Exploring organ-specific features of fibrogenesis using murine precision-cut tissue slices

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\section*{A B S T R A C T}

Fibrosis is the hallmark of pathologic tissue remodelling in most chronic diseases. Despite advances in our understanding of the mechanisms of fibrosis, it remains uncured. Fibrogenic processes share conserved core cellular and molecular pathways across organs. In this study, we aimed to elucidate shared and organ-specific features of fibrosis using murine precision-cut tissue slices (PCTS) prepared from small intestine, liver and kidneys. PCTS displayed substantial differences in their baseline gene expression profiles: 70\% of the extracellular matrix (ECM)-related genes were differentially expressed across the organs. Culture for 48 h induced significant changes in ECM regulation and triggered the onset of fibrogenesis in all PCTS in organ-specific manner. TGF\(\beta\) signalling was activated during 48 h culture in all PCTS. However, the degree of its involvement varied: both canonical and non-canonical TGF\(\beta\) pathways were activated in liver and kidney slices, while only canonical, Smad-dependent, cascade was involved in intestinal slices. The treatment with galunisertib blocked the TGF\(\beta\)/Smad2 signalling in all PCTS, but attenuated culture-induced dysregulation of ECM homeostasis and mitigated the onset of fibrogenesis with organ-specificity. In conclusion, regardless the many common features in pathophysiology of organ fibrosis, PCTS displayed diversity in culture-induced responses and in response to the treatment with TGF\(\beta\)/Smad2 kinase inhibitor galunisertib, even though it targets a core fibrogenic pathway. A clear understanding of the common and organ-specific features of fibrosis is the basis for developing novel antifibrotic therapies.

\section*{1. Introduction}

Excessive scar formation, known as fibrosis, is a common pathological factor in numerous chronic diseases affecting various organs such as the liver, kidneys, intestine, lungs and skin, among others. Pathologies like Crohn’s disease, primary biliary cholangitis and chronic kidney disease cause progressive organ malfunction, resulting in high morbidity and mortality [1–3]. Despite the fact that diverse factors can cause aberrant wound healing, the pathogenesis of fibrosis shares a number of common features across organs [4,5]. Under chronic injury, fibroblasts differentiate into myofibroblasts – contractile, alpha smooth muscle actin (\(\alpha\)-SMA) positive cells – that secrete excessive amounts of extracellular matrix (ECM) proteins and glycoproteins, such as collagen, fibronectin, elastin, biglycan and decorin [6]. ECM remodelling is regulated by matrix metalloproteinases (MMPs) that degrade ECM, and their inhibitors – tissue inhibitors of metalloproteinases (TIMPs). Fibrosis occurs when the balance between MMPs and TIMPs shifts towards the latter, meaning that the synthesis of new ECM by myofibroblasts exceeds the rate at which it is degraded [7]. Other shared characteristics of fibrosis include the release of pro-fibrogenic cytokines and growth factors, impaired angiogenesis [8] and sustained inflammation [9,10].

Furthermore, fibrosis-associated signalling pathways are highly conserved between different organs. For instance, transforming growth factor beta (TGF\(\beta\)) is widely recognized as the key driving force behind fibrogenesis in essentially all organs [11–15]. Excessive release and
sustained activity of TGFβ stimulates cellular differentiation to myofibroblasts and pathological ECM turnover [4]. The TGFβ signalling cascade involves the binding of a ligand to the extracellular domain of the type II receptor (TGFβRII) that recruits and phosphorylates the type I receptor (TGFβRI or activin-receptor-like kinase 5, ALK5). The activated receptor complex triggers the recruitment of SMAD proteins (SMAD2 and SMAD3) that associate with SMAD4 to propagate the signal [16]. Although TGFβ mainly signals via SMADs, it can also activate other, non-canonical pathways such as phosphatidylinositol-3-kinase (PI3K)/AKT, Rho-like guanosine triphosphatases (GTPases) and mitogen activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) [17,18].

Among the available tools to study organ fibrosis, the precision-cut tissue slices (PCTS) model offers several valuable advantages. Opposed to conventional in vitro systems, PCTS preserve complex organotypic architecture and retain cell-cell and cell-matrix contacts [19]. Furthermore, PCTS substantially contribute to the 3Rs by reducing the number of animals needed for research, since slices can be prepared from various tissues, enabling simultaneous use of several organs from one animal. Our group previously demonstrated that PCTS can be used to study the mechanisms of intestinal fibrosis [20], liver fibrosis [21,22], renal fibrosis [23,24] and to test the efficacy of putative antifibrotic drugs.

The aim of this study was to uncover the shared and organ-specific features of fibrogenesis in the intestine, liver and kidney using murine PCTS. We explored ECM regulation and involvement of the TGFβ pathway both on a transcriptional and translational level, and investigated organ-specific responses to the treatment with antifibrotic compound galunisertib. Galunisertib (LY2157299 monohydrate) is a small molecule inhibitor of TGFβRI/ALK5 kinase that specifically downregulates the phosphorylation of SMAD2, and it is currently under clinical development for the treatment of a variety of cancers [25–27]. This study improves our general understanding of organ fibrosis for the purposes of basic research and the development of new therapies.

