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## Precision-cut tissue slices: a novel ex vivo model for fibrosis research

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*  
2016

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Pham, B. T. (2016). *Precision-cut tissue slices: a novel ex vivo model for fibrosis research*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

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# Chapter 1

## General Introduction

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*Adapted from*

### **Evaluation of fibrosis in precision cut tissue slices**

I.M. Westra, B.T. Pham, G.M.M Groothuis and P. Olinga  
*Xenobiotica* 2013, 43(1), 98-112.

### **Results of the 4th Scientific Workshop of the ECCO (Group II): Markers of intestinal fibrosis in inflammatory bowel disease**

F. Rieder, J. R. de Bruyn, B. T. Pham, K. Katsanos, V. Annese,  
P. D.R. Higgins, F. Magro, I. Dotan  
*Journal of Crohn's and Colitis* 2014, 8(10), 1166–1178.

## INTRODUCTION

Fibrosis is characterized by the excessive deposition of extracellular matrix (ECM) proteins, including collagen and this pathological process is the result of chronic inflammation or injury. Fibrogenesis is a physiological phenomenon serving as a part of the physiological repair processes after injury or inflammation. During the repair process, ECM is formed by myofibroblasts to temporarily replace the injured tissue. Afterwards, the inflammatory response will be inhibited, ECM will be degraded and the myofibroblasts will undergo apoptosis. During chronic injury, this repair process can be deregulated, which leads to abnormal accumulation of ECM and culminates into fibrosis (Guarino et al., 2009). Alterations of the original tissue architecture of an organ due to high collagen levels will lead to stiffness and loss of functional cells, resulting in impaired function of the organ (Ghosh and Vaughan, 2012; Wynn, 2008).

Fibrosis has been described in almost every organ, and the fibrotic process shares common mechanisms in different organs (Ghosh and Vaughan, 2012). Inflammation and injury, being essential steps in tissue fibrosis, can trigger profibrotic signaling via activation of cytokines and differentiation of resident fibroblast, endothelial cells, epithelial cells or stellate cells into myofibroblasts, which are responsible for the deposition of ECM (Ghosh and Vaughan, 2012), an indication of the typical multicellular character of the pathology. Transforming growth factor  $\beta$  (TGF $\beta$ ), produced by several tissue-resident and blood-derived cells, is crucial in the development of fibrosis, through its influence on the production of ECM, matrix metalloproteinases (MMP) – enzymes that degrade ECM – tissue inhibitor of metalloproteinases (TIMP) and on T cell function (Hold et al., 2009). Also platelet-derived growth factor (PDGF), produced by macrophages, stellate cells and mesangial cells, is an important factor in the development of organ fibrosis, it plays a vital role in proliferation, migration and survival of myofibroblasts (Bonner, 2004; Trojanowska, 2008). Hence, the levels of PDGF are increased in fibrotic tissue (Trojanowska, 2008).

This thesis focuses on intestinal and renal fibrosis, since the pathophysiological mechanism underlying both pathologies is not fully elucidated. The various chapters delineate the development of a new *ex vivo* model for both intestinal and kidney fibrosis, which is a great tool in unraveling the mechanism of fibrogenesis and can

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be utilized in evaluating the efficacy of potential antifibrotic drugs. This chapter provides a timely overview of the current knowledge regarding intestinal and kidney fibrosis as well as the *in vitro* and *in vivo* models that are used to study fibrosis in these organs.

### ***Intestinal fibrosis***

Intestinal fibrosis (IF) occurs in patients after radiation therapy or as a reaction of intestinal tissue to chronic inflammation. Moreover, IF is a major complication in inflammatory bowel disease (IBD), represented by ulcerative colitis (UC) and Crohn's disease (CD) as the two major types of IBD. Fibrosis is an interaction between the innate immune system and multiple cellular sources of excessive ECM deposition (Fiocchi and Lund, 2011). However, in the intestine, internal factors such as gut microbiota, external and environmental factors also play important roles (Lawrance et al., 2001). Like in other organs, TGF $\beta$  is important in the early onset of fibrosis in the intestine, and is in the inflamed areas in the intestine of patients with CD (di Mola et al., 1999). Moreover, TGF $\beta$  induced a dose-dependent expression of type I collagen and heat shock protein 47 (HSP47) in intestinal fibroblasts (Kitamura et al., 2011; Mulsow et al., 2005). One of the main mysteries in IF is the source of the mesenchymal cells such as fibroblasts and myofibroblasts. A variety of hypotheses exist about the transition and activation of mesenchymal cells into fibroblasts and myofibroblasts during the onset of fibrosis (Flier et al., 2010; Powell et al., 2011; Rieder et al., 2011b). However, none of these hypotheses have been proven, mainly because of the shortage of relevant IF models.

