Cell-specific targeting of renal fibrosis
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Precision-cut kidney slices (PCKS) to study development of renal fibrosis and efficacy of drug targeting ex vivo

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ABSTRACT

Renal fibrosis is a serious clinical problem forming the utmost cause of need for renal replacement therapy. No adequate preventive or curative therapy is available that can be clinically used to specifically target renal fibrosis. The search for new efficacious treatment strategies is therefore warranted. Although in vitro models using homogeneous cell populations have contributed to the understanding of the pathogenetic mechanisms involved in renal fibrosis, these models poorly mimic the complex in vivo milieu. Therefore, here we evaluated a precision-cut kidney slice (PCKS) model as a new, multicellular ex vivo model to study development of fibrosis and the prevention thereof using anti-fibrotic compounds.

Precision-cut slices (200–300 μm thickness) were prepared from healthy C57BL/6 mouse kidneys using a Krumdieck tissue slicer. To induce changes mimicking the fibrotic process, slices were incubated with TGFβ1 (5 ng/ml) for 48 hours in the presence or absence of the anti-fibrotic cytokine IFNγ (1 μg/ml) or an IFNγ conjugate which is targeted to the PDGFRβ (PPB-PEG-IFNγ). Following culture, tissue viability (ATP-content) and expression of α-SMA, fibronectin, collagen I, and collagen III were determined using real-time PCR and immunohistochemistry.

Slices remained viable up to 72 hours of incubation and no significant effects of TGFβ1 and IFNγ on viability were observed. TGFβ1 markedly increased α-SMA, fibronectin, and collagen I mRNA and protein expression levels. IFNγ and PPB-PEG-IFNγ significantly reduced TGFβ1-induced fibronectin, collagen I and collagen III mRNA expression which was confirmed by immunohistochemistry.

The PKCS model is a novel tool to test the pathophysiology of fibrosis and to screen the efficacy of anti-fibrotic drugs ex vivo in a multicellular and pro-fibrotic milieu. Major advantage of the slice model is that it can be used not only for animal but also for (fibrotic) human kidney tissue.
INTRODUCTION

Renal fibrosis is a major contributor to the development of chronic kidney disease (CKD) and is characterized by accumulation of myofibroblasts and excessive extracellular matrix deposition (1, 2). CKD culminates in End-Stage Renal Disease (ESRD) eventually, resulting in significant morbidity and mortality for which the only available therapy is dialysis or renal transplantation (3). The pathophysiology of renal fibrosis is not fully understood and hitherto no adequate preventive or curative therapy is clinically available (4). Research focusing on unraveling the pathophysiology of renal fibrosis is therefore warranted. Traditionally, renal research is performed in *in vitro* cell culture systems or small rodent models as well as on human renal biopsies or explants (5). Despite the fact that *in vitro* experiments using homogeneous single cell preparations allow functional analyses of a specific cell type, the major disadvantage obviously is lack of cell heterogeneity and cellular microarchitecture; phenomena that are undoubtedly involved in determining and driving the fibrotic process (5, 6). Because of ethical constraints, the use of animal models is increasingly discouraged. Furthermore, use of human tissue biopsies is by definition descriptive in nature and prohibits functional studies.

The availability of an *ex vivo* model system that does allow functional analyses on the development of renal fibrosis is therefore highly desired. Recently, precision cut tissue slices (PCTS) have been increasingly used to study the development of liver fibrosis (7-9). In this model cell-cell and cell-extracellular matrix interactions are preserved. As yet it is unknown whether precision cut kidney slices (PCKS) can be used to study the development of renal fibrosis and to screen the efficacy of anti-fibrotic compounds, e.g. IFNγ. IFNγ is a pleiotropic cytokine produced by various activated immune cells including NK cells and T cells (10). In addition to its pro-inflammatory effects, IFNγ has prominent anti-fibrotic effects. IFNγ is able to inhibit fibroblast activation and proliferation, and also reduces extracellular matrix synthesis (11-15). These properties make IFNγ a potential molecule for therapeutic use to target fibrosis. However, the short half-life and undesirable systemic side effects clearly limit clinical utility of IFNγ as an anti-fibrotic drug (16-18). To overcome these problems, we employed a drug-targeting strategy in which IFNγ was targeted specifically to activated myofibroblasts in animal models of liver and kidney fibrosis using the PDGFRβ as docking receptor for the PPB-PEG-IFNγ conjugate (PPB: PDGFRβ-recognizing receptor) (19-23).