2. Methods

2.1. Animals

Adult C57BL/6J mice (Centrale Dienst Proefdieren, University Medical Center Groningen, Groningen, The Netherlands) were housed under controlled conditions with a 12 h:12 h light-dark cycle and free access to water and food. The experiments were approved by the Animal Ethical Committee of the University of Groningen (DEC 6416AA-001).

Organs were harvested via a terminal procedure performed under isoflurane/O2 anesthesia (Nicholas Piramal, London, UK). Freshly excised livers and kidneys were kept in ice-cold University of Wisconsin (UW) organ preservation solution until slicing. Mouse jejunum was preserved in ice-cold Krebs-Henseleit buffer (KHB) supplemented with 25 mM d-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO3 (Merck) 10 mM HEPES (MP Biomedicals, France), saturated with carbon dioxide (95% O2/5% CO2), pH 7.4.

2.2. Preparation of precision-cut tissue slices

Liver, kidney and intestinal PCTS were prepared using a Krumdieck tissue slicer (Alabama Research & Development Corp., Munford, AL, USA) according to the protocol described by de Graaf et al. [28] and Stribos et al. [24], with minor modifications.

2.2.1. Precision-cut liver slices (PCLS)

Liver tissue cores were made using a 6 mm biopsy punch. Slices with a wet weight of 4–5 mg and estimated thickness of 250–300 μm were prepared in ice-cold KHB and transferred to UW directly after slicing, to prevent rapid loss of viability. Slices were incubated individually in 1.3 mL of Williams’ medium E with GlutaMAX (Life Technologies, Bleiswijk, the Netherlands) supplemented with 25 mM d-glucose (Merck) and 50 μg/mL gentamicin (Life Technologies) at 37 °C in an 80% O2/5% CO2 atmosphere while gently shaken at 90 rpm.

2.2.2. Precision-cut kidney slices (PCKS)

Whole mouse kidneys were placed in the coreholder inside the tissue slicer to obtain slices with a wet weight of 4–5 mg and thickness of 250–300 μm. Kidney slices were immediately transferred to ice-cold UW after slicing. Subsequently, PCKS were incubated individually in 1.3 mL of Williams’ medium E with GlutaMAX containing 10 μg/mL ciprofloxacin (Sigma-Aldrich, Saint Louis, USA) and 25 mM d-glucose (Sigma-Aldrich) at 37 °C in a 80% O2/5% CO2 atmosphere while gently shaken at 90 rpm.

2.2.3. Precision-cut intestinal slices (PCIS)

The jejunum was cleaned by flushing KHB through the lumen, the tissue was subsequently divided into 2 cm segments. These segments were filled with 3% (w/v) agarose (Sigma-Aldrich) in 0.9% NaCl at 37 °C and embedded in an agarose core-embedding unit. Intestinal slices with a weight of 1–2 mg were prepared and stored in ice-cold KHB, then cultured individually in 0.5 mL of Williams’ medium E with GlutaMAX supplemented with 25 mM d-glucose, 50 μg/mL gentamicin and 2.5 μg/mL fungizone (ampoterin B; Life Technologies) at 37 °C in an 80% O2/5% CO2 atmosphere while gently shaken at 90 rpm.

2.3. Experimental treatment of PCTS with galunisertib (LY2157299)

Galunisertib (LY2157299) was purchased from Selleckchem (Munich, Germany). Stock solution of 2.5 mM was prepared in DMSO and diluted in the culture medium with a final concentration of the solvent of ≤0.5%. Mouse liver, kidney and intestinal slices were incubated with 10 μM of galunisertib or solvent for 48 h. The tested concentration of galunisertib was in the range of observed plasma exposure [29,30]. Medium was refreshed every 24 h.

2.4. Viability of PCTS

After incubation, slices were transferred to a sonication solution containing 70% ethanol and 2 mM EDTA and snap-frozen [28]. Viability of the slices was assessed by measuring the adenosine triphosphate (ATP) content using the ATP bioluminescence kit (Roche diagnostics, Manheim, Germany). The ATP values (pmol) were normalized to the total protein content (μg) of each slice, estimated by the Lowry assay (Bio-Rad DC Protein Assay, Hercules, USA). Values are displayed as relative values compared to the average of a related control.

2.5. Total RNA isolation cDNA synthesis

Total RNA was isolated from pooled snap-frozen slices (three slices for liver and kidney, six slices for jejunum), using the Qiagen RNAeasy mini kit (Qiagen, Venlo, The Netherlands). The absorbance ratio 260/280 was used to assess RNA purity and was considered satisfactory when values ranged between 1.9 and 2.1. Reverse transcription was performed with 6 ng/μL cDNA and 50 μL Taqman Universal PCR Master Mix.
(Applied Biosystems) was transferred into the loading port of a TLDA card. PCR amplification was performed on the Applied Biosystems ViiA™ 7 Real-Time PCR System. The most appropriate housekeeping gene was identified by NormFinder [31]. Based on these results, Gapdh was used as an endogenous control. Expression values were calculated using the $2^{-\Delta\Delta Ct}$ method [32]. All Ct values above 37 were considered noise and excluded from further analysis. Normalization of the Ct values in each sample was carried out as $\Delta Ct = Ct(\text{target}) - Ct(\text{Gapdh})$. The fold change was calculated comparing the gene expression of each group with the average of the control expression level: fold change $(\text{FC}) = 2^{-\Delta Ct(\text{experimental})/\Delta Ct(\text{control})}$. Gene expression levels were determined relative to 0 h PCIS for baseline expression profiling or to 0 h of respective PCTS for culture and treatment effects. Variance stabilization was managed with $\log_2$ transformation. The heatmap of $\log_2(\text{FC})$ values was generated using the online tool Morpheus (https://software.broadinstitute.org/morpheus/). Hierarchical cluster analysis was performed by average-linkage clustering method using Pearson correlation.