The natural history of IBD is highly heterogeneous and frequently complicated by IF and stricture formation. This appears to be the case for both entities of IBD, UC and CD (Cosnes et al., 2002; Gumaste et al., 1992). More than 30% of CD and about 5% of UC patients develop a distinct fibrostenosing phenotype with progressive narrowing and potential for intestinal obstruction (Gumaste et al., 1992; Louis et al., 2003). Intestinal stricture formation is a frequent indication for surgery in CD (Farmer et al., 1985) and strictures frequently recur leading to repeated surgeries (Fazio et al., 1993). It appears that both entities of IBD and in particular CD exhibit a progressive nature with changes in disease behavior throughout the disease course. In CD, which is a transmural disease, chronic mucosal inflammation

induces remodeling of the entire intestinal wall. This process is a cascade of events that includes epithelial cell and intestinal damage and repair, angiogenesis and lymphangiogenesis and activation of immune cells and mesenchymal cells (MCs). MCs include fibroblasts, myofibroblasts and smooth muscle cells and are the major source of ECM components (Rieder, 2013).

It is difficult to predict which patients will develop a fibrostenosing phenotype (though a majority will do so eventually) and how rapidly they will progress. No specific therapy to prevent or treat IF is known. Therefore, it is of utmost importance to develop (*in vitro*) human and animal models to study the mechanism of IF, which could lead to the discovery of potential targets for therapeutic compounds.

### ***Renal fibrosis***

Almost all progressive chronic kidney diseases eventually develop into renal fibrosis (Boor et al., 2010; Liu, 2011). Renal fibrogenesis is considered as an unsuccessful wound healing process, in which almost all the different cell types in the kidney and also infiltrating lymphocytes, macrophages and fibrocytes are involved (Liu, 2011). After injury, inflammatory cells infiltrate, fibroblasts are activated and ECM is produced (Liu, 2011). The myofibroblast is the key cell type in producing ECM components, however, mature fibroblasts, tubular epithelial cells, macrophages and fibrocytes are also a source of ECM in the kidney (Boor et al., 2010). In fibrosis, the tubular epithelial cells, released from the basement membrane, can undergo epithelial mesenchymal transition, forming fibroblasts (Zeisberg and Neilson, 2010). The interstitial spaces are then filled with matrix components, mostly collagen I, III and fibronectin (Zeisberg and Neilson, 2010). The two main families of proteases involved in matrix degradation in the kidney are MMPs and members of the plasmin-dependent pathway. These proteases not only fragment the ECM but also cleave non-matrix substrates, thereby producing profibrotic growth factors (Zeisberg and Neilson, 2010). Eventually all these cellular and molecular events will lead to renal failure.

To develop therapies for renal fibrosis, detailed knowledge of the disease mechanism is essential. Therefore, adequate *in vivo*, but also *in vitro/ex vivo* models, like kidney slices, are necessary.

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## ***Markers of intestinal fibrosis in IBD***

To enable progress in understanding the mechanism of IF, it is essential to identify markers of IF, in order to (1) stratify patients into different levels of risk before the development of fibrosis, and (2) detect early stages of fibrosis before clinical symptoms have occurred. An optimal fibrosis marker should detect early stages of fibrosis, identify trajectory of fibrosis development, be predictive of future fibrosis, be predictive of and responsive to the effect of antifibrotic therapies and be predictive of non-responsiveness to anti-inflammatory therapies. Accomplishing these goals will open the door for targeted antifibrotic therapy, and the ability to test candidate antifibrotic therapies in clinical trials.

### ***Currently available markers of IF***

No specific and accurate predictors or diagnostic tools for IF exist and to date no marker of fibrosis is in routine clinical use. Several targets have been tested for this purpose (Table 1).

