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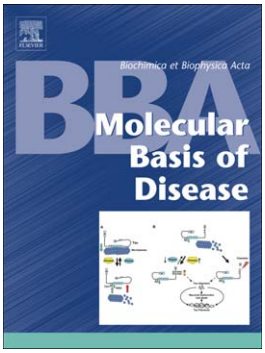


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# TGF-β and fibrosis in different organs – molecular pathway imprints

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## ABSTRACT

The action of transforming-growth-factor (TGF)-β following inflammatory responses is characterized by increased production of extracellular matrix (ECM) components, as well as mesenchymal cell proliferation, migration, and accumulation. Thus, TGF-β is important for the induction of fibrosis often associated with chronic phases of inflammatory diseases. This common feature of TGF-related pathologies is observed in many different organs. Therefore, in addition to the description of the common TGF-β-pathway, this review focuses on TGF-β-related pathogenetic effects in different pathologies/organs, i. e., arthritis, diabetic nephropathy, colitis/Crohn's disease, radiation-induced fibrosis, and myocarditis (including their similarities and dissimilarities). However, TGF-β exhibits both exacerbating and ameliorating features, depending on the phase of disease and the site of action. Due to its central role in severe fibrotic diseases, TGF-β nevertheless remains an attractive therapeutic target, if targeted locally and during the fibrotic phase of disease.

## 1. INTRODUCTION

The fibrotic reaction of the connective tissue following an inflammatory response is mainly characterized by an increased production of extracellular matrix (ECM) components and mesenchymal cell proliferation, migration and accumulation. Despite the existence of numerous distinct causes of chronic inflammatory diseases in different organs and tissues, these diseases are generally characterized by: i) severe and intermittent progression with phases of acute exacerbation and remission; ii) immigration of inflammatory cells (macrophages, granulocytes and T-cells); and iii) increased expression of pro-inflammatory mediators (Figure 1). These processes result in the proliferation of local fibroblasts and, by interaction with epithelial cells, in their differentiation into myofibroblasts and can be regarded as a misguided wound healing. Finally, the inflammatory process comes to rest, but the massive fibrosis prevents a rebuilding of functionally intact tissue and organs.

Transforming growth factor (TGF)-β is an ubiquitously expressed cytokine belonging to a large superfamily of activins/bone morphogenetic proteins [1]. This mediator plays an active role in the processes discussed above, such as proliferation, wound healing [2], and synthesis of ECM molecules [3]. TGF-β, therefore, strongly contributes to fibrotic disorders such as diabetic nephropathy, Crohn´s disease, rheumatoid arthritis, radiation-induced fibrosis, and myocarditis. However, TGF-β is clearly a bi- (or multi-) functional molecule with strong effects on the immune system [4,5].

## 2. TGF-β SIGNALING PATHWAY

TGF-β is synthesized as one part of a large molecule, the pro-TGF-β containing the latency-associated proteins (LAP; Figure 2). The latter is cleaved from TGF-β in the Golgi apparatus, but remains non-covalently associated with the growth factor. The disulfide-bound latent-

TGF-β-binding proteins 1/2 (LTBP 1/2) connect the whole complex to the ECM (details described in [6]). Release of TGF-β from the pro-TGF-β complex can be achieved through proteolytic activity by plasmin [7] or matrix-metalloproteinase (MMP)-2 and MMP-9 [8], through integrins [9,10], recently reviewed in [11], treatment with mild acids [12], or through the action of thrombospondin (THBS; [13]) by disrupting the non-covalent interactions between LAP and TGF-β1. Once released, TGF-β mediates signals through pairs of type I and type II receptors [14]. The type III receptor (betaglycan) acts - probably in conjunction with other heparan sulfate glycans like syndecan [15]- as a co-receptor for binding/presenting TGF and a regulator of TGF-β signaling [16]. The result of ligand binding is the activation of the type II receptor (TGFBR2), which then phosphorylates the type I receptor (TGFBR1). The active receptor complex then phosphorylates the so-called R (receptor)-Smad-2 or Smad-3 that propagates the signal [17]. The phosphorylation of Smad2/3 decreases the affinity for the Smad-anchor for receptor activation (SARA), which in non-stimulated cells mediates the retention of Smad2/3 in the cytoplasma by interaction, and increases the affinity of Smad2/3 for Smad-4 (a so-called co-Smad). This complex is now able to enter the nucleus, to bind transcriptional co-activators like p300 and Creb-binding-protein (CBP) or repressors like SkiL or TGIF [18], and to regulate the transcriptional activity of various genes.