2.7. Quantitative real-time PCR

mRNA expression of key fibrosis markers and genes involved in TGFβ signalling was determined with qRT-PCR. The RT-PCR reaction was performed using the SensiMix SYBR Hi-ROX kit (Bioline,
Luckenwalde, Germany) on a 7900HT Real Time PCR system (Applied Biosystems) with a cycle at 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 25 s followed by a dissociation stage. The mRNA expression values were calculated using the 2-ΔΔCt method, with Gapdh as a reference gene. The primers (50 μM; Sigma-Aldrich) used in this study are listed in Supplementary Table S2.

2.8. Western blotting

Three (for liver and kidney) or six (for jejunum) slices were pooled and lysed in ice-cold RIPA buffer (Thermo Scientific, Waltham, Massachusetts, USA) supplemented with PhoshoStop (Roche Diagnostics, Mannheim, Germany) and protease inhibitor cocktail (Sigma-Aldrich). A total of 90–100 μg of protein was separated by SDS/PAGE on 10% sodium dodecylsulfate polyacrylamide gel, containing 2,2,2-trichloroethanol (TCE; Sigma-Aldrich) for visible detection of total protein load [33], and subsequently transferred to an activated polyvinyl difluoride membrane (Immu-no-Blot PVDF, Bio-Rad). Membranes were blocked in TBST with 5% Blotting-Grade Blocker (Bio-Rad) and incubated with primary antibody (Supplementary Table S3) overnight at 4°C. Immunodetection was performed by incubating the membranes with the appropriate HRP-conjugated secondary antibody. Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad) and ChemiDoc Touch Imaging System (Bio-Rad). Protein expression was corrected for total protein load and expressed as a relative value to the control group.

2.9. Statistics

The aforementioned analyses were performed using three to six pooled slices from the same animal (technical replicates) and repeated with at least three mice (biological replicates). The results are expressed as mean ± standard error of mean (SEM). We used GraphPad Prism 6.0 (GraphPad Software Inc.) to carry out statistical data analysis. Treatment groups were compared by unpaired Student's t-test or one-way ANOVA followed by Dunnett's multiple comparisons test as appropriate. Protein levels determined by Western blot were compared using non-parametric Mann-Whitney test. A p-value of < 0.05 was considered statistically significant.

3. Results

We used murine small intestine (jejunum), liver and kidney to prepare precision-cut tissue slices (PCIS, PCLS and PCKS, respectively) as illustrated in Fig. 1a. Slices were cultured for 48 h under standard conditions or treated with TGFβR1 kinase inhibitor galunisertib. Fig. 1b shows that all slices remained viable at 48 h, in accordance with our previous reports [24,34]. PCIS displayed a slight decrease in ATP content, while the ATP levels of PCLS and PCKS significantly increased at 48 h, indicating that ATP production was restored after the cold ischemia period as a result of slicing. In line with previous reports [22], galunisertib at 10 μM did not elicit toxicity in PCIS, PCLS or PCKS, as it had no impact on ATP content.

3.1. Differential regulation of genes involved in ECM homeostasis in PCTS

To characterize the regulation of ECM homeostasis, we carried out gene expression profiling of tissue slices at 0 h, after 48 h of culture or after the treatment with a TGFβR1 kinase inhibitor galunisertib. We performed TaqMan low density array (TLDA) with a panel of 44 genes related to various ECM components (including six types of collagen), enzymes involved in collagen processing and ECM remodelling and ECM protein receptors.

Fig. 2a illustrates the baseline ECM (regulation) expression profiles of PCIS, PCLS and PCKS, as visualized by a heatmap with hierarchical clustering. Unsupervised cluster analysis of relative expression levels (log2(FC) values) provided a perfect separation of 0 h PCIS, PCLS and PCKS, suggesting substantial differences in the baseline ECM regulation profiles between intestine, liver and kidney. A comparative statistical analysis revealed that only 13 out of 44 transcripts (30%) had similar expression levels in all PCTS (Supplementary Table S4). Furthermore, we identified seven genes (Pld2, Leprel1, Lox2, Mmp13, Bmp1, Fn1 and Ddr2) that were differentially expressed across all PCTS (Fig. 2b).

Fig. 2c–e illustrates the sets of transcripts that represent distinct signatures in PCTS baseline expression profiles. For instance, PCIS at 0 h highly expressed genes encoding collagens (Col1a1, Col3a1, Col6a1) and metalloproteinases (Mmp2 and Mmp9). PCLS, in turn, exhibited characteristically low expression of five transcripts (Col1a2, Col4a1, Lox1, Serpinh1 and Ddr1), while PCKS expressed high levels of other six mRNAs (Leprel2, Pcolce, Slc39a13, Fmod, Bgn and Mrc2) and low levels of P4hb and Dcn.