Genetic signatures are attractive as they are stable, present long before the disease onset and are not affected by alterations in the disease course. Several genes have been evaluated for their association with fibrostenosing CD. Alternations in the nucleotide-binding oligomerization domain containing 2 (NOD2) gene, the first discovered and best explored genetic variant, are weakly associated not only with CD fibrostenosis, but also with ileal disease location and fistulizing disease (Adler et al., 2011) and hence lack specificity. Other genetic variants have been described as being linked to fibrostenosis, such as those in the matrix metalloproteinase (MMP)-3 gene (Meijer et al., 2007) or in the rs1363670 locus near the interleukin (IL)12B gene (Henckaerts et al., 2009). Interestingly, an increasing number of risk alleles, including NOD2, IBD5, diskslarge homolog (DLG)5, autophagy-related protein 16-1 (ATG16L1), and IL23 receptor (IL23R), confer an increasing risk for intestinal resections in CD (Weersma et al., 2009). Gene variants are promising markers, but their population frequency is low and they exhibit incomplete penetrance. The major benefit of genetic biomarkers is their stability over time and independence from environmental factors, though epigenetic modifications at the sites of fibrogenesis might prove important as well.

**Table 1:** Currently available markers of intestinal fibrosis.

	References
<i>Genetic</i>	
NOD2	(Adler et al., 2011)
MMP-3	(Meijer et al., 2007)
rs1363670	(Henckaerts et al., 2009)
Increasing amount of risk alleles for NOD2, IBD5, DLG5, ATG16L1, and IL23R	(Weersma et al., 2009)
<i>Clinical</i>	
Need for corticosteroids during first flare	(Beaugerie et al., 2006)
Early disease onset	(Beaugerie et al., 2006)
Perianal fistulizing disease	(Beaugerie et al., 2006)
Small bowel disease location	(Louis et al., 2003)
<i>Serologic</i>	
Anti-microbial antibodies	(Dubinsky et al., 2006; Rieder et al., 2011a)
ECM molecules (Fibronectin, collagen propeptides, laminin)	(Allan et al., 1989; Kjeldsen et al., 1995; Koutroubakis et al., 2003; Loeschke and Kaltenthaler, 1989)
Growth factors (YKL-40, bFGF)	(Di Sabatino et al., 2004; Koutroubakis et al., 2003)
NOD-2: nucleotide-binding oligomerization domain containing 2, MMP: matrix metalloproteinase, IBD: inflammatory bowel disease, DLG: disks large, ATG: autophagy, IL23R: interleukin 23 receptor, ECM:ECM, YKL-40: tyrosine lysine leucine-40, and bFGF: basic fibroblast growth factor.	

The most widely used criteria to predict fibrostenosing CD are clinical factors. These include the need for corticosteroids, early disease onset, perianal fistulizing disease or small bowel disease location (Beaugerie et al., 2006; Louis et al., 2003). These factors however are encompassing many different disease phenotypes. On the other hand, the Montreal classification (Silverberg et al., 2005) merely identifies fibrosis after it has become clinically apparent and can only be used as a descriptor rather than a predictor. Thus, alternative, noninvasive predictive tools are required. One predictive tool that might be of use as a biomarker is a panel of serologic markers.

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Circulating antibodies against microbial products are found in some patients with IBD, such as anti-*Saccharomyces cerevisiae* (ASCA) among others. These are believed to arise from aberrant immune responses towards the luminal microbiota (Dotan et al., 2006). These antibodies are qualitatively and quantitatively associated with, and predictive of a more complicated disease phenotype, including fibrostenosis (Dubinsky et al., 2006; Rieder et al., 2011a). However, they are not specific for this phenotype, but rather predict complicated CD, including fistulizing disease and the need for surgery. ECM molecules and growth factors, such as laminin, collagens, collagen propeptides or telopeptides (Kjeldsen et al., 1995; Koutroubakis et al., 2003; Loeschke and Kaltenthaler, 1989), basement membrane components or fibronectin (Allan et al., 1989), YKL-40 (also known as human cartilage glycoprotein, a chitinase-like protein), basic fibroblast growth factor (bFGF) and others have been investigated as biomarkers of fibrosis, with inconclusive or negative results. No existing marker of fibrosis showed specific promise in IBD. Evaluating the markers in fibrosis during renal fibrosis, could also be relevant for further study in IBD

### ***Markers of renal fibrosis***

While the inciting agents in renal fibrosis are often different from the liver, lung, and skin, the fibrotic process appears to be shared. While lab markers of renal function can inform about progression of renal disease they do not necessarily reflect fibrotic burden and they can be influenced by a wide variety of factors. Unique to the kidney is the accessibility of urine as a direct, renal-specific read-out for renal fibrosis, allowing the identification of local markers of fibrosis. Urine levels of TGF $\beta$ 1, CTGF and collagen IV increase with progression of chronic kidney disease (CKD) (Cheng et al., 2006; Gilbert et al., 2003; Io et al., 2004; Tsakas and Goumenos, 2006). Urine plasminogen activator inhibitor-1 (PAI-1) has also been shown to correlate with renal fibrosis in patients with diabetic nephropathy (Torii et al., 2004).

