CHRONIC PANCREATITIS FIBROGENESIS: THE ROLE OF TGFβ PATHWAY INHIBITOR SMAD7 IN THE FIBROSIS DEVELOPMENT

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Chronic pancreatitis fibrogenesis: The role of TGFB pathway inhibitor Smad7 in the fibrosis development

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To all researchers who bridge the gap between theory and reality.
ABSTRACT

Chronic pancreatitis is characterized by progressive inflammation, acinar atrophy and fibrosis. Transforming growth factor-B signaling (TGFB) is the most potent fibrogenic cytokine known, and its increased expression is a common denominator for fibrosis in chronic pancreatitis. Smad7 is induced by the TGFB superfamily members as an intracellular inhibitory feedback antagonizing TGFB signaling. To investigate the functional role of Smad7 in vivo, we induced chronic pancreatitis by repeated administration of cerulein in mice that are either ubiquitous or conditional deficient of Smad7. Mice with general deficient of Smad7 showed more severe response to chronic pancreatitis induction as indicated by a stronger accumulation of extracellular matrix (ECM), increased levels of inflammatory cells and mesenchymal cells/myofibroblasts. Mice with fibroblasts-specific deficient of Smad7 showed slightly higher fibrotic index but the production of different ECM proteins and the number of fibroblasts and inflammatory cells were not affected, although in vitro studies confirmed increased activation of mutant pancreatic stellate cells. Mice with myeloid cells-specific deficient of Smad7 exhibited fewer number of macrophages and pancreatic stellate cells and less fibronectin deposition, which might be related to the reduced capacity of Smad7 deficient macrophages to support pancreatic stellate cells’ survival. Thus, targeting TGFB signaling by Smad7 demonstrates diverse effects in different contexts, and Smad7 can exert both exacerbated and protective properties in experimental chronic pancreatitis. Tamoxifen is widely used for the induction of CreERT-mediated genomic recombination in conditional mouse models, but it also affects the fibrotic response in several disease models. In order to investigate a possible effect of tamoxifen on pancreatic fibrogenesis and to evaluate an optimal treatment scheme in an experimental pancreatitis mouse model, we gave administered tamoxifen by oral gavage to both male and female C57BL/6J mice, and then waited for different periods of time before inducing chronic pancreatitis by cerulein. We observed a sex-specific and time-dependent effect of tamoxifen on the fibrotic response both in vivo and in vitro, and a 2-week waiting period before cerulein administration is suggested to reduce tamoxifen side effects to a minimum for the described fibrosis model in female mice.
LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. Cerulein-induced pancreatic fibrosis is modulated by Smad7, the major negative regulator of transforming growth factor-β signaling.
   Li X, Nania S, Fejzibegovic N, Moro CF, Klopp-Schulze L, Verbeke C, Löhr JM, Heuchel RL. Biochim Biophys Acta. 2016 Sep;1862(9):1839-46.

II. Tamoxifen affects chronic pancreatitis-related fibrogenesis in an experimental mouse model: an effect beyond Cre-recombination.

III. Targeting of Smad7 in pancreatic stellate cells does not exacerbate fibrosis during chronic pancreatitis. (Manuscript)
   Li X, Nania S, Kleiter I, Löhr JM, Heuchel R

IV. Macrophage-specific expression of Smad7 is required for normal inflammatory response in experimental chronic pancreatitis. (Manuscript)

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. RCAN1 is a marker of oxidative stress, induced in acute pancreatitis.

II. Fibroblast drug scavenging increases intratumoural gemcitabine accumulation in murine pancreas cancer.

III. Multimodality imaging of pancreatic ductal adenocarcinoma using core-shell structured gold nanorods. (Submitted)
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<tbody>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>Pdx1</td>
<td>pancreatic duodenal transcription factor 1</td>
</tr>
<tr>
<td>Ptf1a</td>
<td>pancreas transcription factor 1 subunit alpha</td>
</tr>
<tr>
<td>Sox9</td>
<td>sex determine region Y-box 9</td>
</tr>
<tr>
<td>Hes1</td>
<td>hairy and enhancer of split-1</td>
</tr>
<tr>
<td>Ngn3</td>
<td>neurogenin 3</td>
</tr>
<tr>
<td>PSCs</td>
<td>pancreatic stellate cells</td>
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<tr>
<td>CP</td>
<td>chronic pancreatitis</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>ADM</td>
<td>acinar to ductal metaplasia</td>
</tr>
<tr>
<td>PDAC</td>
<td>pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>PTHrP</td>
<td>pancreatic parathyroid hormone-related protein</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HSCs</td>
<td>hepatic stellate cells</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>TGFB</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>macrophage chemoattractant protein-1</td>
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<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C motif ligand</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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IFN-γ  interferon-γ
LPS  lipopolysaccharide
iNOS  inducible NO synthase
PDGF  platelet-derived growth factor
Arg1  arginase-1
DBTC  dibutyltin dichloride
PanIN  pancreatic intraepithelial neoplasia
LSL  lox-stop-lox
LAP  latency-associated peptide
LTBP  TGFβ-binding protein
TGFBR1  TGFβ type I receptor
TGFBR2  TGFβ type II receptor
MH1  mad homology-1
MH2  mad homology-2
Treg  regulatory T cells
DCs  dendritic cells
NK  natural killer
MHC  major histocompatibility complex
CTGF  connective tissue growth factor
PAI-1  plasminogen activator inhibitor 1
LRM  Leu-rich motif
PY  Pro-Tyr motif
SBEs  Smad-binding elements
Frox3  forkhead box P3
UUCO  unilateral ureteral obstruction
SERMs  selective estrogen receptor modulators
4-OHT  4-hydroxytamoxifen
ERs  estrogen receptors
BMDMs  bone marrow derived macrophages
WB  western blot
1 INTRODUCTION

1.1 ORGANOGENESIS AND FUNCTION OF PANCREAS

1.1.1 Pancreas organogenesis

Pancreatic organogenesis is a complex dynamic process where signaling events and transcriptional networks coordinate, ultimately resulting in the generation of multiple cell lineages that perform the functions of the mature organ [1]. Embryonically, the mammalian pancreas derives from two parts of posterior foregut endoderm, ventral and dorsal. The ventral pancreas gives rise to the head of the organ, adjacent to the duodenum, whereas the dorsal pancreas gives rise to the tail, adjacent to the spleen [2]. In mouse, the pancreatic domain in the primitive gut is established by receiving the signal from adjacent mesoderm: ventral pancreas is under the control of fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling, and dorsal pancreas is regulated by retinoic acid and FGF2 [3]. Pancreas development is composed of two transitions: the primary transition refers to embryonic day 9 (E9) to E12.5, and the second transition refers to E12.5 until birth [4, 5].

During the primary transition (Figure 1a), several transcription factors and signalings mediate the maintenance of pancreatic progenitors identity, the expansion of multipotent pancreatic progenitor cells into mesenchyme, and the branch of multilayered epithelium [3, 5]. Pancreatic duodenal transcription factor 1 (Pdx1), pancreas transcription factor 1 subunit alpha (Ptf1a), and sex determine region Y-box 9 (Sox9) are the early key transcription factors specifically expressed in this early stage [5]. Signalings involved in early pancreatic development include Notch signaling [6], FGF signaling [7] and Wnt signaling [8]. Hairy and enhancer of split-1 (Hes1) and Sox9 are regulated by Notch signaling [9, 10], which mediates progenitor maintenance. Ptf1a and Sox9 are regulated by FGF and Wnt signaling [11-13], which sends the pro-proliferative signals from the mesenchyme to the epithelium [5].

After the primary transition, pancreatic progenitor cells undergo dynamic morphological changes during the secondary transition (Figure 1b) [5], and it begins with the segregation of a tip and trunk region, where the tips adopt an acinar fate and the trunk gives rise to the endocrine and ductal cell lineages [5, 14]. Transcription factors Nkx6.1/6.2 and Ptf1a mediate the tip/trunk separation process, where Nkx6.1/6.2 promotes trunk identity while Ptf1a favors the tip compartment [15]. Both Nkx6.1/6.2 and Ptf1a are under the control of Notch signaling [16, 17], which promotes the ductal/endocrine progenitor fate while suppresses pro-acinar tip cell identity [5].

Within the trunk domain (Figure 1c), the ductal versus endocrine fate decision is regulated by neurogenin 3 (Ngn3) expression, epithelial cells lack of Ngn3 expression develop to ductal cells while Ngn3 expressing cells give rise to endocrine differentiation [5, 18, 19]. This process is also mediated by Notch signaling. High Notch activity upregulates the expression of Hes1 (Ngn3 repressor) and Sox9 (Ngn3 activator), resulting in Ngn3 repression eventually, therefore the progenitors favor ductal fate. On the other hand, lower Notch activity only
upregulates Sox9 expression, leading to Ngn3 activation and endocrine compartment development [9, 20]. The timing of Ngn3 activation in endocrine precursor cells determines further cell differentiation [5, 21]. Specifically, α-cell determinant Arx and β-cell determinants Pax4, Nkx6.1, and Pdx1 separate these two lineages (Figure 1d) [4, 22-24].

Figure 1. (a) During the primary transition, the transcription factors such as Pdx1, Ptf1a, and Sox9 mediate expansion of pancreatic progenitor cells and maintain pancreatic identity. (b) At the onset of the secondary transition, pancreatic progenitors adopt either tip or trunk identity. (c) Tip cells adopt an acinar phenotype, and trunk cells give rise to both ductal and endocrine cells. High Notch promotes the ductal fate while low Notch activity results in endocrine differentiation. (d) Endocrine precursors further differentiate into five different cell types. Adapt from Shih HP et al. Annu. Rev. Cell Dev. Biol. 2013. 29:81–105. Reprinted with permission from publisher.
1.1.2 Pancreas function

The pancreas, as a gland derived from the endodermal lineage and located in the abdomen behind the stomach, is essential for glucose homeostasis and nutrient digestion [3]. It is mainly composed of acinar cells (98%) with scattered islet cells (2%), exhibiting exocrine and endocrine functions, respectively (Figure 2) [4, 25].

The endocrine pancreas is composed by islets of Langerhans which comprise five endocrine cell types, including glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP-cells, and ghrelin-producing ε-cells [5]. The α-cells comprise 25% of the islets cells, secreting glucagon, which elevates blood glucose level. The β-cells, constituting about 60% of the islets cells, locate mainly in the middle of islet and secrete insulin, which promotes rapid uptake, storage, and use of glucose. The δ-cells, about 10% of the total islets cells, secrete somatostatin, which can inhibit the secretion of both glucagon and insulin and may act as a mediator of metabolic processes. In addition, PP cells secrete a hormone of uncertain function called pancreatic polypeptide, which might be involved in self-regulation of pancreatic secretion activities and hepatic glycogen levels. Last, the ε-cells, composing less than 1% of all islet cells, produce the hormone ghrelin that induces hunger. In addition, islets also secrete several neuropeptides/cotransmitters that modulate exocrine pancreatic function. The close interrelations among these cell types in the islets allow cell-to-cell communication and direct control of hormone secretion. For instance, insulin inhibits glucagon secretion, amylin inhibits insulin secretion, and somatostatin inhibits the secretion of both insulin and glucagon [26].

The functional unit of the exocrine pancreas is composed of acini and ducts (Figure 2b, c). The acinar cells synthesize, store, and secrete pancreatic enzymes such as lipases (fat digestion), amylases (carbohydrate digestion) and trypsin (protein digestion) [5, 27]. Duct cells actively secrete bicarbonate and mucins, and drain acinar digestive enzymes towards the duodenum [4, 28, 29]. Another cell that is situated at the junction of the acini and duct is the centroacinar cell. This cell has ductal cell characteristics but is also likely a progenitor for different cell types for the pancreas [30]. Each pancreatic acinus is surrounded by scarce stroma and pancreatic stellate cells (PSCs). The role of PSCs in healthy organ is probably to maintain and direct proper formation of the epithelial structures [31], whereas in pathologic states, such as chronic pancreatitis (CP) and pancreatic cancer, PSCs have been considered as a central mediator in pathogenesis, which will be discussed in detail in the following section. The function of the exocrine pancreas is tightly regulated by the neuroendocrine system. Endocrine and exocrine pancreas is innervated by central and autonomic nerves. Once these nerves are activated, acetylcholine is released to bind on muscarinic receptors on the acinar cells to stimulate secretion. Cholecystokinin (CCK), secreted by brain neurons and small intestinal cells, is also a very important mediator of pancreatic exocrine secretion. Physiological level of CCK stimulates exocrine pancreatic secretion in humans and rodents. However, rodent pancreatic acini, in contrast to human acini, respond also in vitro to physiological concentration of CCK [32, 33].
1.2 CHRONIC PANCREATITIS (CP)

1.2.1 Definition and classification

CP is defined as a pathological fibro-inflammatory syndrome of the pancreas induced by genetic, environmental and/or other risk factors which develops persistent pathologic responses to parenchymal injury or stress [34-36]. It describes a wide range of progressive exocrine pancreas disorders. The TIGAR-O system for CP classification has been widely used, which is based on various etiologies and categorized into (1) Toxic–metabolic, (2) Idiopathic, (3) Genetic, (4) Autoimmune, (5) Recurrent and severe acute pancreatitis, and (6) Obstructive CP (Figure 3a) [37-39]. Based on clinical features, morphological characteristics and responses to treatment, CP can be broadly classified into three subtypes (Figure 3b): chronic calcifying pancreatitis, chronic obstructive pancreatitis, and steroid-responsive pancreatitis (chronic autoimmune pancreatitis) [36, 40].

Chronic calcifying pancreatitis is normally driven by acinar and duct cell injury or stress, mostly resulted from alcohol abuse and smoking rather than genetic predisposition [34]. It leads to progressive scarring of the pancreatic tissue and pancreatic dysfunction, further
subdivision has been proposed as the typical CP that is dominated by fibrosis, and atypical CP which is dominated by atrophy [34].

Chronic obstructive pancreatitis is a term used for CP that is caused by primary injury to the duct or partial/complete ductal obstruction [40], several factors can induce injury onset, including endoscopic/surgical procedures, gallstones and tumors.

Steroid-responsive pancreatitis, or autoimmune pancreatitis is a unique type of CP for which the etiology can be determined based on histological features [34]. It has been classified into two subtypes: type 1 and type 2 [41]. Type 1 autoimmune pancreatitis is known as immunoglobulin G4 (IgG4)-related disease, which is characterized by intense periductal inflammation, high numbers of IgG4-positive plasma cells, inflammatory changes of venules, multi-organ involvement, peculiar swirling (storiform) fibrosis, and rapid response to corticosteroids and B-cell depletion therapy [34, 40]. Type 2 autoimmune pancreatitis, also called as idiopathic duct-centric chronic pancreatitis, is characterized by neutrophil infiltration within the acinar parenchyma and the ductal epithelium, and the formation of small intraductal abscesses. Both types share similar morphological changes, except for numbers of IgG4-positive plasma cells where type 2 has no serological findings [34, 41].