We next examined the impact of 48 h culture and the treatment with TGFβR1 kinase inhibitor (Fig. 3). Both culture and galunisertib introduced considerable changes in the gene expression profiles of PCIS, PCLS and PCKS, as the cluster analysis clearly separated the samples by time point (0 h vs. 48 h) and treatment (48 h control slices vs. 48 h slices treated with 10 μM galunisertib) (Fig. 3a–c). The effect of culture in PCTS is summarized in Fig. 3d and f, and Fig. 3e, g and h detail the effect of galunisertib. We performed a pairwise comparison of expression levels in slices at 0 h versus 48 h and identified 21 (48%), 26 (59%) and 38 (86%) differentially expressed genes that achieved statistical significance in PCLS, PCKS and M galunisertib, respectively (Fig. 3d). The majority of these transcripts were upregulated in PCLS (92%) and PCKS (89%), whereas in PCIS 10 out of 21 differentially expressed genes (48%) were downregulated during 48 h culture (Fig. 3d), including Col1a1, Col1a2, Col3a1, Pcolce, Pcolce2, Efn and Bgn. The Venn diagram (Fig. 3e) illustrates the numbers of overlapping and unique genes influenced by 48 h culture between PCIS, PCLS and PCKS, followed by the gene lists. Only nine (20%) transcripts were common in all tissue slices: besides altered expression of Col1a1, Col1a2 and Col3a1, the organs showed similar culture-induced increase in P4hb2, Leprel1, Lox2, Fkp10, Mmp13 and Timp1. Furthermore, the number of genes that were shared across pairs of organs dominated over those that were unique within each organ. In particular, PCLS and PCKS exhibited more similar changes in ECM homeostasis at 48 h than their pairwise comparisons with PCIS, as 13 transcripts were commonly affected in PCLS and PCKS, eight transcripts were common between PCIS and PCKS and only three between PCIS and PCLS. As the largest number of differentially expressed genes was observed in cultured PCKS, eight of these genes were unique to PCKS and included Col6a1, Bmp1, P4ha1 (required for proper collagen folding), Lox3 (involved in crosslinking of collagen and elastin), Adamts2 and Adamts3 that are crucial for collagen fibrils formation, and the ECM protein receptors Ddr2 and Mrc2. The top two differentially regulated genes at 48 h in PCIS and PCLS were Mmp13 (fold change 88.29 ± 23.78 in PCIS, 85.96 ± 7.77 in PCLS) and Timp1 (fold change 73.21 ± 8.76 in PCIS, 57.89 ± 16.54 in PCLS), while PCKS showed the most dramatic increase in Mmp9 (fold change 114.34 ± 49.87) and Timp1 (fold change 684.29 ± 86.05) (Supplementary Table S5). Overall, the culture-induced changes in ECM homeostasis indicate the onset of fibrogenesis in PCTS.

When PCTS were treated with 10 μM galunisertib for 48 h, the degree of the response varied between the organs (Fig. 3e). PCKS displayed an altered expression of 29 genes (66%) with statistical significance. The number of genes affected by galunisertib lowered to nine (20%) in PCLS, and only five genes (11%) were significantly affected in PCIS. Notably, the majority of these transcripts were downregulated; however, some genes were upregulated by galunisertib treatment: Pld3 (fold change 1.37 ± 0.09 in PCIS), Lox4 (fold change 1.29 ± 0.12 in PCLS and 1.41 ± 0.14 in PCKS) and Dcn (fold change 3.34 ± 0.12 in PCKS; Supplementary Table S5). Furthermore, statistical analysis revealed that galunisertib significantly downregulated a total of three transcripts across all tissue slices, namely Lox, Col1a1 and
Col3a1 (Fig. 3g), and the level of downregulation was increasing in the order PCIS < PCLS < PCKS. For instance, galunisertib inhibited Col1a1 expression by 48% in PCIS, 87% in PCLS and by 97% in PCKS (Supplementary Table S5). Of the 44 tested genes, 13 genes remained unaffected in PCKS after treatment (Fig. 3h) and included genes encoding prolyl hydroxylases P4ha1, P4ha2, P4hb and P4hc, procollagen N-proteinases Adamts3 and Adamts14, Col4a1 and ECM protein receptors Ddr1 and Ddr2, among others.

3.2. Regulation of selected markers of fibrogenesis in murine PCTS

To confirm the results of the TLDA, we performed single-gene SYBR green RT-qPCR for selected transcripts. Fig. 4a shows the mRNA level of genes encoding for collagen type I (Col1a1), heat shock protein 47 (Serpinh1) and fibronectin (Fn1) in tissue slices at 0 h, after 48 h culture and after treatment with 10 μM galunisertib. We also included a fourth marker of fibrogenesis — Acta2, encoding α-SMA that is expressed by myofibroblasts. The baseline expression of Col1a1, Acta2 and Fn1 was distinctly higher in PCIS as compared to liver and kidney slices, while Serpinh1 mRNA level was the lowest in PCLS. All tissue slices developed a spontaneous fibrogenic response during 48 h culture. We observed a significant upregulation of Serpinh1 and Fn1 in all organs, upregulation of Col1a1 and no change in Acta2 expression in PCLS and PCKS. Of note, the magnitude of culture-induced upregulation varied in an organ-specific manner: the expression level of fibrogenesis markers after 48 h culture were consistently and significantly higher in PCKS than in other tissue slices. In contrast to liver and kidney, PCIS displayed a significant decrease in mRNA levels of Col1a1 and Acta2 at 48 h. Overall, our qPCR results for Col1a1 and Serpinh1 correlated with TLDA, but not for Fn1: in contrast to TLDA results, PCIS (and not PCLS) expressed high baseline levels of Fn1, and there was a significant up-regulation at 48 h in PCLS.