Ultrasound imaging has also entered the field of renal fibrosis. Using the doppler ultrasound technique to calculate the ‘resistive index’ and ‘atrophic index’ has shown promise in predicting time to dialysis and survival in chronic kidney disease (Sugiura and Wada, 2009).

One of the challenges of universal fibrosis markers is that they may be nonspecific, so that a patient with CD may appear to have elevated serum markers of

IF when they have fibrosis in a different organ (skin, liver, lung, kidney, etc.). There may be benefit in obtaining markers of IF from the gut (biopsies) or its output (stool) to increase the specificity of markers for IF.

In summary, we can share the knowledge of fibrotic diseases from various organ e.g., kidney, liver, lung, intestine and skin. Multiple potential biologic and imaging markers are already in clinical use. While organ-specific markers, such as creatinine for renal disease or alveolar epithelial cell specific proteins in the lung, likely will not be helpful markers of IF, multiple markers with a direct link with fibrogenesis have been identified. As fibrotic mechanisms are shared across organs, these provide possible candidates for use in the intestine as well (Table 2). These markers are indispensable for (novel) models in intestinal and renal fibrosis.

**Table 2:** Examples for markers of fibrosis from other fibrotic diseases.

		References
<b><i>Liver</i></b>		
PIIINP, hyaluronic acid, and TIMP1	Serum	(Parkes et al., 2011; Rosenberg et al., 2004)
Cystatin C	Serum	(Ladero et al.)
Transient elastography ultrasound	Liver	(Castera et al., 2008)
Magnetic resonance elastography	Liver	(Yin et al., 2007)
N-cadherin, inter-alpha-trypsin inhibitor heavy chain H4, haptoglobin and serotransferrin	Urine	(van Swelm et al., 2013)
Enolase-1 ( $\alpha$ -enolase) and TSP-1	Serum	(Zhang et al., 2013)
<b><i>Lung</i></b>		
Krebs von den Lungen-6	Serum and BAL	(Hirasawa et al., 1997; Ohnishi et al., 2002)
Surfactant protein-A and -D	Serum	(Greene et al., 2002; Ohnishi et al., 2002)
CCL18	Serum and BAL	(Prasse et al., 2009)
YKL-40	Serum and BAL	(Korthagen et al., 2011)
Osteopontin	Plasma and BAL	(Kadota et al., 2005; Pardo et al., 2005)
Periostin	Serum	(Okamoto et al., 2011)
Napsin A	Serum	(Samukawa et al., 2012)
CTGF	Plasma	(Kono et al., 2011)

HSP-47	Serum	(Huang et al., 2013)
MMP1 & 7	Serum and BAL	(Cicchitto and Sanguinetti, 2013; Rosas et al., 2008)
MMP7, ICAM1, IL8, VCAM1, and S100A12	Plasma	(Richards et al., 2012)
Fibrocytes	Blood	(Moeller et al., 2009)
Peripheral T cell subsets	Blood	(Gilani et al., 2010; Kotsianidis et al., 2009)
<b><i>Skin</i></b>		
miRNAs	Serum and tissue	(Zhu et al., 2013)
ICTP	Serum	(Kikuchi et al., 1994)
PINP and PIIINP	Serum	(Denton et al., 2007; Nagy and Czirják)
Cytokines/chemokines	Serum	(Codullo et al., 2011; Giacomelli et al., 1997; Sato et al., 2001)
CTGF	Serum	(Dziadzio et al., 2005)
Cartilage oligomeric protein	Serum	(Hesselstrand et al., 2008)
Thrombospondin	Serum	(Macko et al., 2002)
Osteopontin	Plasma	(Lorenzen et al., 2010)
MMP9	Serum	(Kim et al., 2005)
Number of myofibroblasts	Skin biopsy	(Kissin et al., 2006)
COMP, TSP-1, IFI44, and SIG1 gene expression	Skin biopsy	(Lafyatis et al., 2009)
Ultrasound	Skin	(Moore et al., 2003)
Magnetic resonance imaging	Skin	(Madani et al., 2008)
<b><i>Kidney</i></b>		
TGFβ1	Urine	(Tsakas and Goumenos, 2006)
CTGF	Urine	(Gilbert et al., 2003)
PAI-1	Urine	(Torii et al., 2004)
Collagen IV	Urine	(Io et al., 2004)