The aim of the present study was to validate the precision-cut kidney slice (PCKS) model for the development of TGFβ1-induced renal fibrosis and to evaluate the potential anti-fibrotic effects of IFNγ and PPB-PEG-IFNγ in this *ex vivo* model.
MATERIALS AND METHODS

Mice
Male C57BL/6 mice weighing 25–30 g were obtained from Harlan (Zeist, the Netherlands). Animals were housed in individual cages with free access to food and water. Before starting the experiment, animals were allowed to acclimatize for at least 7 days. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen (DEC 6427A).

Excision and preparation of mouse kidney slices
The kidney was excised under 2% isofluorane/O₂ anesthesia and placed into ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA). Immediately after harvesting the kidney, mouse PCKS were prepared. In brief, cores were drilled from a whole mouse kidney followed by slicing in ice-cold Krebs-Henseleit buffer saturated with carbogen (80% O₂ / 5% CO₂) using a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) (35). PCKS (200–300 µm thick and 5–6 mg/slice wet weight) were stored in UW until incubation. Slices were cultured in the presence or absence of TGFβ1 as described below. Preparation and culture of kidney slices is illustrated in Figure 1.

Preparation and culture of precision-cut kidney slices (PCKS)

Figure 1: Schematic representation of the experimental approach for obtaining and culturing mPCKS
(1) mouse kidneys (n=3) were excised under isofluorane/O₂ anesthesia and placed into ice-cold UW preservation solution. (2) Cores were drilled and immediately transferred to the cylindrical core holder of the Krumdieck slicer and cut into 5 mm diameter slices. (3) Good quality slices (equal thickness at all sides and smooth edges) were transferred to 12 well plates using a spatula to avoid damaging of the slices, and (4) placed in a shaking (90 times / min) incubator under continuous supply of 80% O₂/5% CO₂ at 37°C.
Experimental setup slice cultures

In this study three independent experiments were performed, each using 4 kidneys (2 mice) from which in total 4 cores were drilled. After slicing, slices were pooled before plating them for culture. ATP measurements were performed on single slices. For mRNA expression analysis, in each experiment slices were cultured in triplicate for each condition after which they were pooled for RNA isolation. Histological analyses were performed on single slices per condition in each experiment.

Incubation of kidney slices

Before adding medium and mPCKS, 12 well plates were coated with 10% bovine serum albumin (BSA) in milliQ water for 30 minutes. Afterwards, the solution was removed and plates were air dried. Slices were then incubated in the coated plates containing per well 1.3 ml Williams Medium E glutamax-I (Gibco, Paisly, Scotland) per well supplemented with 25 mM D-glucose and 50 µg/ml gentamycin. Medium was pre-warmed and gassed with 80% O₂/5% CO₂ before it was added to the wells. Slices were individually transferred to the wells, and plates were then placed in a shaking CO₂ incubator (90 times/min) under continuous supply of 80% O₂/5% CO₂. Slices were incubated for 1, 6, 12, 24, 48, or 72 hours. When appropriate, after 24 and 48 hours slices were transferred to new plates with fresh medium.

Induction of renal pre-fibrosis ex vivo

In order to induce renal pre-fibrosis ex vivo, slices were incubated for 48 hours in the presence of recombinant human TGFβ1 (5 ng/ml, hTGFβ1, Roche, Mannheim, Germany). Medium without TGFβ1 was used for comparison. After 24 hours of incubation, slices were transferred to new coated plates with fresh medium containing TGFβ1.