In this thesis, the term CP only refers to chronic calcifying pancreatitis because it is the most common and classic subtype, unless specified otherwise.
1.2.2 Epidemiology

Although CP displays lower incidence and prevalence than acute pancreatitis, it is a major source of morbidity in the western counties and significantly reduces patients’ life qualities (Figure 4a) [37, 42]. According to population-based studies, the overall incidence rate ranges from 5 to 14 per 100,000 individuals [43, 44]. More specifically, the incidence of early CP has been estimated as 1 per 100,000 individuals, whereas the incidence of established CP is thought to be 14 per 100,000 individuals. Recent studies have shown a modest increasing incidence of CP, which is mainly due to the improvement in diagnosis and changes in disease definition [34, 44, 45]. The prevalence of CP is around 30–50 per 100,000 individuals, and it increases with age with the median age at diagnosis ranging between 51-58 years [44, 46].
The etiology of CP differs with age, sex and races (Figure 4b). Generally, CP mainly affects men with a sex ratio (male/female) of 4.6 [44] with alcohol abuse and smoking as major causes, while women are prone to be affected by idiopathic and obstructive etiologies [34, 47]. Additionally, CP is more common in black individuals than other races. Whether this is associated with socioeconomic status, or results from dietary, genetic, or other factors needs to be further investigated [34, 48].

1.2.3 Risk factors

Alcohol abuse is the single most prevalent cause of CP, which attributes to 40-70% of cases in western countries [34, 47]. Several studies have confirmed direct toxicity of alcohol metabolites to acinar cells via the inhibition of endoplasmic reticulum activity and subsequent increased oxidative stress [49, 50]. The risk of CP increases with heavy alcohol consumption [51], with a threshold of approximately 5 drinks/day (odds ratio, 3.1, 95% CI, 1.87-5.14) based on a case-control study [52], or 4 drinks/day (relative risk, 2.5, 95% CI, 2.0-3.1) based on a meta-analysis [53]. Additionally, alcohol abstinence decreases the progression of recurrent acute or chronic pancreatitis [54, 55].

Smoking is another independent risk factor of CP [56, 57], it is commonly associated with alcohol as cofactors and increases the risk of CP in a dose-dependent manner. In vitro studies showed that nicotine and its metabolites induce oxidative stress in the acinar cells and led to pathogenesis of smoking-related pancreatitis [58, 59]. The overall relative risk estimated for smokers is 2.8 (95% CI, 1.8-4.2). It has been reported that the relative risk is approximately 2.4 (95% CI, 0.9-6.6) for subjects smoking less than 1 pack per day, and 3.3 (95% CI, 1.4-7.9) for those smoking 1 or more packs per day [60]. Once smoking cessation applied, the risk decreases to 1.4 (95% CI, 1.1-1.9), indicating that cessation alters the progression of pancreatitis [47].

Several studies have identified specific genes that predispose to CP by three distinct pathological mechanisms: the trypsin-dependent, the ductal-dependent and the misfolding-dependent pathways [61]. In the category of trypsin-dependent pathway of genetic risk in CP, gain-of-function mutations in PRSS1 (cationic trypsin gene, encoding trypsin 1) lead to premature conversion of trypsinogen to trypsin 1, reduced inactivation of trypsin 1, and pancreas autodigestion [62]. Besides, both SPINK1 (encoding serine protease inhibitor Kazal type 1) and CTRC (encoding chymotrypsin C) are involved in the degradation of prematurely activated trypsin 1. Loss-of-function variants in SPINK1 and CTRC predispose to CP by...
diminishing their protective trypsin-degrading activities [63, 64]. Regarding the ductal-related genetic risk, it has been shown that CFTR (cystic fibrosis transmembrane conductance regulator, encoding anion channel for bicarbonate secretion) mutation impairs flushing of pancreatic ducts and leads to trypsin activation [65, 66]. Additionally, mutation-induced misfolding can also increase risk for CP. For instance, misfolding mutants of PRSS1 [67] and CPA1 (procarboxypeptidases A1) [68] are strong risk factors and may be associated with dominant hereditary pancreatitis. These misfolding enzyme mutants exhibit diminished secretion, intracellular retention and endoplasmic reticulum stress [69].

1.2.4 Pathophysiology

The pathogenesis of CP is still not fully understood. It is a fairly complex process that includes acinar cell injury, acinar stress responses, duct dysfunction, persistent or altered inflammation, and/or neuro-immune crosstalk [34]. Activation of other mechanisms, such as endoplasmic reticulum stress, autophagy, and mitochondrial dysfunction, are also involved in triggering inflammatory responses and extensive pancreatic injury [61]. Generally, it is believed that protein-rich plugs produced by over-secreting acini are not compensated by the increase of ductal bicarbonate flux, which leads to ductal calcification, epithelial lesions and obstruction. Obstruction of the ducts causes ductal hypertension and results in ischemic injury of the acinar cells and inflammation, which subsequently leads to pancreatic parenchymal fibrosis [37, 40].

Five possible pathogenesis mechanisms have been proposed: (1) Necrosis–fibrosis sequence model: after repeated acute pancreatitis, the repair of damaged regions results in the replacement of necrotic pancreatic parenchyma with fibrotic tissue. (2) The “two-hit” model (see details below). (3) Metabolic-toxic effect model: alcohol and tobacco and their metabolites damage the acinar cells directly. (4) Oxidative stress model: oxidative stress leads to intravascular protease activation, acinar cell necrosis, inflammation and fibrosis. (5) Ductal dysfunction model: abnormal ducts lead to protein plugs and ductal obstruction [34].

Among them, the “two-hit” hit model (Figure 5) proposed by Whitcomb has been widely accepted and studied [70]. Briefly, the first hit is the acute pancreatitis, which initiates the injury process by activation of the immune cells through either premature trypsin activation or trypsin-independent mechanisms. The second hit in the progression to CP is a modification of the normal inflammatory response, leading to sustained activation of pancreatic stellate cells and fibrosis. The first hit comes from factors that cause injury, whereas the second hit involves of cell stress responses, cell regeneration and transdifferentiation, tissue remodeling, dysplasia, and altered anatomy [37, 70].
Figure 5. The first hit increases susceptibility to injury, whereas the second hit affects the immune response to promote PSCs associated fibrosis. Alcohol could injure the pancreas via its effects on acinar cells. Altered trypsin functions could also initiate disease. Severe acute pancreatitis induces pancreatic necrosis, which leads directly to scarring and fibrosis in the recovery phase. Other pathways include autoimmune pancreatitis, in which the mechanism to fibrosis is less well understood. Adapted from Whitcomb DC Gastroenterology. 2013; 144(6): 1292–1302. Reprinted with permission from publisher.

1.2.5 Histopathology

The histopathological features of CP vary depending on etiology and stage of disease [34, 71]. Common features of CP include unevenly distributed interlobular/intralobular/periductal fibrosis, progressive atrophy of the acinar parenchyma, duct distortion with calcifications, and inflammatory cell infiltration [72].

During the early stages of CP (Figure 6a), cell-rich fibrosis is more common in interlobular region, resulting in duct distortion with protein plugs. Initially, atrophy of individual acini is characterized by loss of zymogen granulation and flattening of the acinar cells with formation of a central lumen, a phenomenon referred to acinar to ductal metaplasia (ADM). Epithelial metaplasia and moderate inflammatory cell infiltration can be observed as well. In the perilobular tissue, there may be necrotic foci with nearby large numbers of foam cells and cell-rich fibrosis [71, 72].

In advanced CP (Figure 6b), fibrosis extends through atrophic lobules and may form vast sheets of fibrosis and scar tissue with low cellularity and a high collagen content. The perilobular ducts exhibit distortions and dilatations with occasional of cyst, while the interlobular ducts are often filled with protein plugs and calculi (Figure 6c, d). Acinar
compartment is gradually lost and the lobular architecture becomes effaced. Atrophic duct epithelium and enlarged peripheral nerves can be observed (Figure 6e), islets remain present until the late stages, although with reduced numbers (Figure 6f) [71, 72].

Distinguishing the end-stage chronic pancreatitis from well-differentiated pancreatic ductal adenocarcinoma (PDAC) can be difficult sometimes, because they share several similar morphological changes. The differential diagnosis is foremost based on the assessment of two architectural features: lobular architecture and segregation of blood vessels and pancreatic ducts. In CP, the outline of the lobules can still be identified by the distinct quality of the intralobular stroma. Blood vessels and ducts remain separated by acinar parenchyma. In contrast, in PDAC (Figure 6g, h), tumor glands without any associated stroma distribute randomly and sabotage the pancreatic ducts. Ductular structures in close approximation of muscular blood vessels are highly suggestive of invasive adenocarcinoma [72].

Figure 6. Microscopic features of CP. (a) Early CP: interlobular septa are expanded by fibrosis. (b) Advanced CP: inter- and intralobular fibrosis is associated with lobular atrophy. (c) Irregular duct dilatation with protein plugs. (d) Dilated duct with calculus. (e) End-stage CP exhibits interlobular ducts dilation, subtotal acinar atrophy, sheet like fibrosis, prominence of peripheral nerves, and focal mild chronic inflammatory cell in filtration. (f) Islets admixed with ductular structures in the end-stage. (g) Loss of lobular architecture, presence of glandular structures next to a muscular artery in PDAC. (h) Cytological atypia confirms the invasive ductal adenocarcinoma. Adapted from Fiona Campbell and Caroline S. Verbeke, Pathology of the Pancreas, 1st ed., Springer London Ltd, 2013. Reprinted with permission from publisher.

1.2.6 Cells involved in CP development

Acinar cells in chronic pancreatitis

The notion of pathologic autodigestion by the premature digestive enzymes has been linked to development of pancreatitis for long time (Figure 7a), since most mutations that increase the risk for pancreatitis in human locate in genes encoding acinar digestive enzymes or their inhibitors [73, 74]. The digestive enzymes, especially trypsin, is prematurely activated in the acinar cells and activates other digestive proteases pathologically, eventually resulting in
“digestion” of the acinar cells and pancreatic injury [73]. Besides, increased inflammatory signaling, pathologic calcium signaling, autophagy/lysosomal/mitochondrial dysfunction, oxidative stress, and endoplasmic reticulum stress of acinar are also proposed to trigger pancreatitis, in parallel with protease activation [73, 74]. Several experimental models of pancreatitis have been applied to investigate the premature activation theory, including the cerulein model [75], taurocholate model [76], L-arginine model [77], activation of nuclear factor kappa B (NF-κB) [78] and the interleukin (IL) 1β overexpression genetic model [79]. Results from previous studies have confirmed that pathologic calcium signaling [80] and colocalization of zymogen granules with lysosomes [81] were the key steps to induce premature trypsinogen activation [73].

However, recent research challenged the trypsin-centered theory (Figure 7b). By using genetic mouse models specifically lacking pathologic trypsinogen activation, the extent of necrosis in acinar cells was reduced, but did not influence the inflammation level and the development of CP, indicating that the pathogenesis of CP was independent of trypsinogen activation [82, 83]. In contrast to acute injury in which activation of NF-κB pathway is transient [84], sustained intra-acinar activation of NF-κB pathway was observed in both murine and patients CP samples [82]. Furthermore, chronic expression of NF-κB pathway rather than activated trypsin demonstrated the development of CP [83, 85], therefore sustained activation of NF-κB pathway seems to be critical in the pathogenesis of CP [82]. However, by using an elevated intracellular trypsin genetic mouse model, Zhan X et al. demonstrated that early trypsin activation in pancreatic acinar cells resulted in increased fibrosis, acinar cell loss and atrophy, greater inflammatory cell infiltration and prolonged pancreatic injury, indicating a promoted development of CP [86]. Above controversy results suggest that further investigation is still needed to illustrate the complex mechanism of trypsin-centered theory.

Pancreatic parathyroid hormone-related protein (PTHrP) is another factor identified to mediate CP associated inflammation and fibrosis. Acinar cell-specific Pthrp deficient mice demonstrated protective effects in cerulein and pancreatic duct ligation induced CP by suppressing proinflammatory cytokine release and NF-κB activation in acinar cells, and downregulating PSCs activation via a paracrine pathway [87]. Additionally, sustained endoplasmic reticulum stress is also observed in CP and is induced early in pancreatic injury through pathologic calcium signaling, which may be a novel pathogenic mechanism in CP [88].
Figure 7. (a) Trypsin-centered theory: activation of trypsinogen is initiated by colocalization of lysosomal and zymogen, subsequently leads to leakage of cathepsin B in the cytosol resulting in acinar death in the early stages of pancreatitis. Activation of NF-κB leads to release of inflammatory mediators and drives the systemic inflammatory response which causes acinar cell death. Adapted from Saluja A et al. Gastroenterology 2019; 156:1979–1993. (b) Proposed paradigm of CP pathogenesis: intra-acinar trypsinogen activation is not required for CP, while sustained NF-κB pathway in acinar cells results in CP, and chronic pathogenic stimulus may drive the sustained NF-κB response. Adapted from Sah RP et al. Gastroenterology. 2013, 144, 1076–1085. Reprinted with permission from publisher.

**PSCs in chronic pancreatitis**

PSCs are mainly located around acinar cells, compromising 4-7% of all parenchymal cells in pancreas [89]. In normal pancreas, PSCs are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm and expression of desmin and glial fibrillary acidic protein (GFAP). They reside in periacinar and periductal spaces, exhibiting minimal proliferation, supporting normal epithelial cell function and maintaining tissue architecture [90]. The origin of PSCs is still not clear. Transcriptomic analysis revealed a similarity between PSCs and hepatic stellate cells (HSCs) [91] and presumed they share a common origin: septum transversum mesenchyme, which contributes to 64% of HSCs [92, 93]. In addition, several groups have also identified bone marrow as a significant source of PSCs, accounting for approximately 7-9% of the desmin-positive cells in the pancreas [94, 95].