Mitogen-activated protein kinases (MAPKs) and protein kinase C can also interfere with either the nuclear translocation or binding of Smad3/4 complexes to DNA and regulate TGF-β1 signalling [19,20]. Moreover, the serine-threonine protein kinase B can directly interact with Smad3, thereby preventing its phosphorylation and nuclear translocation [21]. Other pathways can be directly activated by TGF-β1. These include components of the MAPK pathway, such as ras, raf, ERK, p38 and JNK, the phosphatidylinositol-3 kinase cascade, as well as the regulators of cadherin junctions, RhoA and Rac ([19,22,23]; Fig. 1). Recently, an involvement of the focal adhesion kinase (FAK) was established in

myofibroblast differentiation and in remodeling of the connective tissue following stimulation with TGF [24,25]. In line with these results, TGF-induced FAK-signaling is required for the activation of TAK [26] or MEKK1 and, subsequently, of JNK [27], all factors shown to be essential for the transcription of pro-fibrotic genes.

Notably, that signal transduction pathways have their own intracellular regulators. An inhibitory Smad (Smad7) blocks TGF-β1 signaling by physical interaction with the activated TGFBR1 receptor and prevents the docking and phosphorylation of Smad2/3 [28,29].

This complex TGF-β signaling pathway (Figure 2) contains numerous ligands, receptors, and signaling molecules which, as potential targets of dysregulation via increased or decreased expression, activation or interaction, may be partially involved in fibrotic reactions in the diseases discussed below.

## 3. TGF-β-RELATED MOLECULES IN RHEUMATOID ARTHRITIS

The importance of TGF-β1 in rheumatoid arthritis (RA) ranges from an association with certain vascularization patterns in the synovial membrane (SM) [30], and an association of TGF-β polymorphisms with the radiological signs of joint destruction [31] to an induction of pro-inflammatory cytokines, MMP [32], aggreccanase [33] and urokinase-type plasminogen activator [34]. In addition, TGF-β plays an important role for the function of regulatory T-cells [5] in the suppression of autoimmunity. Indeed, increased levels of TGF-β1 have been found in the synovial membrane of patients with RA by northern blot [35], immunohistochemistry [36,37], western blot [38] and in synovial effusions [39]. Also TGFBR2 was detected at higher levels than in normal synovial tissue [35].

Using “pathway-directed” software following genome-wide comparison between synovial fibroblasts (SFB) from patients with RA and osteoarthritis (OA) with Affymetrix

arrays, gene expression of TGF-β1, TGF-β3, LTBP1/2, THBS1, TGFBR1, SARA, CBP, SkiL – belonging to the TGF-β-pathway (Figure 2) - was elevated in RA [40]. After validating array data at the mRNA and protein levels using quantitative PCR and western blot/immunohistochemistry, we confirmed an upregulated TGF-β pathway in RA-SFB. The presence of TGF-β1, in conjunction with increased amounts of TGF-β-releasing THBS1 and a higher expression of TGFBR1, may thus lead to an amplified response of RA-SFB to TGF-β. This RA-specific response has been confirmed by increased expression of MMP-11 following TGF-β stimulation, implying a pathogenetic relevance of the TGF-β-pathway for MMP-induced degradation or remodeling processes in RA [40].

The importance of TGF-β for the pathogenesis of arthritis is emphasized by a number of animal models. The abundant expression of TGF-β1, 2, and 3 as well as the TGFBR1 and -2 in rat synovium was increased after onset of the collagen-induced arthritis (CIA) [41], and direct intra-articular injection of TGF-β1 or TGF-β2 induced synovial erythema, swelling, and cellular infiltration resulting in synovial inflammation and hyperplasia [42]. Conversely, neutralization of TGF-β inhibited acute and chronic arthritis induced by streptococcal cell wall (SCW) [43]. In line with these observations, an adenovirus-mediated overexpression of TGF-β1 in rabbit knees led to an increased glycosaminoglycan release, nitric oxide production and, most notably, to fibrosis and muscle edema [44]. The prevention of CIA by administration of a TGFBR1 inhibitor (HTS466284), which concomitantly reduced the expression of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF)-AA, TNF-α, and cellular proliferation [45], further underlines the pathogenetic role of TGF-β in arthritis.

In contrast to the above-mentioned studies, numerous observations show beneficial effects of TGF-β in arthritis. If administered systemically, TGF-β1 suppressed SCW-induced

arthritis, as measured by cellular infiltration and joint erosion [46]. In addition, investigation of the cytokine expression during CIA demonstrated a strong upregulation of TGF-β1/2 in the remission state of disease, possibly refelecting the anti-inflammatory regulation of T-cells by TGF-β in arthritis. If TGF-β signaling was inhibited by expression of dominant-negative TGFBR2 in T-cells [47] the susceptibility and the clinical severity of CIA was strongly increased. Likewise, if TGF-β1 was retrovirally overexpressed in arthritogenic splenocytes, CIA could not be transferred to SCID mice and established disease was ameliorated [48]. Therefore, it may be important to inhibit TGF-β only at the site of inflammation without targeting regulatory lymphocytes at extra-articular sites.