PIIINP: N-terminal propeptide of type III collagen, TIMP: tissue inhibitor of matrix metalloproteinase, CCL18: CC chemokine ligand 18, YKL-40: tyrosine lysine leucine-40, HSP: heat shock protein, MMP: matrix metalloproteinase, ICAM: intercellular adhesion molecule, IL: interleukin, VCAM: vascular cell adhesion molecule, S100A12: S100 calcium binding protein A12, miR: microRNA, ICTP: carboxy terminal telopeptide of type I collagen, PINP: N-terminal propeptide of type I procollagen, CTGF: connective tissue growth factor, COMP: cartilage oligomeric matrix protein, TSP: thrombospondin, IFI44: interferon-induced protein 44, SIG: small inducible gene, TGF: transforming growth factor, and PAI: plasminogen activator inhibitor.

## NOVEL MODELS FOR INTESTINAL AND RENAL FIBROSIS

Numerous *in vitro* and animal models are available to study fibrosis. Cell culture models using fibroblast or myofibroblast cell lines or freshly isolated primary cells, are used to study cell behavior during exposure to fibrotic and antifibrotic compounds, but the interplay of the different cell types involved in organ fibrosis can not be investigated, unless cocultures are used (Iredale, 2007). Furthermore, cell lines can differ from primary cells (Wilkening et al., 2003) and freshly isolated cells can react differently compared with cells in the tissue, because the interplay with other cells and matrix is absent (Snowdon and Fallowfield, 2011). In experimental animal models, fibrosis can be induced by induction of chronic injury or infection, and the detailed cellular and molecular pathways of fibrosis can be studied. Recently, precision-cut tissue slices (PCTS) have shown their use in the study of fibrosis, since the multicellular process of fibrosis can be mimicked in tissue slices and they allow the study of fibrosis in human organs by using human tissue.

In this section the use of PCTS from the kidney and intestine in fibrosis research is evaluated and in particular, the utilization of PCTS in the study of the mechanisms of fibrosis and the effect of antifibrotic drugs is discussed. Most of the techniques to induce fibrosis in PCTS are based on *in vivo* models or techniques that have been used in fibrosis research to induce fibrosis. Therefore, these *in vivo* models are discussed briefly in relation to the possibilities and utilization of PCTS in fibrosis research.

### ***Intestine***

#### *In vivo models for intestinal fibrosis*

Most of the IF animal models available are based on intestinal inflammation typically induced by toxins to mimic CD and UC. In addition, different models based on transgenic animals and gene delivery systems have been developed. To induce IF, 2,4,6-trinitrobenzene sulfonic acid (TNBS) is administered intrarectally, which results in transmural inflammation and ECM deposition. Repeated administration of TNBS produces injury similar to chronic colitis and fibrosis (te Velde et al., 2006). An activation of NF- $\kappa$ B and the production of

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TGF $\beta$ 1 are both strongly involved in the TNBS-induced fibrosis model (Lawrance et al., 2003; Wengrower et al., 2012) again pointing to the strong link between chronic inflammation and fibrosis. The TNBS model has been widely used to evaluate the fibrotic/antifibrotic effect of different drugs and cytokines (Barrett et al., 2012; Lawrance et al., 2003; San-Miguel et al., 2010; Xu et al., 2002). TNBS has also been utilized in transgenic mice to understand the mechanism of TNBS-induced fibrosis. Inokuchi et al. used TNBS in angiotensin knockout mice and found that Ang $^{-/-}$  mice were partly protected against TNBS-induced fibrosis compared to Ang $^{+/+}$  mice (Inokuchi et al., 2005). In Insulin-like growth factor-I (IGF-I) $^{+/-}$  mice TNBS-induced colitis resulted in collagen 1A1 production and fibrosis, which was increased by administration of IGF-I, indicating that IGF-I is a key player in TNBS-induced fibrosis (Mahavadi et al., 2011).

Another well-known IF model is the dextran sulfate sodium (DSS) model. DSS induces acute inflammation in the colon, referred to as colitis. The administration of a repeated dose (3–5 cycles) of DSS results in chronic UC (Okayasu et al., 1990; Suzuki et al., 2011) with increase of extracellular protein and fibrotic cytokines and additionally a thickening of the colon wall (Suzuki et al., 2011). Moreover, mesenchymal cells, such as fibroblasts and myofibroblasts, are increased in the mucosa of the colon of DSS-treated mice (Suzuki et al., 2011). Pucilowska et al. showed that the inflammation is transmural and that collagen production is elevated, both at the gene and protein level (Pucilowska et al., 2010).