Synthesis of conjugates

PPB-PEG-IFNγ (in which PPB is the PDGFR recognizing binding peptide) was synthesized as described previously (19). Briefly, for PPB-PEG-IFNγ, recombinant murine IFNγ (0.256 nmol, Peprotech, London, UK) was mixed with 12.8 nmol of maleimide-PEG-succinimidyl carboxy methyl ester (Mal-PEG-SCM, 2 KDa, Creative PEGworks, Winston-Salem, NC) for 2 hours and dialyzed overnight against PBS using 10 KDa disopodialyzer (Harvard Apparatus, Holliston, Massachusetts, USA). The dialyzed sample was then mixed with SATA (succinimidyl acetyltioacetate)-modified PPB (PPB-ATA, 25.6 nmol) in the presence of a deacetylating reagent. Finally, PPB-PEG-IFNγ was extensively dialyzed against PBS and stored at -80°C.

For PPB-HSA, human serum albumin (HSA, 1.5 µmol dissolved in PBS) was mixed with γ-maleimidobutyryloxy-succinimide ester (GMBS, 30 µmol, dissolved in DMF) for 2 hours and extensively dialyzed against PBS using 10 KDa cut-off dialysis membrane cassette (Thermo Scientific, Rockford, IL, USA). Next, PPB-ATA (34.5 µmol; dissolved in DMF) was added to the GMBS-modified HSA for overnight, and dialyzed against PBS. The final product (PPB-HSA) was freeze-dried for storage at -20°C.
Chapter 6

Anti-fibrotic effects of free IFNγ and PPB-PEG-IFNγ

To determine the anti-fibrotic effects of free IFNγ and PPB-PEG-IFNγ, slices were incubated for 48 hours with free IFNγ, PPB-PEG-IFNγ, PPB-HSA (equivalent 1 µg/ml) or medium alone in the presence or absence of TGFβ1 as described above. After the first 24 hours of incubation slices were transferred to new 12 well plates with fresh medium containing TGFβ1 and the respective anti-fibrotic compounds. TGFβ1 and IFNγ constructs were administered simultaneously. The concentration of 1 µg/ml of IFNγ constructs was chosen based on results from previous in vitro experiments (19, 23).

Viability of mPKCS: ATP and protein content

The general viability of the PCKS during culture was determined by measuring ATP content of the slices. For ATP measurements, single slices were transferred to a sonication solution containing 70% ethanol (v/v) and 2 mM EDTA (pH 10.9), snap-frozen in liquid nitrogen and stored at -80°C until analysis. After homogenization using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA), the samples were centrifuged for 2 min. at 13000 rpm. The supernatant was diluted 10 times with 0.1 M Tris-HCl/2 mM EDTA buffer (pH 7.8) to lower the ethanol concentration and used to measure ATP contents using the ATP Bioluminescence assay kit CLSI (Roche diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. The remaining pellet was used to determine the protein content of the mPCKS by dissolving it in 200 µL of 5 M NaOH for 30 minutes. After dilution with water to a concentration of 1 M NaOH, the protein content of the samples was determined using the Lowry method (Bio-Rad DC Protein Assay, Bio-Rad, Munich, Germany). BSA was used for the calibration curve. ATP values (in pmol) were divided by the total protein content (in µg) of the respective slices and expressed as the ratio ATP/protein.

Quantitative Real-Time PCR

In order to determine TGFβ-induced pre-fibrosis, and to examine the effect of IFNγ and PPB-PEG-IFNγ, gene expression of fibrosis markers (alpha smooth muscle actin [α-SMA], fibronectin [Fn], collagens I & III) was measured using quantitative Real-Time (qRT) PCR. The triplicate slices were pooled and snap frozen. Total RNA from kidney slices was extracted using the RNasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The RNA concentrations were measured on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Single-stranded cDNA was synthesized using Superscript II and random hexamer primers (Invitrogen, Carlsbad, CA) in a volume of 20 µl. cDNA was first diluted to a concentration of 2 ng/µl and 2.5 µl/reaction (5 ng) and was then used for qRT-PCR analysis. PCR reactions were performed in a 10 µl reaction volume containing 1 x qPCR master mix (Eurogentec, Liege, Belgium) and 1x Taqman Gene Expression Assay mix (Applied Biosystems, Forster City, CA). The Taqman assay numbers were as follows: Ywhaz: Mm03950126_s1, Col1a1: Mm00801666_g1, Col3a1: Mm01254476_m1, α-SMA/Acta2: Mm01546133_m1, Fn: Mm01256744_m1, MHCII/CD74: Mm00658576_m1. qRT-PCR reactions were performed on a ABI7900HT
thermal cycler (Biosystems, Forster City, CA). Relative gene expression was calculated using the $2^{ΔΔCt}$ method with Ywhaz (tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein ζ) as housekeeping gene.