## 4. TGF-β-RELATED MOLECULES IN DIABETIC NEPHROPATHY

TGF-β and its signal transduction play a major role in diabetic nephropathy and have therefore been thoroughly studied [49-53]. Among the features of the diabetic milieu, hyperglycemia, increased non-enzymatic glycation of proteins, *de novo* synthesis of diacylglycerol and subsequent activation of protein kinase C, increased intracellular glucosamine production, and enhanced renal production of vasoactive agents (angiotensin II, endothelins, thromboxane) have all been shown to increase the expression of TGF-β in cultured renal cells and animal models of diabetic nephropathy [50,52,54].

The TGF-β level is elevated in the kidneys of insulin-dependent diabetic animals during both early and late stages of disease [53,54]. Treatment of the streptozotocin (STZ)-diabetic rat with sufficient insulin to reduce hyperglycemia suppressed the enhanced expression of TGF-β and matrix components in the glomeruli. In the STZ-diabetic rat and mouse, increased TGF-β1 expression in the renal cortex and glomeruli as well as up-regulation of the TGFBR2 mRNA and protein was noted early after the onset of diabetes

[53].. The *db/db* mouse, a model of type 2 diabetes, characterized by hyperglycemia, obesity, and insulin resistance, develops increased amounts of TGF-β1 localized in the glomerular compartments [55]. In contrast, the mRNA and protein levels of the TGFBR2 are significantly up-regulated in both the glomerular and the tubulointerstitial compartments [55].

The development of diabetic renal hypertrophy and glomerulosclerosis is likely caused by heightened activity of the TGF-β system [56-63]. Short-term treatment of the STZ-diabetic mouse with a neutralizing monoclonal antibody against all three isoforms of TGF-β prevented glomerular hypertrophy, reduced the increment in kidney weight by 50%, and significantly attenuated the increase in TGF-β1, α1(IV) collagen, and fibronectin mRNAs without affecting glycemic control [56]. The results of this study suggested a cause-and-effect relationship between the renal TGF-β system and the development of early structural changes in diabetic nephropathy. Systemic anti-TGF-β therapy for eight weeks prevented the mesangial matrix expansion of diabetic glomerulosclerosis and, most importantly, preserved kidney function, showing for the first time that neutralization of TGF-β activity prevents the progression of renal failure in diabetes [57]. However, the anti-TGF-β antibody did not reduce albuminuria, which itself may promote the progression of renal insufficiency [57]. The paradox of preserved renal function in view of persistent albuminuria may be explained by postulating that the deleterious effects of proteinuria are themselves mediated by the TGF-β system [63-65].

In mouse mesangial and tubular cells, high glucose stimulates the transcription of fibronectin and, in addition, potentiates the transcriptional activation of fibronectin by TGF-β1 [58,59]. This particular effect of TGF-β1 appears to be mediated by Smad3, because over-expression of Smad3 alone was able to induce fibronectin promoter activity [66]. In conjunction with exogenous TGF-β1, Smad3 over-expression synergistically increased

fibronectin expression, as if the extra Smad3 had increased the efficiency of TGF-β signaling [66]. Finally, transfection of a Smad3-dominant-negative construct inhibited TGF-β1 stimulated fibronectin promoter activity [67,68]. However, part of the TGF-β1-induced fibronectin expression may be mediated in parallel by the p38MAPK pathway [68,69]. Finally, there is evidence that Smad3 is a central mediator in the TGF-β1-induced increase of mRNA expression for α1(I) collagen [68,70]. TGF-β-induced MAPK activation also leads to N-terminal phosphorylation of p53 that enables its interaction with TGF-β-activated Smads [64], an indication for cross-talk between the various TGF-β signaling pathways.

TGF-β1 may have additional effects besides the stimulation of extracellular matrix production. In podocytes, TGF-β1 induces apoptosis through Smad7 by inhibiting nuclear translocation of the cell survival factor NF-κB [70]. TGF-β1-mediated activation of Smad7 is specific for podocytes and is not found in mesangial cells in a limited series of biopsies from patients with diabetic nephropathy [64]. Since podocytes are terminally differentiated cells unable to undergo cell division due to the up-regulation of the cell cycle inhibitory proteins p57 and $p27^{Kip1}$, apoptotic loss of cells was not replaced.