IF can also be induced by the administration of peptidoglycan polysaccharide (PG-PS) (Rahal et al., 2012; Theiss et al., 2005). PG-PS directly injected into the intestine of Lewis rats produced a transient acute inflammation (Rahal et al., 2012). After 28 days, PG-PS-injected rats developed IF, measured by elevated procollagen gene expression and histologic scores (Rahal et al., 2012).

Induction of fibrosis after radiation treatment has been known for many years (Donner, 1998; Martin et al., 2000). Small bowel tissue, resected from patients who were treated with radiation therapy showed an increase of the fibrosis marker TGF $\beta$ , elevated levels of ECM proteins, and thickening of submucosal and serosal layer of the intestine (Haydont and Vozenin-Brotans, 2007; Richter et al., 1997). In a rat model of radiation-induced fibrosis, a strong induction of pivotal profibrotic cytokines such as TGF $\beta$ 1 (Langberg et al., 1994) was found. However, up to now, there is no validated protocol for radiation-induced fibrosis that ensures reproducible

elevated fibrosis markers in experimental animals (Langberg et al., 1994; Langberg et al., 1996; Linard et al., 2012).

Transgenic animal models for IF focus on specific genes that may be involved in the different pathways leading to fibrosis. The well-known knockout model for IF is the IL-10<sup>-/-</sup> mouse model (Kühn et al., 1993; Speck et al., 2011). In the IL-10<sup>-/-</sup> mouse, bacterial exposure or surgical intervention results in inflammation and fibrosis that does not occur in the wild-type mouse (Borowiec et al., 2012; Speck et al., 2011). Borowiec et al. reported an accumulation of collagen in the ileocolonic anastomosis of IL-10<sup>-/-</sup> mice 6 weeks after surgery (Borowiec et al., 2012).

IF can also be induced by administration of some specific microflora in normal or IL-10<sup>-/-</sup> knockout mice (Johnson et al., 2012; Mourelle et al., 1998; Rigby et al., 2009; van Tol et al., 1999). As an example, Johnson et al. exposed 8–12-week-old CBA/J mice to a *S. typhimurium* strain to induce fibrosis (Johnson et al., 2012). The described *in vivo* models for IF can be used in precision-cut intestinal slices (PCIS), either to provoke fibrosis or to use slices of fibrotic intestinal tissue to study antifibrotic drugs.

Recently, Rieder et al., summarized the animal models available for IF, which can be categorized into seven groups, including spontaneous, gene targeted, postoperative fibrosis, chemical-, immune-, bacteria-, and radiation-induced. Each group has their own advantages and disadvantages, but none received general acceptance regarding the relevance to human IF (Rieder et al., 2012).

### *Precision-cut intestinal slices (PCIS)*

*In vitro* models for IF are mostly based on fibroblasts using various culture methods and conditions (Agrez and Chua, 1990; Burke et al., 2009; Fritsch et al., 1997; Sellge et al., 2004). Both murine and human intestinal fibroblasts can be used as a model for IF. Kim et al. has successfully evaluated in evaluating the effect of IL-1 $\beta$  on Prostaglandin E2 induction, an antifibrotic mediator, in five primary human fibroblasts cultures (Kim et al., 1998). Simmons et al. showed the induction of type I collagen in a human colon fibroblast/myofibroblast (CCD- 18Co) cell culture model by using TGF $\beta$ 1 (Simmons et al., 2002). However, due to the importance of different cell types in IF, different culture/co-culture methods have been developed. Intestinal epithelial cell and mesenchymal cell co-culture methods were reviewed by Simon-

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Assmann et al. (Simon-Assmann et al., 2007). A three-dimensional culture model of intestinal fibroblasts, macrophages and epithelial cells provides a novel cell culture method to understand IF (Leonard et al., 2010; Spottl et al., 2006). Recently, intestinal organoids have successfully been used as a model to evaluate antifibrotic drugs. The organoids originate from human pluripotent stem cells including myofibroblasts, which can be activated by TGF $\beta$  (Rodansky et al., 2015).

PCIS have been used as a model to study drug metabolism (de Kanter et al., 2005), induction of drug metabolism (van de Kerkhof et al., 2008), regulation of gene expression of metabolizing enzymes and transporters (Khan et al., 2009; Li et al., 2015; Li et al., 2016a; Li et al., 2016b), xenobiotic toxicity and drug transport (Possidente et al., 2011; Vickers and Fisher, 2005).