In response to pancreatic injury or inflammation, quiescent PSCs lose their lipid droplets and undergo morphological and functional changes to become myofibroblast-like cells, which express α-smooth muscle actin (α-SMA), in order to promote a fibro-inflammatory wound-healing response [31]. The origin of activated PSCs under pathological conditions is more complex. Apart from the resident PSCs that proliferate and differentiate into myofibroblasts during fibrosis, it is also suspected that they could be derived from other sources: through the recruitment and differentiation of bone marrow-derived cells [96], via epithelial to mesenchymal transition (EMT) [97] or via endothelial to mesenchymal transition [98].
During pancreatic injury, PSC are activated by responding to factors including oxidant stress, cytokines, growth factors and toxins, which are provided from various cells including damaged acinar cells, neutrophils, macrophages and PSCs themselves (Figure 8a) [99]. Several intracellular pathways are involved in the PSC activation, such as mitogen-activated protein kinase (MAPK) signaling, phosphatidylinositol 3 kinase (PI3K) signaling, protein kinase C signaling, Hedgehog signaling and transforming growth factor-beta (TGFβ) signaling (Figure 8b) [31]. Dysregulated PSCs contribute to pathological inflammation and fibrosis by modulating extracellular matrix (ECM) and secretion of cytokines and growth factors [90, 100, 101].
Like other fibrotic diseases, pathological fibrosis in CP is a result of a skewed balance between fibrogenesis and fibrosis degradation [102]. In addition to the well-established role of ECM deposition to alter the cellular microenvironment, such as collagen type I and III, laminin and fibronectin, activated PSCs also contribute to ECM remodeling through secretion of hyaluronan, matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinases (TIMP), indicating the possibility of fibrosis resolution by specific attenuation of fibrogenic functions of PSCs [90, 103]. Besides the fibrotic role of PSCs, they are also involved in proinflammatory process by secreting various cytokines and growth factors. For example, studies have demonstrated that PSCs could secrete neutrophil chemotactic factor IL-8, macrophage chemoattractant protein-1 (MCP-1) and express intracellular adhesion molecule-1 (ICAM-1) to recruit inflammatory cells [104-106], PSCs are also a source of IL-4 and IL-13 to mediate macrophages activation, which in turn participate in promoting the pancreatic fibrosis [107]. Although the role of PSCs for ECM production and remodeling has been investigated thoroughly, recent studies have demonstrated non-fibroinflammatory functions of PSCs in regulation of epithelial cell fate, immune modulation, and tissue health [90]. For instance, PSCs can recognize pathogen associated molecular patterns via Toll-like receptors [108] and phagocytose necrotic acinar cells [109]. They could also reduce or increase insulin secretion and induce β-cell apoptosis [110, 111].

**Inflammatory cells in chronic pancreatitis**

Acinar cell damages during pancreatitis release inflammatory mediators and trigger innate immune mechanisms that recruit immune cells to the site of inflammation [74]. Pancreatitis is primarily a sterile inflammation, therefore damage-associated molecular patterns rather than pathogen-associated molecular patterns play the role in activation of immune cells [112], and both innate and adaptive immune cell populations were observed in human and murine CP samples [113]. Initially, neutrophils and monocytes are recruited, and dendritic cells, mast cells, T-cells and platelets follow [114]. The major function of neutrophils in pancreatitis is removing pathogens by releasing proteases, antimicrobial peptides, and reactive oxygen species (ROS) [114]. Neutrophils are the major source of ROS production, and they can induce oxidative damage on acinar cells and enhance trypsinogen activation [112, 115]. Depletion of neutrophil or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibited trypsin activation and decreased pancreatic damage [115]. Neutrophils are not typically present in healthy pancreas [74], their adhesion to damaged acinar cells is partly mediated by ICAM-1, which is maintained as low concentration in normal tissue but becomes highly expressed at damaged sites. Therefore, ICAM1-deficient mice are protected from pancreatitis [116].
In addition to neutrophils, monocytes and macrophages undergo marked phenotypic and functional changes and exhibit critical regulatory activity during the initiation, maintenance, and resolution phases of CP [109]. The recruitment and activation of primary monocytes are mediated by chemokines C-C motif ligand 2 (CCL2), CCL3, and CCL5. Afterwards, tumor necrosis factor (TNF), IL-1, IL-6, and ICAM-1 are produced in greater amounts to promote disease progression [114, 117]. Macrophages are either generated from monocytes that differentiate into macrophages when they enter the tissue or from the local proliferation of resident macrophages that originated from yolk sack, fetal liver or hematopoietic stem cells. The main function of macrophages are not limited to phagocytosis of apoptotic/necrotic acini, they are closely involved in supporting and activating stem/progenitor cells, regulating myofibroblasts activation, remodeling ECM for regeneration and promoting angiogenesis [118].

Figure 9. In response to organ injury, an inflammatory response involving of leukocytes, inflammatory monocytes and resident tissue macrophages is activated. During most types of non-chronic tissue injury, inflammatory macrophages are quickly overtaken by pro-wound-healing macrophages that direct tissue repair. Subsequently, macrophages assume a pro-resolving phenotype
that suppresses inflammation, removes excess collagen and fibrin deposits, and helps restore original tissue architecture. However, when tissue injuries become chronic or the wound-healing response itself is dysregulated, persistent activation of wound-healing responses can result in the development of pathological fibrosis. Adapted from Vannella KM et al. Annu Rev Physiol. 2017; 79:593-617. Reprinted with permission from publisher.

Based on patterns of gene and protein expression and function, macrophages are commonly classified as classically activated (M1) macrophages or alternatively activated (M2) macrophages [119]. M1 macrophages are normally induced by interferon-γ (IFN-γ), and lipopolysaccharide (LPS) to release pro-inflammatory factors, such as IL-1β, IL-12, inducible NO synthase (iNOS) and TNF-α. In contrast, M2 macrophage are induced by IL-4, IL-10, IL-13 and TGFB to secret anti-inflammatory factors, such as IL-10, TGFB, platelet-derived growth factor (PDGF) and arginase-1 (Arg1) [120]. Significant macrophages infiltration has been observed in experimental models of CP, where the macrophages were found in proximity to areas of fibrosis [107], indicating a role of macrophages in modulating fibrogenesis (Figure 9). In the early stage of injury, the M1 macrophages amplify the inflammatory response and promote tissue remodeling and fibrosis by directly enhancing the survival and activation of myofibroblasts [121]. During the regeneration phase, M1 macrophages are overtaken by M2 macrophages that direct tissue repair through several distinct mechanisms, including proliferation and expansion of parenchymal cells and fibroblasts [118]. In the final stage of tissue repair, macrophages adopt a remodeling phenotype that suppresses inflammation, removes excess ECM, and helps restore original tissue architecture [121]. However, when tissue injuries become chronic or activation/transformation of macrophages is dysregulated, pathological fibrosis and organ failure will be resulted in [121].

1.3 ANIMAL MODEL

1.3.1 How close do animal models represent human CP?

Several animal models of CP have been developed, including pancreas-intrinsic (e.g., the cerulein model), environmental-toxic (e.g., alcohol model) and genetic model (Table 1). The classic animal models of CP reflect the etiologic factors, such as hyperstimulation of secretion, ductal obstruction and toxic stimuli, while the genetic models mimic the observed mutations, providing new insights of gene regulation and disease mechanism [122]. However, a clinically relevant and satisfactory animal model that reflects all features of human pathogenesis has not yet been established [122, 123].

The most common method to generate CP in rodent is through repeated bouts of AP by injection of cerulein, a CCK analog [124, 125], with a protocol of 50μg/kg/hr×6 hour twice per week for 4-10 weeks for mice and two intraperitoneal injections of 20μg/kg once per week for 16 weeks for rats [123]. Supramaximal doses of cerulein directly lead to accumulation of zymogen granules, enzyme premature activation, and progressive atrophy.
Over time this will lead to an excessive inflammatory response and accumulation of ECM [126, 127]. However, these features regress after 3-6 weeks when the injections are discontinued [128]. Additionally, using the murine cerulein model is under debating since the pathogenesis of human CP is not associated with hyperstimulation by CCK [122]. The L-arginine model is also based on repeated injection to induce acinar necrosis, progressive fibrosis and inflammatory response. The mechanism of L-arginine–induced CP may be due to impaired autophagy caused by mitochondrial dysfunction [129, 130]. However, it takes longer time to exhibit the pathological changes than in the cerulein model. Pancreatic neuropathy, changes of serum glucose and amylase level have not been observed in this model either [131-133].

Although alcohol is one of the major risk factors of CP, alcohol administration alone did not induce CP despite long experimental durations in mice [126]. However, a combination of alcohol and other stimuli such as cerulein led to prominent pancreatic fibrosis, activated stellate cells and inflammatory cell infiltration. Another combination model that has clinical relevance is chronic ethanol feeding or administration of LPS to induce CP by activating innate immune pathways [134]. Overall, alcohol intake is considered as a modifier in these combination models, supporting the observation that alcohol is associated with rapid progression of CP in humans [135]. By performing intravenous injection of dibutyltin dichloride (DBTC) chronic pancreatic injury can also be induced. DBTC is an organotin compound that causes cytotoxic damage on the surface epithelium of the bile duct and leads directly to pancreatic injury [136]. When combined with ethanol intake, DBTC induced acute pancreatic inflammation within 24 hours and extensive inflammation could be observed within 2 weeks, followed by progressive fibrosis after 2 months [137]. The model successfully showed progression from acute pancreatitis to CP and also mimicked the two main etiologies of CP: alcohol intake and duct obstruction [122].

Partial or complete pancreatic duct ligation has been used for CP induction, but pathological features are species-dependent. Duct obstruction alone in rats resulted in acinar cell atrophy and fibrosis without a profound inflammatory reaction, whereas duct ligation in the mouse was technically difficult because of its multiple pancreatic ducts that vary a lot and hard to reproduce uniform induction of fibrosis [126, 127]. However, CP has been induced successfully by using a combination of duct ligation and supramaximal secretagogue stimulation. Mice developed severe pancreatitis after 3 days and maximal fibrosis after 21 days. This is also the first model that recapitulates the features of pancreatic neuropathy [138, 139].

Genetic manipulation, specifically in duct or acinar cells, has been used to produce several CP models. Mouse models with mutated human PRSS1 developed CP spontaneously only up to 10% [140], while with mutated mouse PRSS1, more mice developed pancreatitis spontaneously [141]. The existing studies on the transgenic PRSS1 models are under questions because of the low penetrance of the human PRSS1 mutation and how the results of the murine PRSS1 mutation can be applied to human is still not clear [122]. Overexpression
of human IL-1β under control of the elastase promoter murine pancreas led to prominent histologic features of CP and inflammatory response, but pancreatic exocrine/endocrine insufficiency is still absent. Comparing the histologic changes to the cerulein model, the 20-week-old transgenic mice displayed more severe pancreatitis than mice treated with cerulein for 20 weeks [79, 142]. The involvement of autophagy in pancreatic diseases has attracted attention recently. Autophagy related 5 (Atg5) and Atg7 are central mediators in autophagosome formation. Pancreas-specific depletion of Atg5 resulted in gender-dependent development of CP [143], while Atg7 knockout mice represented more severe CP but without gender difference [144]. However, mutations in Atg genes have not been identified in human CP [122]. The IKK complex is the main effector of NF-κB signaling. Pancreas-specific deletion of IKK resulted in spontaneous development of CP, pancreatic intraepithelial neoplasia (PanIN) like lesions and glucose intolerance were detected in aged mice [145].

<table>
<thead>
<tr>
<th>CP models</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>reflect the common etiology</td>
<td>does not induce CP alone</td>
</tr>
<tr>
<td>DBTC</td>
<td>only one injection needed</td>
<td>more organs damages involved</td>
</tr>
<tr>
<td>Cerulein</td>
<td>• reproducible</td>
<td>• uncertain clinical relevance</td>
</tr>
<tr>
<td></td>
<td>• easy to perform</td>
<td>• fast recovery from damage</td>
</tr>
<tr>
<td>L-arginine</td>
<td>• reproducible</td>
<td>unclear action mechanism</td>
</tr>
<tr>
<td>Duct ligature</td>
<td>• reproducible</td>
<td>• challenging technique</td>
</tr>
<tr>
<td></td>
<td>• time-saving</td>
<td>• species-dependent duct localization</td>
</tr>
<tr>
<td>PRSS1 mutation</td>
<td>clinically relevant</td>
<td>no spontaneous CP</td>
</tr>
<tr>
<td>IL-1β overexpression</td>
<td>spontaneous CP</td>
<td>uncertain clinical relevance</td>
</tr>
<tr>
<td>Atg5/7 deletion</td>
<td>spontaneous CP</td>
<td>mutation not found in human</td>
</tr>
<tr>
<td>IKK deletion</td>
<td>• spontaneous CP</td>
<td>• clinically relevant</td>
</tr>
</tbody>
</table>

Table 1. Advantages and disadvantages of different animal models for CP.

13.2 Cre-LoxP system

Cre-LoxP is a site-specific recombination system, used to conditionally eliminate or activate expression of genes in a tissue/temporal specific manner, thereby enabling the tissue/stage specific functions of genes to be explored in animal models [146]. The system consists of a Cre recombinase and a specific DNA recognition sequence, both derived from the bacteriophage P1. Cre recognizes a 34-bp nucleotide sequence named LoxP and precisely catalyzes the homologous recombination between the two LoxPs. This enables the specific manipulation of DNA based on the direction and location of the two LoxPs [147, 148]. If the two LoxPs are in the same direction on one DNA strand, the DNA between them will be deleted. If two LoxPs are in opposite direction, the DNA fragment between them will be inverted [148].
Figure 10. Cre-mediated recombination in mouse tissues. (A) Conditional gene of interest (GOI) knockout: intercrossing of a Cre mouse with a floxed mouse will lead to flanked exon deletion and loss of a functional protein. (B) Conditional GOI activation: interbreeding of the LSL-GOI allele to a Cre mouse leads to activation of the GOI in a tissue-specific manner. (C) In the absence of tamoxifen, no expression of the reporter gene is observed because of the presence of the stop signal upstream of the reporter gene. (D) When tamoxifen is administered, the Cre is activated and mediates recombination between the LoxP sites in cells. Adapted from Magnuson MA et al. Cell Metab. 2013; 18: 9-20 and Greco V et al. Development 2010; 137: 1586-1594. Reprinted with permission from publisher.

The conditional inactivation of a gene using Cre-LoxP requires e.g. a cell type-specific Cre strain and a target allele strain where the vital gene segment has been flanked with tandemly oriented LoxP sites (Figure 10A-B). Thus, tissue-specific gene knockout mice can be generated by breeding Cre transgenic mice with floxed mice. Similarly, the conditional activation of a gene requires a Cre strain as well, but together with a target allele that is preceded by a lox-stop-lox (LSL) sequence upstream of the coding sequence to be expressed [146, 147, 149]. The Cre-LoxP system can also be used for lineage tracing. Here, a LSL-cassette preceded reporter gene, such as green fluorescent protein or tdTomato, has been
inserted into the ROSA26 locus, which has been found to contain open, accessible chromatin in all cell types in mouse. Such a silenced reporter gene can be activated by Cre recombinase that is under the control of a cell type-specific promoter. These alleles not only enable cell lineage tracing, they can also be used to assess both the sites and efficiency of Cre-mediated recombination [150-152].

Time- and/or stage-specific knockout by using the Cre-LoxP system requires the inducible activation of Cre recombinase, which allows Cre recombination when needed [147]. The Tamoxifen system is one of the most commonly used inducible systems for stage-specific recombination (Figure 10C-D). In this system, the Cre recombinase has been fused to a mutated ligand-binding domain of the estrogen receptor (ER), resulting in a Tamoxifen-dependent Cre recombinase, Cre-ERT, which is activated by Tamoxifen, but not by estradiol. In the absence of Tamoxifen, the Cre-ERT fusion protein is bound to heat-shock proteins such as Hsp90 and located in the cytoplasm. When Tamoxifen is present, it binds to the ER, displacing Hsp90, which exposes the nuclear localization signal of the ER. Under the guidance of the nuclear localization signal, the Cre recombinase can execute its function after moving to the nucleus [147, 153-155].