Studies performed in diabetic patients with various degrees of nephropathy also underline the importance of the renal TGF-β system in disease development [71-73]. All three isoforms of TGF-β are elevated in both the glomerular and the tubulointerstitial compartments of patients with established diabetic nephropathy [71,72]. Furthermore, glomerular TGF-β1 mRNA is markedly increased in biopsy specimens from patients with proven diabetic kidney disease. These investigations suggests that increased renal TGF-β levels closely correlate with the degree of mesangial matrix expansion, interstitial fibrosis, and renal insufficiency.

Another study, designed to assess renal production of TGF-β [73] measured aortic, renal vein, and urinary levels of TGF-β in 14 type 2 diabetic and 11 non-diabetic control patients undergoing elective coronary artery catheterization [73]. Both groups were roughly matched with regard to the range of renal function and the presence of hypertension and proteinuria. Renal blood flow was measured to calculate the net mass balance across the kidney. The gradient of TGF-β1 concentration across the renal vascular bed was negative in the non-diabetic patients indicating net renal extraction of TGF-β1, whereas the gradient was positive in the diabetic patients indicating net renal production of TGF-β1. When the renal TGF-β1 mass balance was calculated, a similar pattern was observed with the non-diabetic kidney removing approximately 3500 ng/min of TGF-β1 from the circulation, and the diabetic kidney adding approximately 1000 ng/min of TGF-β1 [73]. In addition, the level of bioassayable TGF-β was increased four-fold in the urine of diabetic *versus* non-diabetic patients. This was not simply a function of enhanced glomerular permeability to protein since diabetic patients both with and without microalbuminuria displayed similarly high rates of urinary TGF-β excretion [73]. These results demonstrated that the kidneys of diabetic patients overproduce TGF-β1 protein; further details, e. g., the exact contribution of the different renal cell types, need to be investigated.

An interesting *post-hoc* study assessed whether treatment with the angiotensin converting enzyme inhibitor captopril would lower serum TGF-β1 levels in a small subset of patients with diabetic nephropathy who had been enrolled in the Collaborative Study Group [74,75]. After six months, the serum TGF-β1 level decreased significantly by 21% in the captopril-treated group, whereas it increased slightly by 11% in the placebo-treated group [75]. Interestingly, the captopril-treated patients with decreased serum TGF-β1 levels tended to have better preserved renal function over the ensuing two-year period [75]. This association

was even more pronounced in the subset of patients with an initial glomerular filtration rate of less than 75 ml/min. These results suggest that TGF-β1 plays a pivotal role in the progression of diabetic nephropathy and that angiotensin converting enzyme inhibitor therapy may protect the kidney by lowering TGF-β1 production.

## 5. TGF-β-RELATED MOLECULES IN INTESTINAL INFLAMMATION – A DOUBLE-SIDED SWORD

TGF-β is constitutively expressed by epithelial cells, fibroblasts, and mononuclear cells in the gastrointestinal tract [76]. Its critical role in intestinal homeostasis as a negative master regulator of inflammation is well-established [77,78] and indisputable. However, translation of elegant mouse experiments into therapeutic interventions in humans requires a clearer understanding of TGF-β activity in the human gut. Mice with global TGF-β defects, such as TGF-β–null mice or transgenic mice expressing a dominant negative TGFBR2 chain, are unresponsive to TGF-β1 signaling. The former die soon after birth due to systemic inflammation, and the latter develop severe colonic and pulmonary inflammation [79,80]. These manifestations of global TGF-β1 defects are mirrored in mouse models of colitis in that the secretion of TGF-β1 is consistently associated with either the protection from colitis or a greatly diminished severity of colitis. This is seen both in the $T_H1$ model of colitis induced by the haptenating reagent trinitrobenzene sulfonic acid (TNBS), which mimics Crohn's disease (CD), or the $T_H2$ model of colitis induced by the haptenating agent oxazolone, which mimics ulcerative colitis (UC) [81,82]. In addition, it is seen in the colitis of SCID or RAG2-deficient mice receiving $CD45RB^{high}$ (naive) T cells in which the protective effect of the cotransfer of $CD45RB^{low}$ T (memory) cells is abolished by concomitant administration of a neutralizing TGF-β1 antibody [83]. These and other studies quite conclusively establish that TGF-β1 plays

an essential, regulatory role in the control of colitis. On the other hand, in mice TGF-β1 in combination with IL-6 induces a strong pro-inflammatory $T_H17$ cell differentiation [84-86]. Notably, in humans TGF-β1 does not have a direct effect on the development of human $T_H17$ cells, but it can indirectly favour the development of these cells by suppressing the expression of T-bet and selectively inhibiting the expansion of IFN-γ-producing T cells [87-89].