### *Early onset of fibrosis and PCIS*

We recently established that PCIS can be used as a model to study the early onset of fibrosis in the intestine (Chapter 3). PCIS from rat and human small intestine remain viable up to 24 and 48 h, respectively. After incubation for 24 and 48 h, the gene expression of HSP47 was increased in human and rat PCIS (Pham et al., 2015) which is in line with the observation of Honzawa et al. who reported that serum level of HSP47 protein, which is required in the synthesis of normal collagen (Taguchi and Razaque, 2007), is higher in CD patients than in UC patients (Honzawa et al., 2010). When rat PCIS were cultured in the presence of TGF $\beta$ 1, the gene expression of procollagen 1A1 and  $\alpha$ SMA was up-regulated compared with control slices (Pham et al., 2015). These results suggest that the early onset of IF can be mimicked *ex vivo* and that the model is a new tool to unravel the complicated mechanisms of IF.

However, more (human) studies are necessary to validate this *ex vivo* model of fibrosis. It would be interesting to investigate whether the TNBS or the DSS model can be applied *in vitro* to induce the early onset of fibrosis *in vitro*. In addition, the use of animal or human intestine with established IF can be used to prepare fibrotic PCIS to study the mechanism of end-stage fibrosis and to screen potential antifibrotic drugs (Chapter 4 and 5 of this thesis).

### **Kidney**

#### *In vivo models for renal fibrosis*

There are several models that can be used to study renal fibrosis characterized by increased synthesis and accumulation of ECM (Zeisberg et al., 2005). Fibrosis can be induced by exposure of animals to (toxic) compounds. The chemical N-(3,5-dichlorophenyl)-succinimide can be used to provoke tubular interstitial nephritis, which can serve as a model for interstitial renal fibrosis (Barrett et al., 1983). In the renal cortex of rats exposed to this chemical, an increased activity of proline hydroxylase, elevated hydroxyproline content and increased water content was found. All these phenomena are associated with chronic renal fibrosis (Barrett et al., 1983).

Exposure of experimental animals to ochratoxin-A (OA), a mycotoxin found in grain, resulted in renal disease, comparable to endemic nephropathy. Chronic interstitial renal pathology correlated to exposure to OA in grains and animal products (Aukema et al., 2004). Four weeks of exposure to OA in piglets resulted in increased collagen formation and fibroblast proliferation, leading to renal fibrosis (Aukema et al., 2004).

A ligation animal model, like the unilateral ureteric obstruction (UUO) model, in which one of the two ureters is ligated, can be used to induce renal fibrosis in both rat and mouse. In the UUO model urine accumulates in the kidney, which leads to hydronephrosis, progressive alterations of renal parenchyma and development of renal fibrosis (Chevalier et al., 2009; Klein et al., 2011). The proliferation of interstitial fibroblasts and their transformation to myofibroblast results in accumulation of ECM components (Chevalier et al., 2009).

Furthermore, hypertension can lead to chronic renal failure and renal fibrosis (Flamant et al., 2006). Therefore, experimental models of hypertension-induced renal disease were developed. One of them is the NaCl (5%) model, in which the high salt diet induces the development of hypertension (Flamant et al., 2006). Another mouse model for hypertension-induced renal disease is subcutaneous infusion of angiotensin II (Ang II), leading to hypertensive nephropathy which mediates progressive renal injury resulting in renal fibrosis and inflammation (Liu et al., 2012).

Fibrosis can also occur as complication after renal transplantation caused by renal lesions due to immune reactions, drug toxicity of cyclosporine administration and ischemia. Klein et al. developed a rat renal transplant model as model for fibrosis: the Interstitial Fibrosis and Tubular Atrophy (IFTA) model (Klein et al., 2011).

The genetically modified mouse strain COL4A3<sup>-/-</sup> mouse, is used as an

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animal model for the Alport syndrome, which is a type IV collagen disease that leads to hematuria, proteinuria, renal fibrosis and ultimately to renal failure (Gross et al., 2010). The strengths and weaknesses of animal models used in renal fibrosis research were summarized by Meng et al. (Meng et al., 2014). Although, these models are widely used, most of them are limited to some specific species and irrelevant for the human disease.

The above mentioned models for renal fibrosis might be translated into the renal slices model, either to induce fibrosis in slices or to use slices of (fibrotic) renal tissue to test antifibrotic drugs (Chapter 6).

### *Precision-cut kidney slices*

Multiple cell types in the kidney contribute to the development of renal fibrosis. Therefore, precision-cut kidney slices may be used as an *ex vivo* model with all different cell types present, as described for liver (Westra et al., 2014), to study the onset and late stage of renal fibrosis.