To further explore the expression profiles of tissue slices from jejunum, liver and kidney, we measured the expression of fibrosis markers by Western blot (Fig. 4b). We observed an increased protein expression of α-SMA and HSP47 after 48 h in all tissue slices, although the increase of HSP47 in PCLS did not reach statistical significance (fold change 2.77 ± 1.90). In PCIS, even though Acta2 mRNA expression...
was reduced at 48 h, the protein expression of α-SMA showed a significant increase. These results indicate that the fibrogenesis was not only active on a transcriptional, but also on a translational level.

Galunisertib (10 μM) mitigated the culture-induced onset of fibrogenesis in PCKS, as reflected by the dramatically reduced mRNA levels of all tested markers (Fig. 4a). Treatment affected the expression of three markers in PCLS, namely Colla1, Acta2 and Fn1, and it only inhibited Colla1 expression in PCIS. These qPCR results were in line with the TLDA. On a protein level, galunisertib showed less pronounced effects: it only significantly decreased α-SMA expression in PCLS and HSP47 expression in PCKS (Fig. 4b).

To strengthen the evidence that fibrogenic response in tissue slices is influenced by the organ type, we evaluated mRNA expression of Colla1, Acta2, Serpinh1 and Fn1 in slices prepared from fibrotic livers (Supplementary Fig. S1). For this purpose, two mouse models of liver fibrosis were used to generate fibrotic PCLS, namely Mdr2 (Abc4)−/− FVB mice that develop spontaneous biliary fibrosis [35] and Balb/c mice with induced parenchymal liver fibrosis by the administration of CCl4 [36]. We showed that diseased PCLS displayed a clear fibrotic genotype prior to culturing: PCLS prepared from fibrotic livers had significantly higher baseline expression of all four markers (except for Acta2 in PCLS-CCL4) compared to PCLS prepared from livers of control mice (Supplementary Fig. S1a and c). Furthermore, fibrotic PCLS from Mdr2−/− and CCl4-treated mice showed similarities in responses to culture and exposure to galunisertib not only between these two models of liver fibrosis but also compared to the responses in PCLS obtained from healthy mice. For instance, similar to healthy PCLS, 48 h culture induced upregulation of Colla1, Serpinh1 and Fn1, and galunisertib inhibited expression of Colla1 and Acta2, in all fibrotic slices (Supplementary Fig. S1b and d). Some differences were observed as well: in contrast to healthy PCLS, fibrotic slices obtained from CCl4-treated mice showed increased levels of Acta2 during culture, while PCLS from Mdr2−/− mice showed no response in Fn1 mRNA expression to the treatment with galunisertib. These deviations might stem from the differences in the mouse strains, etiology of liver fibrosis and from the pre-existing diseased state of fibrotic PCLS. Nevertheless, the partial overlap in the expression of these four markers between healthy and fibrotic PCLS indicates that organ type largely dictates the responses of tissue slices to pro-fibrotic stimuli as well as to the antifibrotic treatment.

Since inflammation often accompanies fibrogenesis, we investigated the gene expression of several inflammation markers, such as Il-1β, Il-6, Cxcl1 and Tnf (Supplementary Fig. S2). Tissue slices exhibited differential basal expression of these proinflammatory cytokines. For instance, PCIS at 0 h highly expressed Il-6 and Tnf, while PCLS had significantly higher levels of Cxcl1. Culturing for 48 h induced a strong inflammatory response in all organs, while the treatment with galunisertib had no or little effect on the expression of the tested inflammation markers.

3.3. Involvement of canonical and non-canonical TGFβ pathway in fibrogenesis in PCTS

As mentioned above, TGFβ signalling is an essential element of organ fibrosis. Therefore, we investigated the involvement of the TGFβ pathway in the culture-induced onset of fibrogenesis in PCTS by measuring mRNA expression of Tgfb1, Tgfb1 and Serpine1 (Fig. 5a). The latter encodes for plasminogen activator inhibitor 1 (PAI-1), which is tightly regulated by TGFβ and in fibrotic tissues promotes excessive collagen deposition [37].

Baseline expression of TGFβ pathway markers, PCIS showed high levels of Tgfb1, PCKS had distinctly high levels of Serpine1, and there was no difference in expression of Tgfb1 between the organs. As expected, all three markers were significantly upregulated during 48 h culture in PCLS and PCKS, confirming the active involvement of the TGFβ pathway in the onset of fibrosis. PCIS also showed an increase in mRNA levels of Tgfb1 and Serpine1 (at a lower degree as compared to PCKS), but the Tgfb1 expression remained unchanged. Treatment with the TGFβR1 kinase inhibitor galunisertib only effectively blocked the TGFβ pathway in PCKS, while it failed to affect the gene expression of all three markers in PCLS and PCIS.