The important role of Smad3 as an essential mediator of TGF-β1-induced anti-inflammatory and suppressive activities at the mucosal level emerges from studies in mice with targeted deletion of the Smad3 gene. The animals are viable, but die from defects in mucosal immunity at 1–6 months of age. Mutant mice show diminished cell responsiveness to TGF-β1, massive infiltration of T cells, and multiple pyogenic abscesses in the stomach and intestine [87,90]. When Smad signaling was studied in normal human gut mucosa, whole biopsies or isolated lamina propria mononuclear cells, a basal level of phosphorylated (phospho-) Smad3 was observed which was rapidly upregulated by the addition of exogenous TGF-β1 [91].

TGF-β has been also implicated as a key inducer of epithelial-mesenchymal transition (EMT) [92-97]. Amongst others, EMT is an essential component of tissue remodeling and wound repair (reviewed in [98,99]) and fibrosis (reviewed in [100]). During this transition, the epithelial phenotype, characterized by strong cell-cell junctions and polarity, is replaced by a mesenchymal phenotype, with reduced cell-cell interactions, a fibroblastic morphology and increased motility. TGF- β stimulates the proliferation of many cell types, particularly those of mesenchymal origin, and it is also a potent inhibitor of epithelial cell proliferation. EMT in response to TGF-β1 and in fibrosis is mediated predominantly via Smad-dependent (mainly Smad3) pathways [101,102]. A loss of Smad3 in mice blocked both morphological

changes of lens epithelium to a mesenchymal phenotype and expression of EMT markers in response to injury *in vivo* or to exposure to exogenous TGF-β in organ culture [101].

CD is a chronic, progressive disease of the gastrointestinal tract with an unknown etiology. It is characterized by transmural inflammation of all layers of the bowel wall. The formation of stenoses and strictures is common in this disease, which causes abdominal pain, anorexia, and weight loss. Approximately 50% of CD patients undergo surgery for this type of complication during a 10-year course; however, the recurrence rate after surgery is high. In contrast, UC rarely causes intestinal stenosis. Cytokines released from inflammatory cells have long been implicated in the pathogenesis of intestinal fibrosis. TGF-β/Smad signaling plays an important role in CD [103-105]. The transmural infiltrate of CD is responsible for initiating and maintaining a series of connective tissue changes not only involving the mucosa, but also the submucosa and *muscularis mucosae* and *muscularis propria*, where a marked increase of collagen type I, III, and V mRNA is observed [106,107]. TGF-β has been identified as one of the central growth factors/cytokines that specifically induces a fibrotic response after inflammatory injury in the intestinal tract. In CD, there is a marked overexpression of TGF-β1 and TGFBRs in the colonic mucosa [76,108]. Fibrosis in CD can therefore be interpreted as an aberrant healing response to mucosal injury [109]. In addition, TGF-β appears to be involved in intestinal fibrosis in other enteropathies, such as radiation enteritis, collageneous colitis, and intestinal graft-versus host disease [110-112]. Both TGF-β and its receptors are overexpressed in the intestine of patients with CD [113]. Intestinal fibroblast expression of TGF-β isoforms varies according to the nature of tissue. Fibroblasts from normal and inflamed mucosa both express the TGF-β1 and TGF-β3 isoforms, while those from fibrotic tissue show reduced expression of TGF-β3, but enhanced expression of TGF-β2 and TGF-β1 [114]. This is remarkable, since the TGF-β1 and TGF-β2 isoforms have

been specifically implicated in pathogenic fibrosis, while TGF-β3 appears to have antifibrotic properties [115]. Monteleone and colleagues have provided insight into the failure of TGF-β down-regulation in CD. Despite the abundant expression of TGF-β in the mucosa of patients with CD, phospho-Smad3 is diminished in the mucosa compared to control mucosal samples, as is the complex of Smad3 with Smad4. This may be due to the induction and overexpression of Smad7 in the mucosa of patients with CD and UCs [116]. However, upregulation of Smad7 is not specific for inflammatory bowel disease (IBD), but also occurs in *Helicobacter pylori*-induced gastritis [117]. In addition, mucosal T cells in both whole tissue and isolated cells show defective TGF-β1 signaling as measured by reduced immuno-reactivity against phospho-Smad3 [117]. Specific anti-sense oligonucleotides for Smad7 reduce expression of Smad7 in cells isolated from IBD patients which then become responsive to exogenous TGF-β1. TGF-β1 cannot inhibit pro-inflammatory cytokine production in isolated lamina propria mononuclear cells from CD patients, but inhibition of Smad7 with anti-sense oligonucleotides restores TGF-β1 signaling and allows TGF-β1 to inhibit cytokine production. In inflamed mucosal tissue explants from CD patients, inhibition of Smad7 also restores phospho-Smad3 and decreases pro-inflammatory cytokine production, an effect which is partially blocked by anti-TGF-β1. The extension of these studies examined the interactions between Smad signaling and NF-κB activation in inflamed gut: While TGF-β1 is a potent inhibitor of TNF-α-induced NF-κB activation in normal gut, it has no activity in inflamed gut. This can be attributed to over-expression of Smad7, since treatment of cells from inflamed gut with anti-sense to Smad7 allows TGF-β1 to rapidly down-regulate NF-κB activation [117].