### *Early onset of fibrosis and precision-cut renal slices*

Prolonged culture of human kidney slices paves the way to use slices for the study of fibrosis (Vickers and Fisher, 2005). After 72 h of culture, kidney slices show signaling pathways of repair and a fibrogenic response as seen by an increased deposition of collagen IV and increased gene expression of collagens, laminins, contractile proteins and markers of proliferation (Vickers and Fisher, 2005). Therefore, this model is applicable to study mechanisms of fibrotic renal disease (Vickers and Fisher, 2005).

As a model for renal fibrosis, renal cortex slices of transgenic mouse harboring the luciferase reporter gene under the control of collagen I promoter, were used to investigate the mechanisms of collagen up-regulation in renal tissue (Tharaux et al., 2000). In kidney slices incubated with AngII, the procollagen 1 $\alpha$ 2 gene was activated, and by using specific inhibitors it could be shown that MAPK/ERK, AP-1 and TGF $\beta$  are involved in this process (Tharaux et al., 2000). These *in vitro* results indicate that AngII also directly induces collagen 1 gene expression, not only indirectly by the *in vivo* induction of hypertensive nephropathy.

Renal slices from rats recovered for 5 weeks from an acute kidney injury

(AKI) induced by ischemia-reperfusion (I/R) injury, were used to study oxidative stress in renal disease (Basile et al., 2012). *In vivo* experiments showed that AKI enhances the profibrotic response to AngII, which is released during oxidative stress. Dihydroethidium (DHE) incorporation as parameter for oxidative stress, was increased in renal slices recovered after AKI compared with slices from control rats (Basile et al., 2012). These data indicate that the kidney slices are an important model for the study of fibrosis in AKI induced by I/R injury.

In this thesis, chapter 6, the PCKS model was demonstrated as a novel tool to test the pathophysiology of fibrosis and to screen the efficacy of antifibrotic drugs *ex vivo* in a multicellular and profibrotic milieu. An IFN $\gamma$  conjugate targeted to the PDGFR $\beta$  significantly reduced TGF $\beta$ 1-induced fibronectin, collagen I and collagen III mRNA expression (Poosti et al., 2015).

#### *End-stage fibrosis and precision-cut kidney slices*

Nagae et al. used fibrotic kidney to prepare whole kidney, medulla or cortex slices, from UUO rats to study the effect of adrenomedullin (ADM), a vasodilating peptide that reduces renal fibrosis in hypertensive animals on cyclic adenosine monophosphate (cAMP) production (Nagae et al., 2008). In the kidney slices of UUO rats, cAMP production was increased compared with slices of control kidneys and an ADM-neutralizing antibody partially blocked the enhanced cAMP production (Nagae et al., 2008). However, they used rather thick slices (1.5 mm) for a very short incubation time of maximally 45 min. Using thinner, PCKS would probably have prolonged the time period that these kidney slices could be used. Still, even in these relatively thick renal fibrotic slices pharmacological intervention studies were possible.

Flamant et al. studied the effect of discoidin domain receptor 1 (DDR1), a nonintegrin collagen receptor that displays tyrosine-kinase activity, on the development of renal fibrosis, using fibrotic kidney slices besides *in vivo* experiments (Flamant et al., 2006). Renal cortical slices from high-NaCl fed DDR1<sup>-/-</sup> mice showed decreased monocyte chemoattractant protein-1 (MCP-1) secretion following stimulation with lipopolysaccharide compared with slices from high NaCl fed wild-type mice, indicating the importance of DDR1 in the process of renal inflammation and fibrosis (Flamant et al., 2006).

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In conclusion, both normal and fibrotic renal slices can be used in renal fibrosis and antifibrotic pharmacotherapy research (Mutsaers et al., 2015; Poosti et al., 2015; Stribos et al., 2016).

## CONCLUSIONS AND PERSPECTIVES

PCTS from intestine and kidney represent a useful tool to investigate the early onset of fibrosis in both human and experimental animals. Utilizing the slice model can lead to new insights into the (human) mechanisms of the onset of organ fibrogenesis. In addition, in the future, treatment of PCTS with the fibrosis-inducing compounds or factors, used to induce fibrosis *in vivo* in the different organs, may help to further elucidate the mechanisms of human fibrosis. Furthermore, tissue slices from fibrotic intestine and kidney from both human and animals could be used to study the factors that play a role in established fibrosis. Last but not least, human (fibrotic) tissue slices will pave the way for the testing of novel therapeutic pharmacological interventions for human fibrosis, which up till now could only be performed in animal models.

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