Since the activated TGFβR1-TGFβR1/ALK5 complex phosphorylates SMAD2 (pSMAD2) [16], the changes in pSMAD2 can be used to determine the activity of the TGFβ pathway as well as the efficacy of TGFβ inhibitors. Along with pSMAD2, we measured the protein expression of pSMAD1 – the downstream molecule of TGFβR1/ALK1 signalling [38]. As predicted, pSMAD2 was significantly increased during culture to a similar degree in all organs (Fig. 5b). Interestingly, pSMAD1 was also increased in PCKS and PCIS, but not in PCLS. We noticed that in PCLS, 48 h culture induced a much more pronounced change in pSMAD1 protein expression (fold change 92.75 ± 57.72) than in pSMAD2 (fold change 6.72 ± 1.31). Treatment with 10 μM galunisertib inhibited the phosphorylation of SMAD2 in all tissue slices, without affecting pSMAD1.

To explore the activity of non-canonical TGFβ pathways in PCTS, we measured mRNA expression of Traf6, Mapk37, Mapk1, Rock1 and Rock2 (Fig. 6). TGFβ promotes K63 polyubiquitination of tumor necrosis factor receptor-associated factor 6 (TRA6) that activates TGFβ-associated kinase 1 (TAK1) encoded by Mapk37, which are specifically required for activating JNK, p38 and NF-κB [18,39]. In turn, Mapk1 encodes ERK2 – a kinase involved in TGFβ-activated ERK-MAPK signalling. Rho-associated kinases ROCK1 and ROCK2 are the downstream targets of the small GTPases Ras, RhoB, and RhoC. The baseline mRNA expression of tested markers was significantly higher in PCIS compared to other tissue slices, except for Traf6, of which baseline expression was similar in all organs. Furthermore, culture-induced TGFβ-activated MAPK, NF-κB and Rho-like GTPases signalling cascades in PCLS and PCIS, as reflected by significant increase in gene expression of all five markers. The effect in PCIS was the opposite: Mapk1 and Rock1 were downregulated at 48 h, other transcripts also showed a decreased expression, although not statistically significant. As expected, galunisertib had no impact on activation of non-canonical pathways.

3.4. Transcription factors in PCTS

Lastly, we attempted to identify which transcription factors (TFs) are involved in the onset of fibrosis in PCTS. TFs are a large family of proteins that control the transcription of genes, and are often expressed in a tissue and stress-specific manner. We measured mRNA expression of four TFs, namely Yy1, Nfkbia, Stat3, and Egr1 (Fig. 7). These TFs are of interest due to their established connection to ECM regulation or TGFβ signalling. Briefly, Ying Yang 1 (YY1) protein is involved in the transcription of α1 and α2 collagen type 1 gene [40,41]. Early growth
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response-1 (EGR-1) also mediates TGFβ-induced collagen type I transcription, although in a SMAD-independent manner involving MAPK-ERK signalling [42,43]. In turn, TGFβ-mediated activity of NF-kB often requires cooperation with SMADs as transcriptional coactivators to regulate the transcription of its target genes [44], whereas activation of the signal transducer and activator of transcription 3 (STAT3) by TGFβ requires integrated signals from phosphorylated SMAD3 and non-canonical kinases such as JAK1 [45].

Culturing for 48 h resulted in marked upregulation of all tested TFs in PCLS and PCKS. In contrast, PCIS only displayed a culture-induced upregulation of Egr1, while expression of other TFs remained unchanged. The 48 h treatment with galunisertib attenuated culture-induced activation of transcription factors Nfkbi, Stat3 and Yy1 in PCLS and PCKS, but not in PCIS. Gene expression of Egr1 remained unchanged in tissue slices after 48 h treatment.

4. Discussion

In this article, we investigated the heterogeneity of organ fibrosis. To this end, we prepared murine precision-cut tissue slices from intestine, liver and kidneys and studied changes in ECM homeostasis, onset of inflammation and fibrosis and activation of TGFβ signalling prior to culturing, after 48 h of culture or following the treatment with galunisertib, a TGFβRI/ALK5 kinase inhibitor. We provide evidence that, despite the many common features of fibrotic diseases, each organ responds differently to injury and, thus, may not have similar susceptibility to antifibrotic therapy.

At baseline, precision-cut tissue slices displayed substantial organ differences, as a total of 70% of ECM-related transcripts were found to be differentially expressed in mouse intestine, liver and kidney. These differences may arise from fibroblast phenotypic heterogeneity [46,47] or from the fact that cell type composition varies between these organs, as well as the sources of myofibroblasts [48]. The baseline profile may impact further responses of the slices during culture.