If it becomes possible to specifically inhibit Smad7, endogenous TGF-β1 in the inflamed gut may negatively regulate pro-inflammatory cytokine production and NF-κB activation, the major components of the immune overactivity which drives tissue injury in

IBD. At the same time, however, it is important to discover the factors which control Smad7 expression in the inflamed gut. Furthermore, cell-specific expression of Smad7 will be important because TGF-β1 has different effects on different cell types. Thus, while blocking Smad7 will allow TGF-β1 to reduce pro-inflammatory cytokine production by T cells and macrophages, it may allow TGF-β1 to increase collagen production in myofibroblasts, resulting in fibrosis. However, at the moment the relative importance of the known inducers of Smad7 in the gut, such as IFN-γ or TNF-α, or even whether TGF-β1 itself induces Smad7 in a negative regulatory loop, is still unclear.

## 6. TGF-β-RELATED MOLECULES IN RADIATION-INDUCED FIBROSIS

TGF-β1 levels are increased in irradiated mouse skin [118,119] and decrease slowly after irradiation in both pig and human skin [120,121]. Following microvascular hard or soft tissue transfer, TGF-β1 is again upregulated in a biphasic manner. The first expression peak on day 3 post operation is due to enhanced activation of latent TGF-β1 by extracellular enzymes while the second between day 14 and 28 after surgery is a result of *de novo* synthesis [122]. Its most important signaling receptor TGFBR2 is upregulated in irradiated graft beds as well [123]. Signaling leads to increased nucleoplasmatic shuttling of active Smad2/3 and induction of TGF-β1 target genes in fibrotic healing, which is mainly due to decrease in cytoplasmatic levels of the inhibitory Smad7 [124].

As a consequence, the extracellular matrix is qualitatively and quantitatively altered [125]. Prolyl-hydroxyprolinase-β overexpression [126] promotes synthesis of collagen I, III and IV [124], while repression of degrading enzymes, such as MMP-1 and induction of tissue inhibitors [127] suppresses the degrading pathways. Moreover, integrin surface receptors, such as α2β1 integrin are up-regulated as well and modulate transmission of tensile forces

[128]. In the presence of such forces fibroblasts differentiate into myofibroblasts resulting in constrictive fibrosis [129].

Irradiation-induced fibrosis and damage to the microvasculature lead to wound healing disorders following surgery in previously irradiated areas. Such disorders are dependent, in part, on the radiation dosage and the timing of surgery after irradiation [130,131] and reduce the success rate of free flaps to 90% compared with 94% in non-irradiated graft beds [132].

Taking the central role of TGF-β1 in radiation-impaired wound healing into account, the question of whether TGF-β1-levels differ in the healthy tissue between different patients is of utmost importance. It has been demonstrated that patients with an increased TGF-β1 plasma level exhibit an increased risk of developing skin fibrosis following irradiation [133]. To find a particular predictor which sufficiently corresponds with the frequency to develop wound healing complications following surgery in previously irradiated graft beds would give a large clinical impact on planning individual treatment protocols. The availability of reliable markers may eventually allow the prediction of outcome prior to commencement of treatment, and thus allow modification of combined protocols to minimize late adverse effects without compromising tumor control.

## 7. TGF-β-RELATED MOLECULES IN MYOCARDITIS

Heart fibrosis is a hallmark feature of the chronic stage of viral myocarditis [134,135]. Human pathogenic coxsackievirus B3 (CVB3) is considered the most frequent viral cause of chronic myocarditis in men [136]. Clinical manifestations of acute myocarditis vary from flu-like symptoms to the fulminant fatal forms. Frequently, acute myocarditis, with distinct onset, follows a monophasic clinical course, and the majority of patients recover spontaneously after several days of congestive heart failure. Some patients progress into subacute or chronic

forms, which ultimately lead to death. The molecular mechanisms underlying fibrosis development in chronic myocarditis are currently not well understood.

Like humans, mice develop a marked age-related susceptibility to CVB infections [137]. The myocardial lesions in mice closely resemble those seen in human disease [138]; therefore, experimental murine models of coxsackievirus-induced myocarditis have been developed to investigate the pathogenesis of this disease. Although chronic inflammation is characteristic for the human disease as well as for our mouse model, fibrosis is more prevalent in the latter.