1.4 TRANSFORMING GROWTH FACTOR-BETA PATHWAY

1.4.1 Overview of TGFB pathway

The TGFB super family controls many aspects of cell and tissue physiology and be involved different pathological processes [156]. Ligands of this super family include TGFBs, activins, BMPs, growth and differentiation factors, mullerian inhibiting substance and Nodal [157]. There are three different TGFB isoforms: TGFB1-3. All have similar biological activity, although each isoform is expressed in a unique pattern under the control of a specific promoter, and TGFB1 has been primarily linked to fibrogenesis [158]. TGFBs is initially translated into a 50-kDa pro-protein, which is released from cells as an inactive complex formed by binding to a latency-associated peptide (LAP) and a latent TGFB-binding protein (LTBP). Within this tripartite complex, LAP confers latency to the cytokine and the LTBP promotes effective secretion of latent TGFB from cells, and sequesters it into ECM. To achieve an active state, TGFB needs to be dissociated from LAP, a process that can be triggered by various signals including integrins (αvβ6 or αvβ8), proteases and physicochemical factors [158, 159]. The integrin-mediated TGFB activation is well studied, traction-mediated and protease-mediated activation are proposed. Briefly, actin cytoskeleton in cells anchors integrins to exert traction, the traction between cells and ECM deforms LAP and releases active TGFB. On the other hand, the binding between integrins and LAP makes LAP more accessible to be cleaved by proteases, such as plasmin, cathepsin D and MMP, resulting in active TGFB release [159].

Once bound by the dimeric TGFB ligands, two TGFB type II (TGFBRII) and two TGFB type I receptors (TGFBRI) assemble into heterotetrameric complex, which allows the TGFBRII to
phosphorylate the cytoplasmic domains of the TGFBR1. Specifically, TGFB1 and TGFB3 have higher affinity to TGFBR2, whereas TGFB2 requires the presence of TGFB type III receptor (TGFBR3) to efficiently bind with TGFBR2. Activated TGFBR1 phosphorylate two serine residues at the C-terminal of the receptor-regulated Smads (R-Smads: Smad2 and Smad3). This event activates the R-Smads and enables the formation of heteromeric complex between two R-Smads and one common-Smad (Co-Smad: Smad4), which then translocates from the cytoplasm to the nucleus and regulates gene transcription [160, 161]. In addition, the inhibitory Smads (I-Smads: Smad6 and Smad7) antagonize the signaling mediated by R-Smads and Co-Smad (Figure 11a) [162, 163].

TGFB ligands exhibit context-dependent activities mainly by regulating gene expression through receptor-mediated activation of Smad proteins [164]. Both R-Smads and Co-Smad proteins contain two highly conserved domains known as MH1 (mad homology-1) and MH2 domains. The amino-terminal MH1 domain contains nuclear localization signals and a β-hairpin structure that enables Smad binding to DNA. The L3 loop in carboxyl MH2 domain directs the interaction of the R-Smads with TGFBR1, and Smad-Smad interaction in trimeric Smad complex. The R-Smads have a conserved C-terminal SXS motif that is phosphorylated by the activated TGFBR1, resulting in R-Smads activation [156]. The MH1 and MH2 domains are connected by a linker region obtaining phosphorylation sites for several kinases, and the linker serves as the hubs for functional cross-talk with other kinase-driven pathways [156]. Unlike R-Smads, I-Smads only contain a conserved MH2 domain and antagonize TGFB signaling by interfering with the interactions between R-Smads and TGFBR1, down-regulation of TGFBR1 receptors in cooperation with other regulators, prevention of complex formation by R-Smads and Co-Smad, and transcriptional regulation in the nucleus (Figure 11b) [160, 161, 165].
1.4.2 TGFB pathway in immune system

TGFB acts as a potent immunosuppressive cytokine through effects on cell differentiation, proliferation and apoptosis [164]. It controls adaptive immunity by directly promoting the expansion of regulatory T cells (Treg), inhibiting the function of effector T cells and dendritic cells (DCs). In innate immune system, TGFB inhibits natural killer (NK) cells and modulates the phenotype of macrophages and neutrophils, thereby forming a network of negative immune regulatory effects (Figure 12a). Dysregulation of signaling causes congenital defects, fibrotic diseases, immune malfunction and cancer [166].
TGFB has a pleiotropic role in lymphocyte regulation, and this is particularly apparent in T cells [167]. First, TGFB supports the development of the naive T cell pool and the maintenance of immune tolerance [166]. Inhibition of TGFB signaling by deleting TGFBR1 or TGFBR2 in T cells led to enhanced T cells activation and lethal inflammatory disease [164, 168, 169]. In contrast, TGFB exerts an inhibitory role on T cells differentiation. During the priming phase, TGFB limits CD4+ T cell proliferation by reducing autocrine IL-2 production [170] and represses the lytic function of CD8+ T cells by reducing perforin and IFN-γ secretion [171]. Furthermore, TGFB restrains Th1 lineage specification by suppressing key transcription factor T-bet [172], prevents Th2 lineage development by impairing the expression of Th2-specific transcription factor STAT4 [173]. However, TGFB promotes adoption of Th17 cell fate by upregulating lineage-specific transcription factor expression [174]. The induction of T regulatory cells is also controlled by TGFB pathway: activated SMADs in combination with IL-2 induce forkhead box P3 (Foxp3) expression, which is a major determinant of the Treg state [175]. TGFB also modulates B cells activation by inhibiting immunoglobulin synthesis and class switching, it promotes the production of IgA antibodies. Mice with B cell-specific inactivation of Smad2 or deletion of TGFBR2 displayed IgA deficiency [176, 177].

DCs are a central mediator in regulation of Th1 and Treg-dependent immune responses, the antigen presenting function of DCs is inhibited by TGFB via regulating DCs responses to inflammatory stimuli [178]. Suppression of TGFB signaling by deleting TGFBR2 in DCs induced autoimmune pancreatitis, indicating the role of TGFB in maintaining immune homeostasis and preserving the integrity of pancreatic acinar cells [179, 180]. NK cells respond rapidly to stress cells in the absence of an adaptive response, allowing a rapid immune reaction. TGFB signaling blocks their functions at multiple levels, it silences IFN-γ and T-bet expression in NK cells, thus inhibiting Th1 responses [166].

TGFB exerts distinct programs in monocyte/macrophage lineage depending on the differentiation state and context [166]. For monocytes, TGFB acts as chemoattractant to recruit cells to the site of injury or inflammation [181], upregulates adhesion molecule expression and facilitates monocyte transmigration by inducing MMPs to dissolve vascular membranes [182]. In macrophages, TGFB reduces their phagocytosis function and antigen presentation ability by decreasing the expression of IgG receptors and major histocompatibility complex (MHC) class II molecules, respectively [183, 184]. Macrophage activation is also under the control of TGFB pathway. First of all, TGFB inhibits the expression of pro-inflammatory mediators, such as TNF-α, IL-12 and iNOS, in order to resolve inflammation and prevent the development of immunopathology [185, 186]. Apart from the role of downregulating M1 macrophage marker, TGFB also upregulates anti-
inflammatory mediators, such as IL-10, arginase, mannose receptor, CCL17, YM1, and FIZZ1, indicating its role in polarizing macrophages towards a M2 phenotype [187, 188]. Additionally, there is a regulatory feedback mechanism between macrophages and TGFB: macrophages containing phagocytosed apoptotic cells can induce TGFB secretion [189], which in turn restrains the inflammatory response. M2 macrophages activated by a variety of anti-inflammatory cytokines also serve as a major source for TGFB production during the tissue repair phase [121]. Therefore, as both producer and recipient of TGFB, macrophages exhibit diverse roles in tissue injury, fibrogenesis and regeneration by modulating the proliferation and activation of fibroblasts through TGFB signaling.

1.4.3 TGFB pathway in fibrogenesis

The fibrotic process is characterized by inflammation altered epithelial-mesenchymal interactions and proliferation/activation of fibroblasts [190]. TGFB has been considered as the critical mediator to regulate the interplay among multiple cell types via interacting different pathways [191]. The intricate nature of TGFB signaling in fibrogenesis is highlighted by the differentiation from fibroblasts to myofibroblasts and the following ECM accumulation [191].

The main fibrotic effector cells are myofibroblasts, and the roles of TGFB in fibroblasts activation have been well investigated: increased α-SMA and ECM production were observed in TGFB treated fibroblasts [192]. In addition, TGFB induces growth factors expression, such as connective tissue growth factor (CTGF), FGF and PDGF, to promote fibroblasts proliferation in autocrine or paracrine manner [191, 193, 194], it also upregulates the expression of PDGF receptors on fibroblasts to enhance the pro-proliferation capacity [195]. Apart from promoting fibroblast proliferation, TGFB prevents fibroblasts from apoptosis through p38 MAPK and PI3K/AKT signaling and cell-cycle regulators [191, 196, 197]. On the other hand, fibroblasts are not the only source for myofibroblasts differentiation. Epithelial derived cells can contribute to the pool of activated myofibroblasts by undergoing EMT, and this process is activated by TGFB mediated induction of mesenchymal transcription factors, such as Snail1 and Twist [191]. EMT transcription factor deletion in hepatocytes or tubular epithelial cells has demonstrated that both liver and renal fibrosis were attenuated [198, 199].

The role of TGFB in promoting ECM deposition is via upregulating protein expression, secretion, and stabilization [191]. For instance, transcription of Col1a2 gene is dependent on the nuclear translocation of Smad3 and Smad4 [200], and the posttranslational assembly is also facilitated by TGFB induced collagen-specific molecular chaperone [201]. In addition to supporting fibril assembly, TGFB also induces the expression of protease inhibitors, including plasminogen activator inhibitor 1 (PAI-1) and TIMPs, to attenuate the breakdown of newly produced collagens [191]. Besides the Smad-mediated signaling, TGFB can also regulate the expression of fibronectin and CTGF via p38 MAPK signaling [202, 203].
Together with other ECM proteins upregulated by TGFB, such as laminin and elastin, the collagen-based fibrosis increases tissue stiffness which accelerates fibroblasts collagen production and expression of contractile proteins such as α-SMA [204]. Increased α-SMA further enhances integrin-mediated TGFB activation and provides a feed-forward loop for ECM accumulation [191].

1.5 THE ROLE OF SMAD7 IN TGFB SIGNALING

1.5.1 Smad7 as the modulator of TGFB signaling

Smad7 was identified almost simultaneously by two groups, through PCR using degenerated oligos targeting an R-Smad consensus sequence and as an expressed sequence tag related to known SMAD proteins. Smad7 was the first Smad to be reported as a negative feedback regulator, i.e. a TGFB-inducible antagonist of TGFB mediated signaling [163, 205, 206]. Smad7 lacks the typical MH1 domain as R-Smads and Co-Smad, it is referred to as N-terminus. Both N-terminus and MH2 domain of Smad7 are indispensable for its optimal inhibitory function: the N-terminus determines the subcellular localization of Smad7 and the MH2 domain is responsible for interaction with the activated TGFBR1 and R-Smads [157, 207]. Several motifs are closely related to inhibitory function, for example, the Leu-rich motif (LRM) and the Pro-Tyr motif (PY) in the N-terminus are required for E2 ubiquitin-conjugating enzyme UbcH7 recruitment and recognition of Smurfs, respectively [157, 208]. The L3 loop in the MH2 domain is required for the interaction between Smad7 and the TGFBR1 (Figure 13) [209].

Figure 13. Structure of Smad7 gene and functional motifs.

Smad7 can inhibit TGFB family signaling by competing with R-Smads for association with TGFBR1, mainly through the interaction between the MH2 domain and TGFBR1. However, the MH2 domain itself is not sufficient to exhibit full inhibition, it also requires the cooperation with the N-terminus to facilitate and stabilize its interaction with TGFBR1 [207, 210]. Moreover, Smad7 leads to the proteasomal degradation of the TGFBR through recruitment of the E3 ubiquitin ligases Smurf1 and Smurf2 to the receptor [157]. Smad7 associates with the Smurfs’ WW-domain via the PY motif and assists in ubiquitylation by
recruiting the E2 ubiquitin-conjugating enzyme UbcH7 [208]. Smurfs were originally identified to exert degradation of R-Smads [211], it has also been demonstrated that Smurfs could accelerate the export of Smad7 from the nucleus to the cytoplasm [212], and the complex composed of Smad7, Smurf2 and the salt-inducible kinase facilitates the interaction with TGFBR1 and promotes TGFBR1 degradation [213, 214]. Additionally, Smad7 can induce TGFBR1 dephosphorylation by recruiting protein phosphatase 1 [215] and translocate ubiquitylated TGFBR1 to the endosomal compartment for lysosomal degradation [216]. Another important mechanism of Smad7 inhibitory activity is though interfering Smad2/3–Smad4 complex formation. The Smad7 MH2 domain competes with Smad4 to associate with activated Smad2/3 and recruits the E3 ubiquitin ligase to facilitate the ubiquitylation and degradation of phosphorylated Smad2/3 [217]. Apart from the interaction with TGFBR1 and R-Smads, Smad7 can also suppress TGFB signaling by interacting with the Smad-binding elements (SBEs) to confer transcription repression [218].

The control of Smad7 function is vital for maintaining the TGFB signaling in the normal physiological range. Posttranslational modifications, interactions with other proteins and the regulation of Smad7 expression are normally involved in this process [157]. Arkadia, a RING type E3 ubiquitin ligase, physically interacts with Smad7 and induces its poly-ubiquitination and degradation [219]. Other factors including RNF12, Jab1/CSN5, and Cbl-b were also reported to enhance ubiquitin-dependent degradation of Smad7 [220-222]. Besides regulating Smad7 stability, ubiquitin-conjugating enzyme E2O and AMSH-related protein were shown to interfere with the interaction between Smad7 and TGFBR1, thus attenuating its inhibitory activity [223, 224]. Transcription of Smad7 is regulated by various transcription factors. Not only TGFB, but also other growth factors and cytokines like BMPs, TNF-α, IFN-γ and IL-1 etc. can induce Smad7 expression [225-227], while Foxp3 and TGF-b-inducible early gene-1 suppress the expression of Smad7 [228, 229]. c-Ski and SnoN are further important TGFB signaling inhibitors acting by preventing Smad dependent target gene expression and regulating Smad7 transcription [230]. At the base level, c-Ski and SnoN inhibit the Smad7 promoter basal activity in a SBE-dependent manner. Upon stimulation, c-Ski and SnoN dissociate from the Smad7 promoter region, resulting in de-repression/induction of Smad7 expression [231, 232].

1.5.2 The role of Smad7 in homeostasis and pathology

Smad7 plays a key role in the control of various physiological events and some pathological processes, and a balanced expression level of Smad7 is necessary for maintaining homeostasis during embryonic development of many organs [233, 234]. Mice with entire Smad7 deletion (Smad7Δ/Δ) were embryonic lethal [235], and mice lacking the MH2 domain (Smad7ΔMH2) on a C57BL/6 background died before weaning with cardiac defects [236, 237], but when deleted on an outbred ICR (Institute for Cancer Research) background, mice developed to adulthood with a smaller body size [237]. This indicates that the phenotype of Smad7 inactivation is also dependent on the mouse strain background [157]. Deleting exon 1,
coding for half of the Smad7 protein (Smad7ΔexI), resulted in a hypomorph, meaning that the knockout was not complete, but still had some Smad7 inhibitory functions though in a weakened form [238]. Furthermore, these mice exhibited alterations in B cells responses such as Ig class switching [238] and impaired skeletal development [239].