Slice preparation, which entails cold ischemia and mechanical trauma, and culturing have a great impact on tissue slices: mouse intestinal, liver and kidney slices show extensive changes in ECM regulation and develop early fibrogenic as well as inflammatory responses after 48 h incubation. Similar culture-induced responses were previously reported in PCIS [20], PCLS [49] and PCKS [24]. In this study, we demonstrate that the magnitude of these changes varies by tissue type: culture-induced effects were most pronounced in PCKS and the least in PCIS. Matrix remodelling is a critical component of fibrosis [4], and our results show that all tested organ slices shared culture-induced changes in the gene expression of collagen type I (α1 and α2) and III, fibronectin, LOXL2, an enzyme that promotes collagen production [50], as well as HSP47 and FKBP65 that act as chaperones of type I collagen [51,52], among others. All tissue slices also showed a dramatic upregulation of Mmp13 and Timp1, further suggesting extensive matrix remodelling during culture, as it has been shown that Timp1 is upregulated during fibrogenesis in mouse models and in humans [53–55] and increased levels of MMP13 promote fibrosis [56–58]. Exposure of PCTS to 80% oxygen during culture also leads to an increased production of reactive oxygen species (ROS). In this case, it is likely that the oxidative stress also contributes to the onset of spontaneous inflammation and fibrogenesis in PCTS; however, the exact role of ROS and oxidative stress in the PCTS model requires further investigation.

Despite the shared similarities in ECM regulation, culture-induced changes in intestinal slices markedly differed from the other tissue slices. The majority of the studied genes were upregulated in PCLS and PCKS, whereas nearly half of the transcripts were alternatively regulated in PCIS. For instance, genes encoding collagen type I (α1 and α2), type III and alpha-SMA were among those with reduced expression in PCIS during culture. Collagen is secreted as a soluble procollagen molecule with an NH2-(N) and a COOH-(C)-terminal propeptide, and the removal of these propeptides is essential for the formation of insoluble collagen fibers [59]. Bone morphogenic protein (BMP) 1 enzymatically cleaves the C-terminal propeptide [60], while two enhancer proteins — procollagen C-endopeptidase enhancer (PCOLCE) 1 and 2 – increase catalytic activity of BMP-1 for fibrillar procollagens in vitro [61,62]. BMP-1 expression increases in response to fibrotic deposition of collagen [63]; however our study showed that intestinal expression of Bmp1 remained unchanged during culture. Furthermore, expression of both Pcolce and Pcolce2 were downregulated in PCIS at 48 h. These observations contradict the published work that reported on increased mRNA levels of collagen type I, III and V in fibroblasts isolated from patients with Crohn’s disease [64,65]. Regarding the expression of α-SMA in PCIS, the discordance between its gene and protein levels at 48 h could be attributed to the differences in rate of transcriptional and translational processes and/or the half-lives of transcripts and proteins [66]. Further elucidation of the mechanisms of collagen type I and α-SMA transcriptional regulation in PCIS is, therefore, needed.

As a part of our interest in elucidating inter-organ differences, we investigated one of the core fibrosis pathways – the TGFβ pathway. TGFβ pathway was activated during culture in all tissue slices; however, the involvement of canonical (SMAD-dependent) and non-canonical (SMAD-independent) signalling cascades was different across the organs. In PCLS and PCKS, all tested TGFβ-mediated pathways were activated during culture, namely TGFβRI/ALK5/SMAD2, MAPK-JNK/p38/ERK, NF-kB and Rho-like GTPases. In contrast, in PCIS, only the canonical TGFβ signalling cascade was actively involved in the onset of fibrosis. Furthermore, among all tissue slices, PCIS displayed a stronger culture-induced activation of TGFβRI/ALK1/SMAD1 than of ALK5/SMAD2. There is an increasing number of studies reporting that the TGFβRI/ALK1/SMAD1 pathway is involved in organ fibrosis [38]. TGFβ signalling via ALK1 receptor and SMAD1, SMAD5 and SMAD8 promotes endothelial proliferation and migration and acts as an antagonist mediator of ALK5/SMAD2/3-induced ECM protein expression [67,68]. It has been reported that ALK1 inhibits, while ALK5 potentiates, TGFβ-induced SMAD2-dependent transcriptional activity and the expression of ECM components in human chondrocytes and endothelial cells [69,70]. Therefore, it might be possible that high activity of TGFβRI/ALK1/SMAD1 signalling in PCIS plays a role in the observed downregulation of ECM- and fibrosis-related genes in PCIS during culture.

The treatment with galunisertib selectively blocked SMAD2 phosphorylation in all tissue slices, regardless of the organ of origin, without affecting pSMAD1 or non-canonical TGFβ signalling cascades. However, galunisertib only reduced the expression of the TGFβ pathway markers (Tgfβ1, Tgfβ1 and Serpine1) in PCKS, but not in PCLS or PCIS. Recently, Luangmonkon et al. reported that 10 μM galunisertib blocked SMAD2 phosphorylation in rat and human PCLS, but affected Tgfβ1 expression only in human liver slices [22]. Of note, this study also investigated the effects of TGFβ1 on rat and human PCLS.
during culture and in presence of galunisertib. Studies on hepatocellular carcinoma also found a significant reduction of Tgfb1 and Tgfb1 mRNA levels by galunisertib in vivo in mice and ex vivo in human tumor tissue [71,72]. These findings suggest that along with organ-differences, the effect of galunisertib varies between species and is possibly more pronounced in tissues with an established pathological state.