**Histology of mPKCS**

Study of histological changes was performed on 2 µm sections from formalin-fixed paraffin-embedded slices. For immunohistochemistry the following primary antibodies were used: anti-alpha smooth muscle actin (α-SMA, clone ASM-1, Progen Biotechnik, Heidelberg, Germany); anti-fibronectin (ab6584, Abcam, Cambridge, UK); and anti-collagen III (s1330-01, SouthernBiotech, Birmingham, Alabama, USA). Sections were deparaffinized in xylene and rehydrated in graded alcohol and distilled water. Antigen retrieval was achieved by overnight incubation at 60ºC in 0.1 M Tris/HCl buffer (pH 9.0) for fibronectin and collagen III staining. No antigen retrieval was performed for α-SMA staining. Endogenous peroxidase activity was blocked with 0.03% H$_2$O$_2$ (in PBS) for 30 min. Primary antibody binding was detected by sequential incubations with horseradish peroxidase (HRP)-labeled appropriate secondary and tertiary antibodies (obtained from DAKO, Glostrup, Denmark). Peroxidase activity was visualized using 3,3’-diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark) as chromogen (10 min incubation). Sections were counterstained with haematoxylin for 1 minute and mounted with Kaiser’s glycerin gelatin.

**Quantification of immunostaining**

To quantify immunostaining of fibronectin and collagen III, sections were first scanned using a NanoZoomer HT (Hamamatsu Photonics K.K., Shizuoka Pref., Japan). Next, the extent of fibronectin and collagen III positive staining was measured (number of positive pixels) using Aperio ImageScope software (version 9.1.772.1570, Aperio Technologies Inc, Vista, CA, USA).

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA). Data are expressed as mean ± standard error of the mean of three independent experiments. Differences between multiple groups were calculated using ANOVA. Comparisons of two groups were performed using Student’s t-test. p<0.05 was considered statistically significant.

**RESULTS**

**Viability of mouse kidney slices (mPKCS) during incubation**

In order to determine viability of mPKCS, ATP levels per mg of protein were assessed directly after preparation of slices, and after 1, 6, 12, 24, 48, and 72 hours of incubation (Figure 2A). Kidney slices remained viable up to 72 hours of incubation; however, a minor,
Chapter 6

A non-significant decrease in ATP levels was observed after 24 hours of incubation. Up to 72 hours, slices retained constant ATP levels. Since the morphology of kidney slices started to deteriorate after 72 hours of incubation (based on PAS staining, data not shown), the experiments with TGFβ1 and anti-fibrotic compounds were conducted with a maximal culture time of 48 hours. In a separate experiment, we first examined the viability of slices after 48 hours of incubation with or without TGFβ1 in the presence of free IFNγ, PPB-PEG-IFNγ or PPB-HSA. As shown in Figure 2B, TGFβ1 did not significantly alter ATP content. Furthermore, free IFNγ, PPB-PEG-IFNγ, or PPB-HSA did not affect ATP levels independent of presence or absence of TGFβ1.

**Figure 2**: Effect of incubation time and conditions on mPCKS viability

(A) Non-cultured normal kidney slices (n=3) directly after the slicing procedure (0) and after different incubation intervals.

(B) Effect of 48 hours of incubation with TGFβ1 on ATP content of mPCKS in the presence of IFNγ, PPB-PEG-IFNγ, and PPB-HSA with or without TGFβ1.