Challenged by different stimuli, Smad7ΔexI mice showed enhanced tissue injury, fibrosis and inflammatory response in different fibrotic disease models, including CCL4-induced chronic liver fibrosis [240] and unilateral ureteral obstruction (UUO) induced renal fibrosis [241]. In contrast, overexpression of Smad7 prevented bleomycin-induced lung fibrosis [242], and hepatocyte-specific Smad7 overexpression showed attenuated CCL4-induced liver fibrosis [243], while alcohol-induced liver fibrosis was profoundly aggravated in hepatocyte-specific Smad7 deficient mice [244]. Collectively, these studies have demonstrated the protective function of Smad7 in attenuating TGFB-mediated fibrosis in multiple organs [245].

Regarding the pancreas, conditional Smad7 overexpression in embryonic β-cells results in β-cell hypoplasia and neonatal lethality, whereas overexpression of Smad7 in adult pancreatic β-cells leads to reduced insulin production [246]. Smad7 overexpression restricted to acinar cells by the elastase I promoter led to PanIN, which underscores the role of TGFB tumor suppressor activity [247]. When CP was induced in the same mouse strain, pancreatic fibrosis was reduced, possibly due to a decrease of PSCs activation and inhibition of TGFB induced ECM deposition [248].

The roles of Smad7 in the regulation of immune response and inflammation vary depending on the tissues/cells type [245]. For instance, overexpression of Smad7 in the kidney could attenuate autoimmune renal inflammation [249], it can also suppress TNF-α signaling and NF-κB activation to promote anti-inflammatory effects [250]. However, mice with overexpression of Smad7 in T cells developed an enhanced autoimmune encephalomyelitis [235]. CD4+ T cells with high expression of Smad7 display accelerated T cell proliferation and activation, resulting in severe colitis in mice [251]. Airway reactivity and related cytokine production were enhanced in Smad7 overexpressing transgenic mice as well [252]. Together, these results hint to a pro-inflammatory effect in Smad7 overexpressing mice.

1.6 TAMOXIFEN

Tamoxifen, a non-steroidal triphenyl ethylene derivative, was used as the first-line therapy for breast cancer since the 1970s, and categorized as selective estrogen receptor modulators (SERMs) [253]. In the beginning, the function of tamoxifen was simply considered as a competitive antagonist of estrogen: the prodrug tamoxifen was metabolized by cytochrome P450 enzymes in liver, and the metabolite 4-hydroxytamoxifen (4-OHT) exerted much higher affinity to estrogen receptors (ERs), including ERα and ERβ. In contrast to estrogen-ERs complex, tamoxifen-ERs complex recruits corepressors, rather than coactivators, to inhibit estrogen-responsive gene transcription [254, 255]. However, more studies have confirmed that tamoxifen has mixed antagonist and agonist properties in a tissue/cell-specific manner.
For instance, tamoxifen functions as an antiestrogen in the breast but also exhibits predominant estrogenic activity in the uterus, skeletal, liver and cardiovascular systems [253]. These dual functions are mainly dependent on differentially expressed ERs, ligand-dependent receptor conformational changes, various interactions with co-activators and co-repressors expressed in a tissue-specific manner [256, 257].

Apart from the clinical role, tamoxifen has also been exploited for developing genetic engineered mouse model. The tamoxifen-inducible Cre-LoxP systems is widely used for the generation of conditional gene-modified mice in a tissue and stage-specific way. The efficiency of Cre recombinase is influenced by different factors, such as the Cre expression level, the chromatin structure, epigenetic status of the floxed alleles, and the administration schemes of tamoxifen [258, 259]. Diverse tamoxifen administration protocols have been reported, describing different dosages, application routes and treatment time schedules [260]. However, tamoxifen is not just an inducer for Cre recombinase, it also exhibits some unexpected “off-target” effects in different tissues. For instance, tamoxifen could inhibit bone resorption and influence bone homeostasis [261], exposure of a single dose of tamoxifen in pubertal mice was able to induce adverse effects to testis and reproductive endocrine system [262]. If the side effects last for a longer period, it might confound the interpretation of time-sensitive studies. Evidence was provided by a CCL4-induced liver injury model, where tamoxifen could strongly attenuates hepatotoxicity even after a 10 days tamoxifen exposure-free period [263]. Therefore, optimizing the tamoxifen treatment scheme is necessary to minimize these confounding “off-target” effects when applying inducible Cre-LoxP system. Last but not least, since tamoxifen is an estrogen analog, one might expect different effects on male and female organisms.
2 RESEARCH QUESTIONS

Although the pathogenesis of CP has been widely investigated in previous studies, the exact mechanism of irreversible fibrogenesis and inflammation response is still not fully understood. The TGFB pathway is considered as the center mediator for fibrotic disease via regulating diverse pathological activities. In addition, both fibroblasts and inflammatory cells, as the effector cells for fibrosis and inflammatory response, exhibit important roles during CP progression. However, the role of TGFB signaling and specifically its inhibitor Smad7 in fibroblasts and inflammatory cells during CP initiation and progression is still unknown. Although the use of the tamoxifen-inducible Cre-LoxP system has facilitated the investigation of many genes, the side-effects of the inducer tamoxifen have only recently come into focus. Without optimized tamoxifen administration protocol, the readout of experiments might be confounded. Therefore, testing of possible side effects is mandatory before embarking on specific research questions.

Specific questions:

- What is the role of TGFB pathway/Smad7 in fibrogenesis during CP development in general?
- What is the role of TGFB pathway/Smad7 in fibroblasts during CP development?
- What is the role of TGFB pathway/Smad7 in myeloid cells during CP development?
- What is the optimal tamoxifen administration scheme for cerulein-induced CP mouse model?
3 METHODS AND MATERIALS

3.1 ANIMAL MODELS

3.1.1 Smad7ΔexI mouse model
The generation of the Smad7 mutant mice was described previously by our group [238]. Briefly, the coding region of the first exon of Smad7, including the starting ATG codon and a part of intron I, was replaced with a neomycin selection cassette. A hypomorphic mutation rather than a complete null mutation was conducted because only the first half of the Smad7 protein (the N-terminus) was deleted.

3.1.2 Col1a2-CreERT;Smad7fl/fl mouse model
Col1a2-CreERT;Smad7fl/fl mice were generated by breeding B6.Cg-Tg(Col1a2-cre/ERT,-ALPP)7Cpd/J mice [264] with B6.Cg-Smad7tm1.1lnk/J (Smad7fl/fl) mice [235] on C57BL/6J strain background to achieve the conditional Smad7 knockout in fibroblasts.

Col1a2-CreERT-tdTomato mice were generated by breeding the B6.Cg-Tg(Col1a2-cre/ERT,-ALPP)7Cpd/J mice [264] with B6.Cg-Gt(ROSA)26SorImo(CAG-tdTomato)Hze/J mice [265] on C57BL/6J background to be used as the fibroblasts reporter strain.

Col1a2-CreERT-tdTomato;Smad7fl/fl mice were generated by mating Col1a2-CreERT-tdTomato mice and Smad7fl/fl mice [235] to be used as the mutated fibroblasts reporter strain.

3.1.3 LysM-Cre;Smad7fl/fl mouse model
LysM-Cre;Smad7fl/fl mice were generated by mating LysM-Cre strain [266] and Smad7fl/fl mice [235] on C57BL/6 strain background to achieve the conditional Smad7 knockout in myeloid cells.

LysM-Cre;tdTomato mice were generated by mating LysM-Cre strain [266] and B6.Cg-Gt(ROSA)26SorImo(CAG-tdTomato)Hze/J mice [265] to be use as the myeloid cells reporter strain.

LysM-Cre;Smad7fl/fl;tdTomato mice were generated by mating LysM-Cre;tdTomato mice and Smad7fl/fl mice [235] to be used as mutated myeloid cells reporter strain.

3.1.4 C57BL/6J mouse
C57BL/6J were purchased from PKL4 animal facility at Karolinska Institutet, Huddinge to conduct the optimization of tamoxifen administration experiment.
3.1.5 Protocol for cerulein induced CP and tamoxifen administration

*Smad7ΔexI* mice between 2-3 months of age were subjected to CP by the well-established 4-week cerulein injection protocol, while the control group was administrated with only saline. Cerulein was used to induce CP as following: 50μg/kg cerulein (Sigma C9026) dissolved in saline was injected intraperitoneally every hour, 6 times per day, 2 days per week for 4 weeks in total [124]. Mice were sacrificed on the third day after the last cerulein injection. A total of 6 mice were treated per group.

Male mice from *LysM-Cre;Smad7fl/fl*, *Smad7fl/fl*, *LysM-Cre;Smad7fl/fl;tdTomato* and *LysM-Cre;tdTomato* strains between 2-3 months of age were subjected to CP as described above to investigate TGFB pathway/Smad7 in myeloid cells during CP development. Female mice from *Col1a2-CreERT;Smad7fl/fl* and *Smad7fl/fl* strains between 2-3 months of age were subjected to tamoxifen oral gavage ([200mg/kg] Sigma T5648 dissolved in corn oil Sigma C8267) once per day for 5 consecutive days to activate Cre recombination, two weeks later cerulein injection were performed as described above to investigate the role of TGFB pathway/Smad7 in fibroblasts during CP development. All mice were sacrificed on the third day after the last cerulein injection. A total of 8 mice were treated per group.

For experiments aiming for optimizing tamoxifen administration, following scheme was conducted (Figure 14): C57BL/6J mice between 2-3 months of age were treated with tamoxifen oral gavage once per day for 5 consecutive days. 3 days, 1 week, 2 weeks or 3 weeks after the last oral gavage, mice were subjected to CP by 2-week cerulein injection, respectively. In the cerulein control group, mice were administrated with corn oil instead of tamoxifen, followed by 2-week cerulein injection 3 days after the last corn oil treatment. In the saline control groups, mice were pre-treated with 5-days tamoxifen, followed by 2-week saline i.p injection 3 days after the last tamoxifen treatment. Mice were sacrificed on the third day after the last cerulein/saline injection. A total of 10 mice were treated per group consisting of 5 female and 5 male mice.

All animal experiments were performed with the approval of the local animal ethics committees (Stockholm Södra djurförsöksetiska nämnd: S63-14; Linköping djurförsöksetiska nämnd: ID 91-15, ID 2-17).

![Figure 14. The administration scheme of tamoxifen and cerulein for the optimization experiment.](image-url)
3.2 CELL CULTURE

3.2.1 PSCs and tissue-resident macrophages isolation and culture

In order to obtain pure primary PSCs for in vitro experiments (see below), *Col1a2-Cre<sup>ERT</sup>-tdTomato;Smad7<sup>fl/fl</sup>* mice and *Col1a2-Cre<sup>ERT</sup>-tdTomato* mice (n=3 for each strain) were sacrificed for PSCs isolation. The collagenase digestion method was used for isolation of PSCs as described before [267]. Briefly, pancreas tissue was cut into small pieces and digested by collagenase. The resulting cell suspension was washed with HBSS and filtered through 70 um cell strainer for preparative FACS, and tdTomato expressing PSCs were then sorted by BD FACSARia based on the phycoerythrin (PE) channel. Similar FACS method was used for the isolation of pancreatic tissue-resident macrophage from *LysM-Cre;Smad7<sup>fl/fl</sup>;tdTomato* mice and *LysM-Cre;tdTomato* mice.

The primary PSCs were expanded in Dulbecco’s modified Eagle’s medium/F12 medium (DMEM/F12) supplemented with 10% FBS and 0.5% penicillin-streptomycin (PS) until passages 5-6. Then, 2*10<sup>5</sup> PSCs/well, 5*10<sup>3</sup> PSCs/well and 2*10<sup>6</sup> PSCs/dish were seeded in 6-well plates, 96-well plates and 10-cm petri dishes, respectively, and cultured with 10% FBS + 0.5% PS supplemented DMEM/F12 medium. Pancreatic tissue-resident macrophages were harvested directly after FACS for RNA isolation and PCR.

3.2.2 Bone marrow derived macrophages (BMDMs) isolation and polarization

BMDMs were obtained by rinsing opened tibia and femur with PBS+10% FBS to get bone marrow from *Smad7<sup>fl/fl</sup>* mice and *LysM-Cre;Smad7<sup>fl/fl</sup>* mice. Cells were filtered through cell strainer and collected for centrifugation. Supernatants were discarded, and cells were lysed in red cell lysis buffer, then filled up with PBS+10% FBS for centrifugation. Afterwards, cells were plated in RPMI complete medium (ThermoFisher-31870-025, with 10% FBS, 1% PS and 1% glutamine) supplemented with M-CSF (50ng/ml, Miltenyi, #130-101-706) with following numbers: 2*10<sup>5</sup> cells/well, 1*10<sup>4</sup> cells/well and 2*10<sup>6</sup> cells/dish in 6-well plates, 96-well plates and 10-cm petri dishes, respectively. After one week of differentiation and expansion, BMDMs were polarised on day 7 by culturing them in the presence of TGFB (10 ng/ml) with/without IL-4 (20ng/ml, Peprotech-200-04) for 20 hours, or LPS (100ng/ml, Sigma) with/without IFN-γ (200U/ml, Peprotech-300-02) for 6 hours in RPMI medium supplemented with M-CSF (50ng/ml).

3.2.3 Tamoxifen/ cerulein/TGFB in vitro treatment

PSCs were cultured with 0.5% FBS + 0.5% PS supplemented DMEM/F12 medium for starvation overnight. Afterwards, cells were treated with different combination of cerulein (final 10<sup>8</sup>M, Sigma C9026), 4-hydroxytamoxifen (final 5*10<sup>6</sup>M; Sigma H7904) and TGFB
(final 5ng/ml, PeproTech-100-21) for 24h or 48h, then harvested for quantitative real-time PCR (RT-PCR) and western blot (WB) as described in the results section.

### 3.2.4 Preparation of conditioned media from polarized macrophages

BMDMs were induced with LPS/IFN-γ or IL-4/TGFB1 as described above and the cytokine-supplemented medium was discarded. After washing the cells with PBS, fresh RPMI complete medium supplemented with M-CSF were added for overnight incubation. PSCs were rinsed with PBS and cultured in the respective conditioned media from polarised BMDMs for 24h, followed by RT-PCR, viability assay and WB. Viability assay was performed for PSCs by using CellTiter-Glo® 3D Cell Viability Assay Kit (Promega-G9683) according to the manufacturer’s instruction.