Excessive fibrosis, as it occurs under conditions of chronic myocarditis, can be classified as either replacement fibrosis, when functional tissue is replaced by connective tissue, or as reactive fibrosis, which is part of an adaptive process [139]. In addition to collagen, tenascin C, and fibronectin, splice variants of these molecules are often part of the fibrotic tissue [140,141]. Various cytokines and growth factors are believed to contribute to the induction of fibrosis, including TGF-β [142], IL-1, [143], TNF-α [144,145], and PDGFs [146,147]. For example, persistent expression of cytokines in the chronic stage of CVB3-induced myocarditis has been described for various mouse models [148-150], as well as for human dilatative cardiomyopathy [151]. New observations suggest that sustained pro-inflammatory signaling is associated with a pro-fibrotic phenotype based on TGF-β–mediated signaling [152].

For fibrogenesis in the heart, members of the PDGF family are apparently important mediators. Transgenic overexpression of PDGF-C and PDGF-D, two more recently discovered PDGF isoforms [153], in the heart leads to massive cardiac fibrosis [146,147]. Therefore, the importance of PDGF for the pathology of chronic myocarditis was investigated in mice with CVB3-induced myocarditis. Interestingly, all analysed isoforms of PDGF-A, -B, and -C were upregulated in close correlation with the inflammatory process. High levels of

399 the growth factors persisted only in MHC class II knockout mice, which develop a chronic
400 myocarditis upon CVB3 infection, whereas immunocompetent C57BL/6 wild-type mice
401 exhibit only an acute, completely reversible myocarditis [154,155].

402 Furthermore, it has been shown that the PDGF-receptor (PDGFR) blocker Imatinib
403 inhibits activation of resident PDGFR, and attenuates fibrosis in this mouse model
404 significantly [156]. These data strongly suggest that elevation of PDGF levels and subsequent
405 activation of PDGFR causally contribute to the type of cardiac fibrosis which occurs in this
406 model. Efficacy of Imatinib for attenuation of fibrosis has recently been reported also for
407 other organs, i.e., liver, joints, and kidney [157-159]. It can therefore be assumed that
408 Imatinib-sensitive tyrosine kinases play a more general role in fibrogenesis. In addition to the
409 PDGFR [157,160], the Abelson tyrosine kinase, a mediator of TGF-β-signaling (c-Abl) [161],
410 has also been proposed as a relevant target for Imatinib in the inhibition of fibrogenesis
411 [159,162]. While our data [156], and data of others [146,147] strongly suggest a causal
412 involvement of the PDGFR in fibrogenesis, they do not exclude that c-Abl activity is also
413 involved in fibrogenetic signalling. It could function downstream of TGFBRs, but also
414 partially mediate signalling of the activated PDGFR [163]. It is known that TGF-β can drive
415 cardiac fibrosis when overexpressed in the mouse heart [164] and the use of genetic mouse
416 models to understand the role of TGF-β signalling in the heart is reviewed in [165]. But the
417 relative contribution of the different TGF-β-mediated signalling mechanisms to fibrosis in our
418 model of CVB3-induced chronic myocarditis remains to be fully elucidated.
419

420 ## 8. DIFFERENCES AND SIMILARITIES OF TGF-β IN DIFFERENT ORGANS

421 As presented in Table 1, numerous molecules have similar expression patterns in the diseases
422 mentioned in this review. Due to limited information from the human system, some data were
423 replaced by results derived from the respective animal model (fibrosis following irradiation).

TGF-β1 is elevated in most pathologies in its active or latent form and shows similarities with the isoform TGF-β2 in most cases. In contrast, the expression of TGF-β3 varies from down-regulation during wound healing and CD, abundance without regulation in rheumatoid arthritis and UC, to up-regulation during diabetic nephropathy. If determined, the expression of TGFBR2 is often enhanced during disease. The widespread appearance of the term "n.d." points out the urgent need for further investigation of the expression and the effects of TGF-β-related molecules in human diseases associated with fibrosis, particularly at the stage of signaling and transcriptional regulation.

## 9. THERAPEUTIC STRATEGIES

As either increased or decreased activity of the TGF-β pathway has been implicated in the pathogenesis of different human diseases, methods for increasing or decreasing signaling through these pathways are required [166]. There are some tools for enhancing TGF-β signals, e. g., direct administration of the ligand, usage of agonists, and increased expression of receptors or decreased expression of signaling antagonists.

On the other hand, there are different ways to inhibit TGF-β signaling, e. g., administration of neutralizing antibodies, application of soluble receptors, usage of antisense nucleotides, and chemically synthesized inhibitors of the receptor serine/threonine kinases.