**Biological activity of IFNγ and PPB-PEG-IFNγ ex vivo**

To determine the biological activity of free IFNγ and PPB-PEG-IFNγ, we assessed major histocompatibility class II (MHC II) expression which is known to be upregulated by IFNγ (Bansal et al., 2011a). Expression of MHC II in response to IFNγ was analyzed in TGFβ- and non-TGFβ-incubated slices. qRT-PCR results showed that incubation of kidney slices in the presence of both free IFNγ and PPB-PEG-IFNγ caused a significant upregulation of MHC II expression compared with medium, effects that were not influenced by TGFβ1 (Figure 3A). However, PPB-HSA (the PDGFRβ-specific drug delivery carrier without IFNγ) did not induce MHC II expression. These data indicate that IFNγ and PPB-PEG-IFNγ retained their biological activity in kidney slices.
**Figure 3:** MHC II and PDGFRβ expression in mPCKS

(A) mRNA expression of MHC II in pre-fibrotic (+ TGFβ1) and non-fibrotic (- TGFβ1) kidney slices treated with IFNγ, PPB-PEG-IFNγ or PPB-HSA. (B) mRNA expression of PDGFRβ in pre-fibrotic (+ TGFβ1) and non-fibrotic (- TGFβ1) kidney slices treated with IFNγ, PPB-PEG-IFNγ or PPB-HSA. (*p<0.05 versus medium).

**PDGFRβ expression in mPCKS**

We recently showed increased interstitial PDGFRβ expression in both human and mouse fibrotic renal tissue which co-localized with α-SMA positive interstitial myofibroblasts (23). High expression of PDGFRβ on interstitial myofibroblasts indicates that this receptor is an appropriate target for fibroblast-specific delivery. Therefore, we next analyzed whether cultured mPCKS in the presence of TGFβ1 are also characterized by PDGFRβ expression. As shown in Figure 3B, mPCKS cultured with medium but without TGFβ1 clearly expressed PDGFRβ which was not increased by TGFβ1. A clear, but non-significant reduction in PDGFRβ expression was observed after incubation in the presence of free IFNγ and PPB-PEG-IFNγ (Figure 3B).
Figure 4: TGFβ1 (5 ng/ml) induces expression of fibrotic markers in mPCKS
mRNA expression of (A) α-SMA, (B) fibronectin, (C) collagen I, and (D) collagen III in mouse kidney slices after 48 hours of incubation. (*p<0.05, ***p<0.001 versus medium).

Induction of α-SMA and ECM expression in mPCKS by TGFβ1
To study development of (pre)fibrosis in mPCKS in the presence of TGFβ1, mRNA expression of fibrosis markers was determined after 48 hours of incubation with TGFβ1 (5 ng/ml). As shown in Figure 4, we observed significant upregulation of α-SMA (A), fibronectin (B), and collagen I (C) mRNA expression upon incubation with TGFβ1 compared to non-TGFβ1 incubated control (medium) slices. TGFβ1 also increased expression of collagen III, however without reaching the level of statistical significance (p=0.08). We next checked by immunohistochemistry whether increased mRNA expression of fibrosis markers also translated into altered protein expression levels. As shown in Figure 5A (two upper rows), incubation with TGFβ1 indeed markedly increased α-SMA, fibronectin and collagen III expression.
Figure 5: Protein expression of α-SMA, fibronectin and collagen III in mPCKS cultured without TGFβ (medium), or with TGFβ in the presence of free IFNγ, PPB-PEG-IFNγ or PPB-HSA (A) Photomicrographs of immunohistochemistry for α-SMA, fibronectin and collagen III on mPCKS after 48 hrs of culture (magnification: 200x). Quantitative analysis of fibronectin (B) and collagen III (C) expression in mPCKS cultured under various conditions. (*p<0.05, **p<0.01, Student’s t-test)

Effect of free IFNγ and PPB-PEG-IFNγ on the expression of fibrosis markers
To investigate whether free IFNγ and PPB-PEG-IFNγ treatment ameliorates fibrosis, qRT-PCR analysis was performed on fibrotic and healthy control slices. As already shown in Figure 4, TGFβ1 induced α-SMA, fibronectin, collagen I, and collagen III mRNA expression. Figure 6A depicts a schematic representation of the targeting strategy in which IFNγ is targeted towards PDGFRβ-expressing myofibroblasts using the PPB-PEG-IFNγ conjugate. In cultured slices incubated with TGFβ plus either IFNγ or PPB-PEG-IFNγ, mRNA expression levels for fibronectin, collagen I, and collagen III were significantly reduced compared to slices incubated with TGFβ alone (Figure 6C-E). IFNγ and PPB-PEG-IFNγ also showed somewhat reduced α-SMA expression (Figure 6B), however without reaching the level of statistical significance. To determine whether IFNγ, PPB-PEG-IFNγ, and PPB-HSA have protective effects in spontaneous induction of fibrosis, kidney slices were also incubated with the aforementioned compounds in the absence of TGFβ1. In this condition, we did not observe significant reduction of fibrosis maker expression in the presence of free IFNγ or PPB-PEG-IFNγ.
IFNγ, although clear trends were observed for collagen I (Figure 6D) and collagen III (Figure 6E).