### 3.3 HISTOLOGICAL ANALYSES

Upon sacrifice of the mice, the head of the pancreas was fixed in buffered 4% formalin followed by 70% alcohol, embedding in paraffin for sectioning. Determination of the collagen-based fibrotic index was performed with the help of Sirius red (HistoLab, Cat. No. HL27150.0500)/Fast green counterstaining; Certistain®, Merck, Cat. No. 1.04022) stain in combination with ImageJ. Five fields per section were selected randomly at 200X magnification. The images were converted into separated channels using ImageJ’s color deconvolution plugin (http://fiji.sc/Colour Deconvolution) [268]. The fibrotic index was calculated as the percentage of collagens area in the total tissue area. The severity of pancreatitis using a set of modified scoring criteria described previously [269, 270]. This set included three histological parameters, inflammation, atrophy and fibrosis with an assessment range from 0 to 3, representing the severity of pathology as no changes, minor changes, moderate changes and severe changes, respectively. Representative images were taken from each tissue sample and then evaluated and graded for the different assessment criteria.

The tail of the pancreas was embedded in OCT (HistoLab, Cat. No. 45830) on dry ice for cryosectioning. Cryosections were fixed with acetone/methanol (1:1) for 15min at -20°C. Primary antibodies were used as follows: collagen I (1:200, ab34710), collagen III (1:200, ab7778), collagen IV (1:200, ab6586), fibronectin (1:200, ab2413), vimentin (1:100, Cell Signalling-5741), CD11b (1:50, BD Pharmingen-550282), F4/80 (1:200, Bio-rad-Cl:A3-1), MRC1 (1:200, Bio-rad-MR5D3), CD11c (1:200, BD Pharmingen-550283). Nonspecific binding sites and endogenous biotin were blocked with PBST containing 10% FBS and 1% BSA or goat serum (1:10, Dako-X0907) with/without Avidin/Biotin Blocking Kit (Vector Laboratories-SP-2001). The tissues were incubated with primary antibodies overnight at 4°C, and subsequently with a secondary antibody (biotinylated goat-anti-rabbit immunoglobulins, 1:200, Dako-E0432; biotinylated goat-anti-rat immunoglobulins, 1:200, BD pharmingen-559286; DyLight488-conjugated streptavidin, 1μg/ml, Jackson ImmunoResearch; goat anti
rabbit-Alexa Fluor 488, 1: 500, Invitrogen A-11034; goat anti rat-Alexa Fluor 488, 1: 500, Invitrogen A-11006; goat anti rat-Alexa Fluor 546, 1:200, Invitrogen A-11081; goat anti hamster-Alexa Fluor 546, 1:500, Invitrogen A-21111) for 1 hour at room temperature. Nuclei were stained with DAPI. Images were captured using a Zeiss epi-fluorescence inverted microscope (Axiophot).

The morphometric analyses of collagen I, collagen III, collagen IV, fibronectin, vimentin, CD11b, F4/80, MRC1 and CD11c were also performed with the help of ImageJ software [268]. Five fields per section were selected randomly at 200X magnification. The relative extent of extracellular matrix accumulation, mesenchymal cell and inflammatory cell occurrence were determined by the percentage of positive (green or red fluorescence) staining in the total pancreatic tissue area within the cryosection.

3.4 POLYMERASE CHAIN REACTION
RNA was extracted from cells or tissue using the Qiagen RNeasy kit (74104), and iScript™ cDNA synthesis kit (Bio-red, 1708891) was used for cDNA preparation as described before [271]. RT-PCR was performed by using SYBR green kit (Thermofisher Scientific, K0243), TaqMan Master Mix (Thermofisher Scientific, 4444964) and the Bio-Rad CFX96 thermal cycler.

3.5 IMMUNOBLOTTING
Cells were lysed in lysis buffer (ThermoFisher-78510) containing proteinase inhibitor (ThermoFisher-87786) and phosphatase Inhibitor cocktails (ThermoFisher-78420) and clarified by centrifugation. Protein concentrations were determined by Bradford protein assay (Bio-rad-5000205), and 30μg proteins were separated by using 4-20% TGX gels (Bio-rad-4568094) and blotted onto a PVDF membrane (Bio-rad-1620177). Membranes were blocked for 1 hour in 5% milk in TBST buffer (ThermoFisher-28360) and incubated overnight at 4°C with primary antibody: collagen I (ab34710, 1:1000), fibronectin (ab2413, 1:1000), α-SMA (ab5694, 1:1000), p-Smad2 (cellsignaling-3101, 1:1000), Smad7 (LS-C313088, 1:500). Following three washes in TBST, horseradish peroxidase-conjugated secondary antibody (GENA934, 1:10000) was added and samples were incubated for 1h at room temperature. β-actin (Sigma-A2228, 1:8000) and α-tubulin (ab7291, 1:5000) in combination with corresponding HRP-conjugated secondary antibody (GENXA931, 1:10000, GENA934, 1:10000) were used as loading controls. Antibody binding was visualized with the enhanced chemiluminescence detection system (Bio-Rad-1705060). Images were captured and analyzed with Bio-Rad Gel Doc™ XR+ Gel Documentation System.
3.6 STATISTICAL ANALYSIS

Data are presented as mean ± 95% confidence interval for RT-PCR and quantification of fibrotic index and immunofluorescence staining, and mean ± SE for viability assay. Mann-Whitney U test was used for immunofluorescence analysis for two-sample comparisons, and two-sided paired student’s t-test was used for viability assay. For RT-PCR, the ΔΔCt equation was used to determine relative expression, which was normalized to controls, and the p-value was calculated by two-sided paired student’s t-test based on ΔCt values.
4 RESULTS

4.1 SMAD7ΔEXI EXACERBATES CP DEVELOPMENT

Following repeated cerulein injection, we observed histological changes in the pancreas of both wild-type and Smad7ΔexI mice, including fibrosis, inflammation, atrophy and ADM. Overall, the Smad7ΔexI mice showed a significantly increased response to cerulein stimulation compared to wild type controls, and the lack of functional Smad7 gene seemed to considerably aggravate the severity of CP. By evaluating the relative fibrillar collagen amount visualized by Sirius Red/Fast Green staining, it was shown that there was significantly more fibrillar collagen interspersed between the acini in Smad7ΔexI mice. To further support this observation, we quantified the levels of collagen type I, and Smad7ΔexI mice presented with significantly higher density of fiber staining in the interlobular area after cerulein injection. Additionally, there were more vimentin positive interstitial mesenchymal cells and CD11b positive inflammatory cells surrounding acini in Smad7ΔexI mice compared to wild-type mice after cerulein induction. Taken together, we conclude that lack of a functional Smad7 gene results in more severe damage in CP (Table 2) due to elevated TGFB signalling. However, the role of Smad7/TGFB signaling in the different pancreatic compartments, such as fibroblasts and myeloid cells needs further investigation.

<table>
<thead>
<tr>
<th>CP parameters</th>
<th>Comparing with WT mice, Smad7ΔexI mice demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity of CP</td>
<td>Trend: higher</td>
</tr>
<tr>
<td>Fibrotic index</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>Collagen I deposition</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>Fibroblasts infiltration</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>Inflammatory cells infiltration</td>
<td>Significantly higher</td>
</tr>
</tbody>
</table>

Table 2. Summary of histological analyses in CP tissue from Smad7ΔexI and control mice. Severity of CP was evaluated by inflammation, atrophy and fibrosis level, see page 33.

4.2 CONDITIONAL GENE TARGETING OF SMAD7 IN FIBROBLASTS AND MYELOID CELLS

4.2.1 Col1a2-CreERT;Smad7fl/fl mouse model

Successful recombination in the sorted Col1a2-CreERT-tdTomato;Smad7fl/fl PSCs was verified by RT-PCR which showed significantly lower Smad7 mRNA expression level. PCR using primers that amplify the sequence resulted from Cre-mediated Smad7 deletion occurred only in Col1a2-CreERT-tdTomato;Smad7fl/fl PSCs. PSCs derived from Col1a2-CreERT-tdTomato;Smad7fl/fl mice had no detectable Smad7 protein expression, whereas Col1a2-CreERT-tdTomato PSCs expressed low amounts of Smad7 that could be increased by TGFB1 stimulation. Additionally, isolated Col1a2-CreERT-tdTomato;Smad7fl/fl PSCs had upregulated
levels of mRNA expression of TGFB target genes Serpine1 (PAI-1), Cnn1 (Calponin), Tagln (SM22) both before and after TGFB1 or cerulein treatment in vitro. These results went in hand with increased protein expression of collagen I, fibronectin and α-SMA. Thus, Col1a2-CreERT-tdTomato;Smad7fl/fl PSCs lacked appreciable Smad7 expression and demonstrated increased TGFB signaling resulting in the increased expression of several TGFB target genes, both at mRNA and protein level in vitro. (Table 3).

<table>
<thead>
<tr>
<th>Investigated parameters for in vitro experiments</th>
<th>Comparing with control PSCs, Smad7-deficient PSCs demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad7 mRNA level</td>
<td>Significantly lower</td>
</tr>
<tr>
<td>Smad7 protein level</td>
<td>No detection</td>
</tr>
<tr>
<td>TGFB target genes mRNA level without treatment</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>TGFB target genes mRNA level after 1h treatment</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>TGFB target genes mRNA level after 24h treatment</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>Collagen I/α-SMA protein level without treatment</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>Collagen I/α-SMA protein level after 24h TGFB1 treatment</td>
<td>Significantly higher</td>
</tr>
</tbody>
</table>

Table 3. Result summary of in vitro experiments using PSCs isolated from Col1a2-CreERT-tdTomato;Smad7fl/fl PSCs and Col1a2-CreERT-tdTomato pancreata.

4.2.2 LysM-Cre;Smad7fl/fl mouse model

To assess the recombination efficiency of the floxed Smad7 allele in macrophages, we employed different strategies. Using antibodies for FACsorting which recognize CD11b, F4/80 and Ly6G we found that 65% of the myeloid cell population isolated from pancreata of LysM-Cre;Smad7fl/fl;tdTomato mice were macrophages and 95% of those were positive for the red fluorescent reporter protein tdTomato indicating recombination activity by LysM-Cre. 95% of blood monocytes from the same mouse strain were positive for tdTomato. Cre-mediated recombination was also confirmed by PCR, indicated by a 286-bp amplicon in the LysM-Cre;Smad7fl/fl BMDMs. Smad7 mRNA expression was markedly reduced by 98% in LysM-Cre;Smad7fl/fl BMDMs, and no amplification could be detected from LysM-Cre;Smad7fl/fl;tdTomato macrophages isolated directly from pancreas tissue by FACS. Additionally, LysM-Cre;Smad7fl/fl M2 BMDMs (treated by TGFB1 and IL-4) exhibited higher levels of phosphorylated Smad2, indicating an enhanced TGFB signaling activity (Table 4).

<table>
<thead>
<tr>
<th>Investigated parameters for in vitro experiments</th>
<th>Comparing with control macrophages, Smad7-deficient macrophages demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad7 mRNA level</td>
<td>Significantly lower</td>
</tr>
<tr>
<td>P-smad2 protein level</td>
<td>Significantly higher</td>
</tr>
</tbody>
</table>

Table 4. Result summary of in vitro experiment for Smad7-deficient macrophages.
4.3 THE EFFECT OF CONDITIONAL TARGETING OF SMAD7 ON EXPERIMENTAL CHRONIC PANCREATITIS

4.3.1 The effect of fibroblasts-specific Smad7 deletion in CP fibrogenesis

To determine whether elevated TGFB signaling in PSCs would lead to increased fibrotic response, experimental chronic pancreatitis was induced by cerulein in both Col1a2-Cre\textsuperscript{ERT};Smad7\textsuperscript{fl/fl} and Smad7\textsuperscript{fl/fl} mice. To our surprise, there was only a slight trend towards a higher fibrotic index in the Col1a2-Cre\textsuperscript{ERT};Smad7\textsuperscript{fl/fl} mice but no statistically significant difference compared to Smad7\textsuperscript{fl/fl} control mice. Supporting this observation, the immunofluorescence staining for collagen I, collagen III and collagen IV showed comparable levels between mutant and wild type mice. Only fibronectin expression was slightly, but significantly higher in the Col1a2-Cre\textsuperscript{ERT};Smad7\textsuperscript{fl/fl} group. Similarly, no statistically significant difference in vimentin and CD11b expression, surrogate markers for PSCs/myofibroblasts and macrophages, could be detected by immunofluorescence staining either. RT-PCR quantification of fibrogenesis associated marker genes also confirmed that Col1a2-Cre\textsuperscript{ERT};Smad7\textsuperscript{fl/fl} mice did not exhibit increased ECM gene mRNA expression, although Asma and Ctgf mRNA expression, genes indicative of PSC activation, were significantly increased. Thus, deleting Smad7 using Col1a2-Cre\textsuperscript{ERT} did not exacerbate fibrosis or alter the number of PSCs and inflammatory cells infiltration (Table 5).

<table>
<thead>
<tr>
<th>CP parameters</th>
<th>Comparing with control mice, Col1a2-Cre\textsuperscript{ERT};Smad7\textsuperscript{fl/fl} mice demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrotic index</td>
<td>Trend: higher</td>
</tr>
<tr>
<td>Collagen I deposition</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Collagen III deposition</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Collagen IV deposition</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Fibronectin deposition</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>Fibroblasts infiltration</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Inflammatory cells infiltration</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Asma mRNA level in CP tissue</td>
<td>Significantly higher</td>
</tr>
<tr>
<td>Ctgf mRNA level in CP tissue</td>
<td>Significantly higher</td>
</tr>
</tbody>
</table>

Table 5. Result summary of histological analyses in CP tissue from Col1a2-Cre\textsuperscript{ERT};Smad7\textsuperscript{fl/fl} and control mice.

4.3.2 The effect of myeloid cells-specific Smad7 deletion in CP fibrogenesis

As macrophages produce significant amounts of pro-fibrotic TGFB after phagocytosis of apoptotic cells [272], we hypothesized that myeloid-specific deletion of Smad7 would exacerbate the fibrogenic response following repeated cerulein injections. Surprisingly, the collagen-based fibrotic index of the LysM-Cre;Smad7\textsuperscript{fl/fl} and control mice (Smad7\textsuperscript{fl/fl}) was comparable after CP was induced. Consistent with this histologic finding, the collagen I and
collagen IV deposition analyzed by immunofluorescence revealed no difference either. However, other fibrosis parameters including abundance of fibronectin, number of vimentin-positive PSCs/myofibroblasts and number of F4/80+ macrophages were decreased in LysM-Cre;Smad$^{7\beta/\beta}$ pancreatic tissue compared to control. As fibrogenesis has been shown to be altered by the macrophage phenotype, we next investigated if the phenotype of macrophages was altered by Smad7 deletion. To this end, we performed immunofluorescence for MRC1, preferably expressed on anti-inflammatory “M2”-like macrophages and CD11c, preferably expressed on “M1”-like macrophages. In fact, Smad7 deletion resulted in decreased accumulation of M2-like macrophages in LysM-Cre;Smad$^{7\beta/\beta}$ mice, but did not have an effect on M1-like macrophage accumulation when compared to controls (Table 6). In addition, analysis of whole LysM-Cre;Smad$^{7\beta/\beta}$ pancreatic tissues revealed significantly less transcription of the fibrosis marker genes Ctgf and Tgfb1 compared to controls.