Following the observation that galunisertib downregulated Tgfb1, Tgfb1 and Serpine1 only in kidney slices, our results demonstrate that galunisertib mitigated culture-induced changes in ECM homeostasis and early fibrogenesis most effectively in PCKS, showed moderate activity in PCLS and had only limited effect in PCIS. Most of the tested ECM- and fibrosis-related genes seem to operate in a TGFβRI/ALK5-dependent manner.

Fig. 5. Baseline expression, culture-induced effects and impact of TGFβRI/ALK5 inhibitor on TGFβ signalling in murine precision-cut tissue slices. (a) Expression of Tgfb1, Tgfb1 and Serpine1 mRNA levels were measured by RT-qPCR in PCIS, PCLS and PCKS at 0 h (baseline), after 48 h culture or treatment with 10 μM galunisertib. Results are shown as 2^ΔCt values normalized to Gapdh. Data are presented as mean ± SEM, n = 3. (*) denotes statistical differences between slices from different organs at 48 h; p < 0.05. (b) Protein expression levels of pSMAD2 and pSMAD1 were examined in by Western blot. Representative gel electrophoresis bands are shown along with the quantification results of densitometry. Protein expression was normalized for total protein load and expressed as relative value to the control group. Data are presented as mean ± SEM, n = 4–5, *p < 0.05.
Traf6 baseline

Map3k7 baseline

Mapk1 baseline

Rock1 baseline

Rock2 baseline

Fig. 6. Baseline expression, culture-induced effects and impact of TGFβRI/ALK5 inhibitor on non-canonical TGFβ signalling in murine precision-cut tissue slices. Expression of Traf6, Map3k7, Mapk1, Rock1 and Rock2 mRNA levels were measured by RT-qPCR in PCIS, PCLS and PCKS at 0 h (baseline), after 48 h culture or treatment with 10 µM galunisertib. Results are shown as 2^ΔCt values normalized to Gapdh. Data are presented as mean ± SEM, n = 3. (*) denotes statistical differences in baseline levels or differences between 0 h and 48 h slices of the same organ, while (#) denotes statistical differences between slices from different organs at 48 h; p < 0.05.

SMAD2 dependent manner in kidney, but not in intestine, emphasizing that ECM homeostasis and early fibrogenesis are regulated differently in these organs. Notably, ex vivo activity of galunisertib in PCLS (healthy and fibrotic) falls in line with recent in vivo studies that showed antifibrotic potency of galunisertib in liver fibrosis, which was mainly associated with ECM remodelling [73,74]. We speculate that similarly, antifibrotic effects of galunisertib in PCKS might translate into mitigation of renal fibrosis in vivo.

Interestingly, while galunisertib inhibited mRNA expression of collagen type I (α1) and III in all tissue slices, the treatment had no impact on collagen type IV as well as on Leprel1 gene, encoding P3H enzyme responsible for modifying type IV collagens [75]. Different types of collagen vary in their structure, assembly and function. Types I and III collagens belong to the family of fibril-forming collagens that are largely present in ECM, whereas type IV collagen is the main structural component of basement membranes [76]. The regulation of these
Guo et al. demonstrated that TGFβ factors (TFs) in PCTS during culture and after galunisertib treatment. Lung fibroblasts, which in turn can directly regulate Yy1 in lung fibroblasts, which in turn can directly regulate αSMA and collagen expression [77]. We did not observe an increase in Yy1 expression in PCIS during culture; this could explain the absence of induction of the expression of Col1a1 and Acta2 during culture. In general, TFs that operate in a SMAD-dependent manner (Yy1, Stat3, Nfkb1) were affected by galunisertib treatment in both PCLS and PCKS, while those that are SMAD-independent (Egr1) were not.

5. Conclusion

Taken together, our study details the organ-specific features of fibrosis in murine intestine, liver and kidney slices. PCTS, as an ex vivo fibrosis model, reflects the diversity of the responses that are specific to the organ and species. Furthermore, our results revealed that treatment with TGFβRI/ALK5 kinase inhibitor elicits varying effects in PCTS, confirming that organs do not display similar susceptibility to antifibrotic therapy, even though it targets a core fibrosis pathway. The following limitations of the PCTS model have to be considered: (1) relatively short culture period might not fully demonstrate changes on a translational level; (2) influence of the immune system cannot be directly assessed and (3) complexity of a multi-organ system cannot be replicated. Our findings provide the foundation for future investigations of the unique organ features within the common pathology – fibrosis. A better understanding of the processes and mechanisms that contribute to organ fibrosis may stimulate a hybrid approach for the drug development – targeting core fibrosis-regulating factors in an organ-specific manner. Therefore, new in-depth studies of precision-cut tissue slices of mouse and human origin using advanced genomic and proteomic technologies, among others, are needed.

Transparency document

The Transparency document associated this article can be found, in online version.
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

E.B., M.B. and P.O. designed the study; E.B., and E.G. carried out experiments with the help of D.O. and analyzed the data; D.S. and Y.O.K. provided access and assisted with mouse models of liver fibrosis; R.A.B provided custom designed TLDA; E.B. wrote the manuscript with critical review from H.A.M.M., D.S., R.A.B., M.B. and P.O. All of the authors approved the final version of the manuscript for publication.

Appendix A. Supplementary data

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References