**Figure 6**: Effect of IFNγ and PPB-PEG-IFNγ on the expression of fibrotic markers in mPCKS.
(A) Schematic representation of targeted delivery of IFNγ to PDGFRβ-expressing myofibroblasts in mPCKS. Relative gene expression of (B) α-SMA, (C) collagen I, (D) fibronectin, and (E) collagen III. (*p<0.05 versus medium, §p<0.05 versus PPB-HSA).
To determine whether mRNA expression profiles also translated into differential protein expression levels, immunohistochemistry for α-SMA, fibronectin and collagen III was performed. Staining for collagen I was technically not feasible. Representative photomicrographs of α-SMA, fibronectin and collagen III staining are shown in Figure 5A. Microscopic analysis revealed no clear differences in α-SMA expression (similar to mRNA expression, Figure 4B). Quantitative analysis for fibronectin (Figure 5B) showed significantly (p<0.01) increased expression levels in slices cultured in the presence of TGFβ1 (compared with medium) which was reduced by IFNy (p<0.05) and PPB-PEG-IFNy (p=0.05). Similar results were observed for the expression of collagen III (Figure 5C). PPB-HSA did not show any significant inhibitory effect on neither mRNA nor protein levels.

**Figure 7:** The ex vivo PCKS model bridges the gap between basic research performed in *in vitro* cell culture models and translational *in vivo* human studies on pathophysiology and therapy of renal disease. *In vitro* cultured cells displayed are primary human tubular epithelial cells (PTECs).

**DISCUSSION**

The present study reveals the applicability of precision-cut kidney slices (PCKS) as: 1) an *ex vivo* model to study renal fibrogenesis, and 2) to test anti-fibrotic effects of IFNy and targeted IFNy aiming at reducing renal fibrosis. Specifically, the results demonstrate that incubation of mPCKS with TGFβ1 resulted in upregulation of fibronectin, collagen I, and α-SMA expression which indicates that mPCKS represent an ideal model to study the onset of early fibrosis. Preserved expression of α-SMA suggests that fibroblasts remained active.
during culture, still intervention with free IFNγ and PPB-PEG-IFNγ clearly dampened TGFβ1-induced expression of fibronectin, collagen I and collagen III, thereby demonstrating the anti-fibrotic potential of both free and targeted IFNγ.

Renal fibrosis results from excessive extracellular matrix accumulation and fibroblast proliferation (24-26). In order to develop an efficacious anti-fibrotic therapy detailed knowledge of the underlying pathophysiology is necessary. It is already recognized for long time that in vitro studies can provide useful information on the mechanisms of disease (5, 8). Precision-cut tissue slices (PCTS), a three-dimensional multicellular environment, is a powerful model in order to provide insight into mechanisms of organ injury (27-31). Tissue slices have the biologically relevant structural features of in vivo tissues (32). PCTS have been widely used as ex vivo model to study development of liver and intestinal fibrosis (9, 33-35). Ex vivo liver and intestinal slices maintain cell heterogeneity and tissue architecture, providing an appropriate tool to study multicellular processes such as fibrosis (9, 33-35).

In this study mouse kidney slices remained viable during the incubation period of 72 hours as determined by ATP content, a parameter generally used as indicator of viability. In addition, the increased expression of fibrosis markers in response to TGFβ1 also indicated that the relevant cells involved in fibrogenesis (i.e., fibroblasts) remained viable. Moreover, activation and/or proliferation of fibrogenic cells, which was accompanied by increased production of extracellular matrix, indicates that kidney slices could be a useful tool to study the effects of anti-fibrotic compounds in a multicellular environment.