<table>
<thead>
<tr>
<th>CP parameters</th>
<th>Comparing with control mice, LysM-Cre;Smad$^{7\beta/\beta}$ mice demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrotic index</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Collagen I deposition</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Collagen IV deposition</td>
<td>Comparable level</td>
</tr>
<tr>
<td>Fibronectin deposition</td>
<td>Significantly lower</td>
</tr>
<tr>
<td>Macrophages infiltration</td>
<td></td>
</tr>
<tr>
<td>M1 macrophages infiltration</td>
<td>Comparable level</td>
</tr>
<tr>
<td>M2 macrophages infiltration</td>
<td>Significantly lower</td>
</tr>
<tr>
<td>Tgfb1 mRNA level in CP tissue</td>
<td>Significantly lower</td>
</tr>
<tr>
<td>Ctgf mRNA level in CP tissue</td>
<td>Significantly lower</td>
</tr>
</tbody>
</table>

Table 6. Result summary of histological analyses in CP tissue from LysM-Cre;Smad$^{7\beta/\beta}$ and control mice.

4.3.3 Conditional Smad7 deletion in myeloid cells affects macrophages polarization

TGFB signalling plays a critical role in promoting an anti-inflammatory “healing” macrophage phenotype [273]. To investigate this in more detail, BMDMs from LysM-Cre;Smad$^{7\beta/\beta}$ and Smad$^{7\beta/\beta}$ mice were subjected to RT-PCR for factors that may be involved in exerting the anti- and pro-inflammatory macrophage functions. Surprisingly, unpolarized LysM-Cre;Smad$^{7\beta/\beta}$ BMDMs displayed an upregulation of both pro-inflammatory factors including IL6, iNOS and anti-inflammatory factors Ym1 and Mrc1, whereas the expression level of Vegfr1, Vegfa, Vegfc, Pdgfa and Pdgfb were downregulated in LysM-Cre;Smad$^{7\beta/\beta}$ BMDMs. Interestingly, when LysM-Cre;Smad$^{7\beta/\beta}$ BMDMs were polarized towards pro-inflammatory phenotype (IFN-γ and LPS treatment), they displayed a decrease in the mRNA levels of the pro-inflammatory cytokines CXCL9, CXCL10, CXCL11 and Tnfα, when compared with the controls. On the other hand, deletion of Smad7 did not skew BMDMs towards an anti-inflammatory phenotype upon subjection to TGFB1 and IL-4 treatment. In summary, Smad7 deletion in myeloid cells led to a reduced pro-inflammatory response. This,
however, did not affect the direction of commitment towards the anti-inflammatory phenotype (Table 7).

<table>
<thead>
<tr>
<th>Investigated parameters for in vitro experiments</th>
<th>Comparing with control BMDMs, Smad7-deficient BMDMs demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpolarized condition</td>
<td>Mixed M1/M2 markers with lower Pdgfa/b expression</td>
</tr>
<tr>
<td>M1 condition</td>
<td>Decreased pro-inflammatory commitment</td>
</tr>
<tr>
<td>M2 condition</td>
<td>Comparable anti-inflammatory commitment</td>
</tr>
</tbody>
</table>

Table 7. Result summary of in vitro experiments for macrophage polarization.

### 4.3.4 The crosstalk between macrophages and fibroblasts

Macrophages are activated by damaged cells/tissue and in turn activate resident fibroblasts. Since we found lower numbers of myofibroblasts in LysM-Cre;Smad7fl/fl mice, we performed viability assays to assess the interaction between Smad7-lacking macrophages and PSCs. Normal PSCs (from Col1a2-CreERT-tdTomato mice) were cultured in conditioned media from control (Smad7fl/fl) and LysM-Cre;Smad7fl/fl unpolarized(M0)/M1/M2 BMDMs. The results revealed that no matter which polarisation was executed, PSCs cultured in LysM-Cre;Smad7fl/fl BMDM media were always less viable than the ones cultured in control BMDM media. Moreover, this difference was also related to PSCs activation, as the expression levels of the myofibroblast activation markers, such as Acta2, Colla1, Fn, Ctgf and PAI-1, were decreased in PSCs treated with LysM-Cre;Smad7fl/fl BMDM M2 media, compared to the ones treated by Smad7fl/fl BMDM M2 media. The corresponding M2 LysM-Cre;Smad7fl/fl BMDMs also showed lower expression of Pdgfa and Pdgfb mRNAs. Comparable results were obtained on the protein level by WB. This indicated that both proliferation and activation of PSCs were affected by Smad7-mutant macrophages (Table 8).

<table>
<thead>
<tr>
<th>Investigated parameters for in vitro experiments</th>
<th>Comparing with PSCs treated by control BMDM media, PSCs treated by Smad7-deficient BMDM media demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability</td>
<td>Significantly lower</td>
</tr>
<tr>
<td>ECM deposition level</td>
<td>Significantly lower in M2 condition</td>
</tr>
<tr>
<td>Growth factors in BMDMs</td>
<td>Significantly lower expression of Pdgfa/b mRNA in M2 BMDMs</td>
</tr>
<tr>
<td>PSC activation level</td>
<td>Significantly lower in M2 condition</td>
</tr>
</tbody>
</table>

Table 8. Result summary of in vitro experiments investigating the crosstalk between macrophages and PSCs.

### 4.4 OPTIMIZATION OF THE TAMOXIFEN ADMINISTRATION SCHEME

#### 4.4.1 The sex- and time-dependent effect of tamoxifen in CP development

When we utilized the Col1a2-CreERT;Smad7fl/fl mouse model to investigate the fibroblasts-specific role of Smad7 during CP development, tamoxifen had to be administrated to activate
Cre recombinase. Results of a pilot experiment using only female control mice ($\text{Smad}^{\text{fl/fl}}$) showed that mice treated with tamoxifen for 5 days plus cerulein after a 3-day waiting period had a significantly higher fibrotic index than mice only treated with corn oil (vehicle for tamoxifen) plus cerulein following the same scheme (Figure 15). In order to test if tamoxifen has a time- and possibly also a sex-specific influence, we induced chronic pancreatitis by a two weeks cerulein treatment in female and male mice following different waiting periods after the last tamoxifen pretreatment (Figure 14, 16).

![Figure 15](image1.png)

Figure 15. Mice treated by cerulein intraperitoneal injection plus tamoxifen pre-administration showed significantly higher fibrotic index than the group receiving only cerulein treatment. N=8 for each group, (**p<0.01

![Figure 16](image2.png)

Figure 16. Representative images of H&E staining of CP tissue in female mice (A) and male mice (B) from each group. Magnification: 200X.
Like in the pilot experiment, we observed a higher fibrotic index in female mice after a 3-day waiting time before cerulein induction. This finding was corroborated by staining for collagen I, and the effect of tamoxifen on collagen I seemed to be oscillating, which was indicated by another increase in expression if pancreatitis was induced 3 weeks after tamoxifen application. Interestingly, tamoxifen generally decreased the fibrotic index and collagen I deposition in male mice. In addition to collagen I, we observed less collagen IV both in female and male mice. For fibronectin, we observed yet another response to tamoxifen, namely a generally increased deposition of fibronectin in both, female and male mice.

In order to investigate whether tamoxifen also had an effect on fibroblasts and inflammatory cells, immunofluorescence staining of vimentin, F4/80 and CD11b was performed. Similar to the collagen I expression levels, pancreata from female mice presented with a higher number of PSCs/myofibroblasts when CP induction was started only 3 days after tamoxifen pretreatment compared to vehicle pretreatment plus cerulein. This difference disappeared after longer waiting periods. However, the pancreata of male mice had generally lower numbers of PSCs/myofibroblasts when pretreated with tamoxifen compared to vehicle. Moreover, tamoxifen pretreatment resulted in mostly increased numbers of macrophages in female mice. In male mice on the other hand, we observed a trend of less macrophages. Similar results were observed by CD11b staining, which in addition to macrophages also detects other granulocytes (Table 9).

<table>
<thead>
<tr>
<th>CP parameters</th>
<th>Comparing with mice without tamoxifen pretreatment, mice with following tamoxifen-free period demonstrated:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-day waiting time</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>Fibrotic index</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen I deposition</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen IV deposition</td>
<td>→</td>
</tr>
<tr>
<td>Fibronectin deposition</td>
<td>↑</td>
</tr>
<tr>
<td>Fibroblasts infiltration</td>
<td>↑</td>
</tr>
<tr>
<td>Macrophages infiltration</td>
<td>↑</td>
</tr>
<tr>
<td>Inflammatory cells infiltration</td>
<td>↑</td>
</tr>
</tbody>
</table>

Table 9. Result summary of histological analyses in pancreatic tissue from C57BL/6J mice treated with different tamoxifen plus cerulein schemes. ↑: Significantly higher. ↑: Higher trend. ↓: Significantly lower. →: Lower trend. ⇪: Comparable to controls.

4.4.2 The effect of tamoxifen in fibroblasts

In order to study the tamoxifen-related mechanisms in modulating pancreatic fibrogenesis, we investigated whether co-treatment of tamoxifen and cerulein in primary mouse PSCs would reflect the differences observed in our in vivo experiments, and whether TGF-β and/or ER signaling were involved. Both Col1a1 and Ctgf mRNA were significantly upregulated after 24h co-treatment of tamoxifen and cerulein in female PSCs, compared with vehicle plus cerulein treatment (Figure 17A). Surprisingly, Tgfb1 and PAI-1/Serpine1 mRNA levels were
not changed in female PSCs by tamoxifen co-treatment at the 24h time point. On the other hand, the ERα (Esr1) mRNA expression level was dramatically reduced (Figure 17A). After 48h co-treatment, there was no more difference for these parameters with the exception of PAI-1/Serpine1 that was downregulated (Figure 17B). In male fibroblasts, Col1a1 mRNA was significantly reduced after 24h and 48h co-incubation, going in hand with reduced Ctgf mRNA expression. Interestingly, there was an upregulation in both Tgfb1 and PAI-1/Serpine1 whereas Esr1 expression levels were unchanged (Figure 18).

Figure 17. The mRNA expression level of Col1a1, Ctgf, Tgfb1, Serpine1 and Esr1 was evaluated by RT-PCR in female primary PSCs after (A) 24h and (B) 48h co-incubation with 4-OHT and cerulein. N=3 for each group, (*) p<0.05, (**) p<0.01, (***) p<0.001.
Figure 18. The mRNA expression level of Col1a1, Ctgf, Tgfb1, Serpine1 and Esr1 was evaluated by RT-PCR in male primary PSCs after (A) 24h and (B) 48h co-incubation with 4-OHT and cerulein. N=3 for each group, (*) p<0.05, (**) p<0.01, (*** p<0.001.
5 DISCUSSIONS

5.1 THE COMPLEXITY OF SMAD7-DEFICIENT MOUSE MODEL

More recent work in genetically engineered mouse models have indicated that Smad7 is associated with tissue fibrosis in different organs. In our Smad7\textsuperscript{ΔexI} mouse model, which is characterized by a general/ubiquitous targeting of Smad7, we observed a significant increase in myofibroblasts and inflammatory cells, resulting in more pronounced collagen accumulation and pancreatitis severity. However, Smad7\textsuperscript{ΔexI} stain is a hypomorph, most probably producing a truncated Smad7 protein consisting of the MH2 domain, which can still fulfill some inhibitory function. In such a mouse, all cells normally expressing Smad7, express the mutation. In all published experimental fibrosis models using the Smad7\textsuperscript{ΔexI} strain, the expected increase in the fibrotic response is observed due to increased TGFB signalling in all participating cell types. In order to further elucidate the role of TGFB signaling/Smad7 in specific cells, a conditional Smad7 knockout mouse model was utilized in the following studies.

The Smad7\textsuperscript{fl/fl} strain used in our experiments might not generate complete null knockout either, because only the proximal promoter region (1kb) and exon 1 of Smad7 are flanked by LoxP sites [235]. Since we were unable to generate a WB for Smad7 from BMDMs/macrophages, we cannot exclude that a truncated Smad7 protein, exhibiting some residual inhibitory effect, is still generated in the LysM-Cre;Smad7\textsuperscript{fl/fl} model, leading to a mild, hypomorph phenotype [238]. On the other hand, we only detected traces of increased pSmad2 levels in mutant M2-polarised macrophages, supporting the idea of increased TGFB signaling. Interestingly, similar results were also found in CD4-Cre;Smad7\textsuperscript{fl/fl} mice, where Smad7 mRNA expression was not detectable, and TGFB-induced Smad2 phosphorylation was augmented compared with wild type cells [235].

In Col1a2-Cre\textsubscript{ERT};Smad7\textsuperscript{fl/fl} PSCs, Smad7 protein was not detectable in contrast to wild type PSCs, and ECM deposition and fibroblast activation were accordingly increased. However, we failed to demonstrate upregulated Smad2/3 phosphorylation. Similar unexplainable/confusing results were reported from Smad7-deficient murine embryonic fibroblasts, where neither Smad7 mRNA nor Smad7 protein was detected but Smad2/3 phosphorylation was surprisingly decreased [237]. The possible explanations can be that Smad7 is not actively involved in the regulation of TGFB signalling in fibroblasts, which is unlikely, or loss of Smad7 may be compensated for its functions by other pathways.

Taken together, we can also not exclude the generation of a truncated protein in the conditional strain, and we don’t know what transcriptional activity the residual promoter is, which could be also different in different cells types. In order to obtain a complete knockout one would have to flank exon-I and -IV with LoxP-sites to remove all coding sequences. However, due to the long distance between LoxP-sites, the efficiency of recombination might be rather low. Another general problem for the Smad7 is that there are no antibodies available which reliably recognize endogenous levels of Smad7 protein.
5.2 NEW INSIGHTS INTO THE MECHANISMS OF TGFB SIGNALING MEDIATED FIBROGENESIS

5.2.1 Diverse roles of TGFB signaling in fibrogenesis

TGFB has long been considered as the central mediator of fibrotic disease via activation of both canonical (Smad-dependent) and non-canonical (non-Smad-dependent) pathways, resulting in activation of myofibroblasts, ECM deposition and inhibition of ECM degradation [274]. The pivotal role of TGFB in fibrogenesis has been confirmed in different disease models. Overexpression of active TGFB1 in lung by adenoviral vectors induced prolonged pulmonary fibrosis [275], and transgenic mice with hepatic overexpression of TGFB1 was sufficient to induce multiple tissue fibrotic lesions [276]. Similar results were also found in CP condition: TGFB1 overexpression in murine pancreas resulted in enhanced fibrosis and PSC activation [277, 278]. Accordingly, neutralization of TGFB1 demonstrated protective effect in myocardial fibrosis and lung fibrosis [279, 280].

