 4.3.3. Effect of Comp23 on cytoskeletal and cell adhesion damage

Oxidative stress leads to structural and functional damage of intracellular macromolecules. $H_2O_2$ treatment destroyed the filamentous actin network of the epithelial cells and disrupted healthy localisation of cell adhesion molecules (CAM). Our results showed that Comp23 increased the expression of CAMs and preserved the architecture of the actin cytoskeleton-CAM complexes in the epithelial cell exposed to oxidative stress.

### 4.3.4. Effect of Comp23 on intestinal permeability

To investigate the significance of PARK7 in the maintenance of the mucosal integrity we examined its effect on $H_2O_2$ treated small intestine *ex vivo*. We found that Comp23 greatly decreased the permeability of the small intestinal sacs.

## 5. Conclusions

In this dissertation the cellular and molecular mechanism of tissue remodeling was discussed. In our experiments, the determinant role of actin involved in several cell functions was demonstrated.

- We developed a set of carefully designed PCR primer pairs to determine the expression of mouse, human and rat α-SMA and ß-actin without cross reactions with other actin isoforms.
- Our study gives an experimental explanation, how the improper primer design can alter the results of related measurements due their unspecific binding of to the various actin isoforms.
- IL-24 caused significant alteration in morphology and stress fiber displacement of myofibroblast *in vitro*.
- IL-24 plays a role in the pathomechanism of renal and intestinal tissue remodeling, leading to increased myofibroblast accumulation and ECM production *in vivo*.
- PARK7 plays a role in the defence of intestinal epithelial cells against oxidative stress via increasing the expression of antioxidant elements, moderating the disruption of actin cytoskeleton and supressing cell death.
- PARK7 contributes to the maintenance of mucosal integrity and the barrier function of the small intestine.

## 6. Bibliography of the candidate's publications

### 6.1. Research articles related to the theme of the PhD thesis

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