IFNγ could be a useful therapeutic target to attenuate the development of renal fibrosis. Despite its potential effectiveness, application of IFNγ in clinical trials resulted in negative data (16-18). Main reasons for the lack of clinical effects is the poor pharmacokinetic profile of IFNγ and severe side effects due to ubiquitous expression of IFNγ receptors. Therefore, targeted delivery of IFNγ to key cells is thought to be a prerequisite to enhance its therapeutic efficacy and at the same time reduce systemic side effects. Recently, we studied anti-fibrotic effects of IFNγ and PEGylated IFNγ (PPB-PEG-IFNγ) targeted to PDGFRβ-expressing myofibroblasts in an animal model of renal fibrosis (23). Our strategy was to conjugate the cyclic PDGFRβ-binding peptide (PPB) to mouse IFNγ via a PEG linker (PPB-PEG-IFNγ) to achieve specific delivery of IFNγ to PDGFRβ-expressing cells, due to upregulation of this receptor in fibrotic diseases. In order to check the applicability of the PCKS model for testing anti-fibrotic compounds, we examined IFNγ and targeted IFN (PPB-PEG-IFNγ) on TGFβ-activated pre-fibrotic slices and on non-activated slices. Contrary to our expectation, PDGFRβ expression was not affected by TGFβ1. This might be explained by the fact that fibrosis is a multifactorial process, and therefore induction of PDGFRβ expression in vivo might be dependent on presence of different pro-fibrotic cytokines rather than TGFβ1. Interestingly, in the group treated with IFNγ and PPB-PEG-IFNγ, we noticed a trend towards reduction of PDGFRβ expression which might explain their anti-fibrotic effects via down regulation of the PDGF/PDGFRβ signaling pathway. We observed that both IFNγ and PPB-PEG-IFNγ exert anti-fibrotic effects as they significantly dampened TGFβ-induced fibronectin, collagen I and
collagen III expression after 48 hours incubation. These observations confirmed our recent \textit{in vivo} data (23).

To determine whether the anti-fibrotic effects were not due to PPB-induced blockade of PDGFRβ-signaling, we coupled PPB to albumin (HSA). We did not observe remarkable anti-fibrotic effects of PPB-HSA \textit{ex vivo}; only on the mRNA level a (non-significant) effect of PPB-HSA on collagen III expression was observed, indicating that most of the observed anti-fibrotic effects are IFNγ-mediated. Biological effects of IFNγ take place via the nuclear signaling sequence (NLS) which is present in the C-terminus region of IFNγ. IFNγ containing NLS is capable to bind to IFNγ receptor1 (IFNyR1) and initiate a cascade of events which is required for nuclear import of STAT1 and generation of biological activity. Activation of STAT1 by PEG-PPB-IFNγ was previously shown (19). We propose that PEG-PPB-IFNγ is taken up via PDGFRβ and the internalized construct next releases IFNγ or its metabolite which then binds to the intracellular part of IFNyR1 and activates the JAK/STAT pathway. However, this premise needs to be further explored.

In summary, the mPCKS model is a novel tool to study the pathophysiology of early fibrotic processes not only in animal tissue, but also in (fibrotic) human kidney tissue. Importantly, from the results of this study we conclude that the \textit{in vivo} observed anti-fibrotic effect of IFNγ and PPB-PEG-IFNγ can be successfully reproduced using mPCKS, indicating that this \textit{ex vivo} model is a useful tool for preclinical studies to test the efficacy of potential new anti-fibrotic drugs on fibroblast activation in a multicellular, pro-fibrotic milieu. In addition, the use of this model can contribute substantially to the reduction, refinement, and potential replacement of animal experiments. Further studies are ongoing to investigate the application of non-fibrotic and fibrotic human PCKS in order to validate our targeting strategy in the human setting. We believe the PCKS model is able to bridge the gap between basic research performed using \textit{in vitro} cell culture systems and translational human \textit{in vivo} studies as schematically represented in Figure 7. The preclinical studies using PCKS should then pave the road towards clinical studies on cell-specific targeting of renal fibrosis.
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