Most fibrogenic effects are thought to be mediated by the direct effect of TGFB on fibroblast-like cells via the Smad-dependent pathway, leading to cell proliferation, migration and activation [274]. The heterotrimeric complex of Smad2/3/4 is the regulator of profibrotic genes transcription cooperating with both co-activators and co-suppressors. Several transgenic models have been generated to investigate the specific role of effector R-Smad proteins. However, only Smad3-knockout mice are viable while deletion of Smad2 or Smad4 in mice is embryonic lethal [274, 281], and Smad3 deficiency indeed attenuated bleomycin-induced pulmonary fibrosis and UUO-induced renal fibrosis [282, 283]. With the help of Cre-LoxP system, the role of Smad2 and Smad4 has also been uncovered: tubular epithelial cells-specific Smad2 deletion accelerated UUO-induced renal fibrosis by enhancing Smad3 activation [284] while tubular epithelial cells-specific Smad4 deletion reduced UUO-induced renal fibrosis without affecting Smad3 activity [285]. These results suggest that Smad3 is the main effector for profibrotic transcription regulation via binding to SBEs, while Smad2 is required for full Smad3 activity in the interaction with Smad4.

Besides the role of TGFB ligands and effector Smad proteins, the TGFB receptors have also been investigated. Inhibition of TGFBRII by adenoviral vector infection decreased pancreatic fibrosis in mice [286], and conditional overexpressing a dominant-negative mutant form of TGFBRII under the control of pS2/TFF1 promoter led to ameliorated CP fibrosis upon cerulein injections [287]. However, TGFBRII ablation in pancreatic epithelial cells resulted in increased inflammatory cell infiltration in early disease stage and PSC activation in the late stage [288]. Controversial results were also reported in renal fibrosis, where conditional deletion of TGFBRII in tubular epithelial cells inhibited severe UUO-induced renal fibrosis [289] but deletion of TGFBRII in renal collecting duct cells resulted in exacerbated UUO-induced renal fibrosis [290]. Therefore, the role of TGFB signaling in mediating fibrogenesis is far more complex than we thought, and it exhibits diverse effects in a cell-type dependent way [274], highlighting the necessity to investigate its specific function in different contexts.
5.2.2 The role of fibroblasts mediated by TGFB signaling

The profibrotic effects of TGFB involve a combination of mechanisms and cell types, and the role of fibroblasts draws much attention since they are the main effector cells to produce ECM [164]. The central role of activated PSCs in CP fibrogenesis was confirmed, and TGFB signaling is one of the secreted most potent fibrogenic cytokines inducing PSCs activation through downstream Smad pathways. These lead to induction of a profibrogenic transcriptional program including expression of laminin, fibronectin, collagen I, collagen III and other ECM proteins [192, 291, 292]. In our study, conditional Smad7 deletion was under the control of the COL1A2 promoter. There was surprisingly no difference in fibrogenesis after experimental CP was induced by cerulein injection, although the isolated PSCs lacking Smad7 had a significantly higher induction of collagen I production and higher level of activation towards the myofibroblast phenotype in vitro. Similar results were also reported in a renal fibrosis model, where conditional deletion of TGFBR2 under the control of COL1A2 or tenascin promoters led to impaired collagen I production in vitro and in vivo, but overall tissue fibrosis was unchanged in the knockouts after UUO-induced renal injury [293]. These unexpected results challenge the traditional concept of TGFB-driven fibroblasts involvement in fibrogenesis.

It is important to notice that PSCs cultured in vitro may display differently compared to cells residing in the pancreatic microenvironment, and the interaction of PSCs with other cells types is difficult to fully represent in vitro [294]. Additionally, different in vitro culturing conditions used for PSCs culture including plastic, Matrigel and collagen I, significantly influence PSCs gene expression patterns [295]. Therefore, our Smad7-deficient PSC phenotype in vivo still needs further investigation. Moreover, in the previous UUO-model, tamoxifen was administrated to activate the Cre recombinase after the renal injury was induced. As our results from the tamoxifen study indicated, the possible “off-target” effects of tamoxifen makes it difficult to draw solid conclusion on how this conditional TGFB2-deficiency could affect renal fibrogenesis.

Apart from PSCs, other non-fibroblast cells can also attribute to CP fibrotic response, several studies have reported that bone marrow-derived stem cells were able to contribute to the activated PSCs population in CP [94, 96]. Additionally, there is evidence of the existence of α-SMA positive renal interstitial cells that does not produce collagen I, these non-collagen-producing cells might modulate the fibrotic response through effects independent of direct collagen I production [293]. Not only these mesenchymal cells are involved in fibrogenesis, studies also confirmed a role of TGFB affecting epithelia cells [288, 290], endothelia cells [296] and inflammatory cells [297] in different fibrotic disease model. Moreover, modifying the TGFB signaling activity may have adverse consequences. There were studies showing that excessive TGFB signaling is deleterious [298] and compensatory signaling of other growth factors can be triggered as well [293]. Thus, modulating the TGFB signaling in fibroblasts alone might not be sufficient to affect the whole fibrogenesis process, and the interplay among different cell types and pathways contributes to the final fibrotic scenario.
5.2.3 The role of macrophages mediated by TGFB signaling

CP is initiated with acinar cell damage followed by inflammatory cell infiltration. Continuous fibroblasts activation and excessive ECM deposition and insufficient degradation are the subsequent responses to irreversible inflammation. Macrophages play critical roles in both maintenance and resolution of tissue damage by undergoing marked phenotypic and functional changes [121]. Known as an immunosuppressive cytokine, TGFB has been shown to suppress or alter the activation, maturation and differentiation of macrophages, dendritic cells and neutrophils [299]. Lack of TGFBR2 in macrophages resulted in a reduced anti-inflammatory M2 phenotype [273]. However, our results did not indicate a shift towards an M2 phenotype after Smad7 deletion in unpolarized macrophages. This might be due to that the native macrophages were quiescent unless stimulated/polarised, and the lack of Smad7 in the absence of other external factors may not be sufficient to induce distinct polarisation. Another study has shown that mixed macrophage phenotypes normally coexist under different physiological and pathological conditions [107], because the microenvironment during tissue repair was highly dynamic, and macrophages might exist at any point in the continuum of polarisation states [300]. For instance, phagocytosis of apoptotic damaged acinar cells induced a prominent M1/M2 mix-phenotype, which persisted throughout the whole regenerative process [301]. Furthermore, the current classification of M1/M2 macrophages is primarily based on activation stimuli in vitro, rather than the functions of the polarized cells [302]. The dynamic changes in macrophage functions are much broader than the dichotomy of M1/M2 macrophage subsets [2]. Therefore, these may account for the mixed expression level of M1/M2 markers in Smad7-lacking macrophages at the basal, non-polarized condition.

Upon M1/M2 polarization in vitro, our experiments also indicated that deletion of Smad7 contributed to a less pro-inflammatory M1 phenotype rather than a shift towards an M2 phenotype. This might be due to the suppressive effect of TGFB signalling on M1 polarisation. On the other hand, although Smad7 is considered as a major inhibitor for TGFB superfamily, it is also capable of affecting other pathways. For instance, it has been shown that Smad7 suppresses pro-inflammatory TNF-a signaling and NF-κB signaling [250, 303], it also functions as a scaffold protein to facilitate p38 MAPK pathways activation [304]. Thus, deletion of Smad7 in the myeloid lineage might have consequences outside canonical TGFB signalling.

Tissue injury triggers a complex series of cellular responses, and macrophages have been considered as the bridge between initial inflammation and later tissue regeneration and repair [305]. Our in vivo data indicated that Smad7 deletion in macrophages did not affect the collagen-based fibrotic index in experimental CP, although several fibrosis- and inflammation-related markers had lower expression levels, such as fibronectin, vimentin, F4/80 and MRC1. These results can be explained by the reduced M1 pro-inflammatory phenotype displayed by LysM-Cre;Smad7fl/fl M1 BMDMs, because M1-like macrophages at early phase might also contribute to ECM deposition by producing pro-fibrotic cytokines [119, 306]. A CCL4-induced liver fibrosis model confirmed the profibrotic role of IL-1 and
TNF, which normally produced by M1-like macrophages [306]. On the other hand, we observed that there were less M2-like macrophages in LysM-Cre;Smad7^{fl/fl} pancreata, which could lead to fewer PSCs and less fibronectin deposition because of less production of TGFβ and PDGFB, and whether the lower number of M2-like macrophages was due to the specific microenvironment created by initially less activated M1-like macrophages is unclear yet.

In addition, the ex vivo IL-10/TGFβ polarised M2 macrophages did not induce profibrotic responses [187], and depletion of Arg1 from macrophages did not affect lung fibrosis either [307]. Macrophage-specific TGFBR1 deletion did not prevent renal fibrosis [308], while macrophage-specific TGFBR2 deletion protected against the development of tubulointerstitial fibrosis by decreasing macrophages infiltration rather than altering macrophage phenotype [297]. These results suggest that the role of M1/M2 macrophages reflects dynamic properties rather than being limited to either pro- or anti-fibrotic phenotype. Modulating different part of TGFβ signaling can lead to diverse responses, indicating a possible crosstalk with other pathways. Aside from TGFβ, there are variety of potential factors produced by macrophages and these growth factors and cytokines can affect the macrophages phenotype in turn. Therefore, the final fibrotic response might attribute from interplay of multiple molecules and signaling instead of TGFβ alone.

Of note, LysM is highly expressed in mature tissue-resident macrophages but is expressed at lower level in recruited monocytes [121], and not all macrophages originate from the myeloid lineage [309]. Therefore our LysM-Cre;Smad7^{fl/fl} model might not fully reflect the role of Smad7 in all macrophages and distinguish the specific role of tissue-resident macrophages and monocytes-derived macrophages. Moreover, normal pancreas contains two macrophage subsets in the stromal compartment, which are derived from primitive hematopoiesis and definitive hematopoiesis, respectively. Both subsets, located in distinct intrapancreatic microenvironments, exhibit M2 profiles, suggesting the macrophage phenotype is educated/directed by the local milieu [310, 311]. Thus the LysM-Cre;Smad7^{fl/fl} macrophage phenotypical profile observed in our study might be influenced by both the physiological local milieu and Smad7-deletion.

5.3 THE EFFECT OF TAMOXIFEN DURING FIBROGENESIS

The effects of tamoxifen in fibrotic diseases have been reported, it can ameliorate renal fibrosis [312, 313] and peritoneal fibrosis [314, 315]. A sex-dependent effect of tamoxifen was also described in obstructive nephropathy-induced fibrosis model. Tamoxifen reduced fibrosis, accompanied by a reduction in nuclear Esr1 positivity in female mice in contrast to male mice [316]. Our data indicate an opposite tamoxifen effect on cerulein-induced fibrogenesis in pancreas with a higher fibrotic index and amounts of collagen I in female pancreata and reduced fibrotic index/amounts of collagen I in male pancreata compared to vehicle plus cerulein-treated mice of the respective sex. The mechanism of tamoxifen modulating fibrogenesis is constituted from different aspects. First, tamoxifen, as a SERMs
can regulate ECM through ERs signalings. For instance, collagen content was significantly increased in the skin of Esr1\(^{-/-}\) female mice but decreased in Esr2\(^{-/-}\) female mice [317]. Specific activation of Esr1 and Esr2 prevented cardiac fibrosis development, while Esr2-deletion increased cardiac fibrogenesis [318-320]. In our in vitro experiments, the Esr1 mRNA level was significantly downregulated after 24h tamoxifen plus cerulein treatment in female PSCs, accompanied by increased collagen I deposition. In male PSCs the Esr1 mRNA level was not affected and the collagen I expression was greatly decreased. Together, these observations indicate that the effect of tamoxifen through ERs signaling on the fibrogenic process is not alike in all cell types, and the nature and extent of that response is determined by the combination of different transcription factors and co-activators/co-repressors with the ERs on a given promoter [321].

In addition, tamoxifen can regulate ECM synthesis and degradation via TGFB [313, 315, 322, 323] or ERK1/2 pathway [324, 325]. However, the influence of tamoxifen on TGFB signaling is still under debate, as opposing effects have been observed in different species and cell types [324, 326]. Tamoxifen/ER\(\alpha\) can either inhibit TGFB signaling [312] or enhance it [327], while other studies demonstrated that it did at least not interfere with the canonical Smad signaling [328]. In our in vitro experiments, it seems that tamoxifen modulates the activity of classical TGFB response genes/promoters in opposite directions depending on the sex of the PSCs, and similar results were reported in other studies as well [315, 329]. One possible explanation might be that tamoxifen enhances the TGFB induced expression of the inhibitory Smad7 to exert suppression of certain TGFB response genes [315], or higher levels of MMPs are generated, tipping the balance towards ECM degradation [330].

Moreover, tamoxifen plays a role in regulating fibroblasts and inflammatory cells. Tamoxifen has been demonstrated to inhibit the activation and proliferation of fibroblasts in vivo and in vitro [313, 316, 331]. With respect to immune cells, tamoxifen can result in reduced macrophage infiltration and proinflammatory cytokine expression in male mice [312]. On the other hand, increased recruitment of Kupffer cells and leucocytes to damaged regions of tamoxifen pretreated livers of male mice was observed [263]. Pro-inflammatory cytokine production was found to be either enhanced or dampened by ER\(\alpha\) activity [332-334]. The change in the number of PSCs and macrophages in our in vivo study seems to support the sex-specific collagen I accumulation/fibrotic index following tamoxifen pretreatment.

The oscillating results from our in vivo experiments might be related to tamoxifen tissue-specific pharmacokinetics, as well as its effect on ER\(\alpha\) protein stability [335]. Tamoxifen plasma levels have been reported to be undetectable 2 and 4 weeks after the treatment in mice [316]. Another study reported that there was residual tamoxifen in the pancreatic islets up to 4 weeks after subcutaneous injections of tamoxifen [336]. These different kinetics might relate to the distribution and metabolism of the lipophilic substance tamoxifen in different tissues. Specifically, adipose tissue contained high concentration of the parent tamoxifen molecule and low concentrations of metabolites thereof because of the low activity of metabolizing enzymes in fat tissue [337]. Thus, adipose tissue might represent a preserving
peripheral compartment, which could interfere with tamoxifen plasma/tissue levels during and after tamoxifen administration.
6 CONCLUSIONS

- General/ubiquitous Smad7 deficiency induce more severe damage in CP with evidence of stronger accumulation of ECM, increased levels of inflammatory cells and an elevated number of mesenchymal cells/myofibroblasts. Thus, Smad7 has a protective effect in CP development.

- Fibroblasts-specific Smad7 deficiency does not exacerbate CP indicated by comparable ECM deposition and the number of fibroblasts and inflammatory cells. Therefore, targeting fibroblasts-derived TGFB signaling by Smad7 deletion is not sufficient to increase CP associated fibrosis in our model.

- Myeloid cell-specific Smad7 deficiency attenuates CP suggested by reduced number of macrophages and PSCs in CP, translating into a reduced fibronectin deposition. Thereby, targeting macrophage-derived TGFB signaling by Smad7 deletion provides an unexpected mild protective effect for CP development in our model.

- Tamoxifen administration has unneglectable sex-specific and time-dependent side effects, which affecting the experimental outcome in a cerulein-based CP model in mice. A 2-week waiting period before cerulein administration is suggested to reduce side effects to a minimum for the described fibrosis model in female mice.
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