Regarding TGF-β inhibition, some clinical trials have been performed with neutralizing antibodies, especially in fibrotic diseases, including a TGF-β2 neutralizing antibody (lerdelimumab) which effectively decreased the amount of scarring after glaucoma surgery [167]. A TGF-β1 neutralizing antibody (CAT-192, metelimumab) has been administered intravenously to patients with systemic sclerosis, which causes scarring in skin and internal organs, however, without any evidence of efficacy and with more adverse events

and serious adverse events in *verum* than in placebo patients [168]. This raises the question of whether broader or even complete blockade of the TGF-β axis will be safe or rather associated with substantial toxicity [168]. Despite these concerns, a pan-TGF-β (GC-1008) neutralizing antibody has recently being administered to patients with idiopathic pulmonary fibrosis in a phase I clinical trial for safety evaluation [169] and is being administered to patients with focal segmental glomerulosclerosis [170], renal cell carcinoma, and malignant melanoma [171]; however, safety and efficacy data from these studies are not yet available.

Similar to neutralizing antibodies, soluble TGF-β superfamily receptors abrogate signaling at the ligand level by binding the ligand and preventing it from binding to its cell surface receptors. Due to the lack of studies in humans, only data from animal models are available. For example, soluble TGFBR3 has demonstrated efficacy against renal damage progression associated with diabetes in mice [172]. Similarly, soluble TGFBR2 has anti-cancer effects in mice, as it suppresses the growth and metastasis of pancreatic cancer cells [173] and also inhibits breast cancer cell growth, migration, invasion, and metastasis [174].

Using anti-sense oligonucleotides to reduce the expression of TGF-β superfamily members is a relatively new therapeutic tool that has been already successful applied in clinical trials for cancer treatment [175,176]. Also, small molecule inhibitors have been introduced to block TGF-β signaling. The TGFBR1-inhibitor Ly573636, which blocks intrinsic receptor-kinase activity, is currently being assessed in patients with certain cancers (see [176]), whereas other TGFBR1-inhibitors have shown to be efficient in mouse tumor models [177].

## 10. CONCLUSIONS

Due to the central role of TGF-β in severe fibrotic diseases (with similarities and dissimilarities among organs/diseases) it is an attractive therapeutic target, as already shown in several clinical trials (see above).

The strongly bifunctional role of TGF-β, however (pro-fibrotic, but anti-inflammatory), requires great care for the application of TGF-β-directed treatments. Future strategies will therefore have to focus on developing suitable tools to address this bifunctionality such as: 1) locally or regionally restricted administration of agonists or antagonists (in particular, broad TGF-β blockade by agents such as TGF-β antibodies, TGF-β receptors (soluble/anti-sense), latency-associated peptide or Smad7; 2) phase-dependent application in those periods of disease dominated by the critical pathogenetic features of TGF-β; and 3) development of local or systemic biomarkers indicative of a future favorable response. The systemic applicability of broad TGF-β blockade will have to await the safety date from ongoing trials.

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**Table 1. Expression of TGF-β related proteins in human disease compared to controls (normal or inflammatory)**

| Organ | Irradiated skin | Heart | Synovial membrane | Gut | | | Kidney |
|---|---|---|---|---|---|---|---|
| Disease | Wound healing | Fibrotic heart diseases* | RA | CD inflamed | CD fibrotic | UC | Diabetic nephropathy |
| Molecule | | | | | | | |
| THBS1 | **n.d.** | ↑[178,179] | ↑[40,180] | ↓[181] | **n.d.** | ↔[181] | ↑[182] |
| Latent-TGF-β | ↑[122] | **n.d.** | ↔[183], ↑[39] | **n.d.** | **n.d.** | **n.d.** | ↑[182] |
| TGF-β1 | ↑[122] | ↑[184,185] | ↔[35,36,40,183], ↑[37,38] | ↑[76,108,114] | ↑[108,114] | ↑[76,108,114] | ↔[186], ↑[53,72] |
| TGF-β2 | ↑[122] | ↔[185] | ↔[35,183] | ↑[114] | ↑[114] | ↔[114] | ↑[72] |
| TGF-β3 | ↓[187] | ↑[185] | ↔[35,183] | ↓[114] | ↓[114] | ↔[114] | ↔[186], ↑[72] |
| TGFBR1 | **n.d.** | ↓[184] | ↑[40] | ↔[113] | **n.d.** | **n.d.** | **n.d.** |
| TGFBR2 | ↑[188] | ↔[184] | ↑[35,183] | ↑[113] | **n.d.** | **n.d.** | **n.d.** |

↑ increased, ↓ decreased, ↔ no change, **n.d.** not determined; *mostly following myocardial infarction

## FIGURE LEGENDS

**Figure 1:** TGF-β1 induces different, but overlapping response in different organ systems.

**Figure 2:** Molecules of the TGF-β-pathway ranging from the release of TGF-β, binding of receptors, signaling through Smads, FAK and MAPK to its entry into the nucleus and the regulation of the transcriptional activity (adapted from [40]).

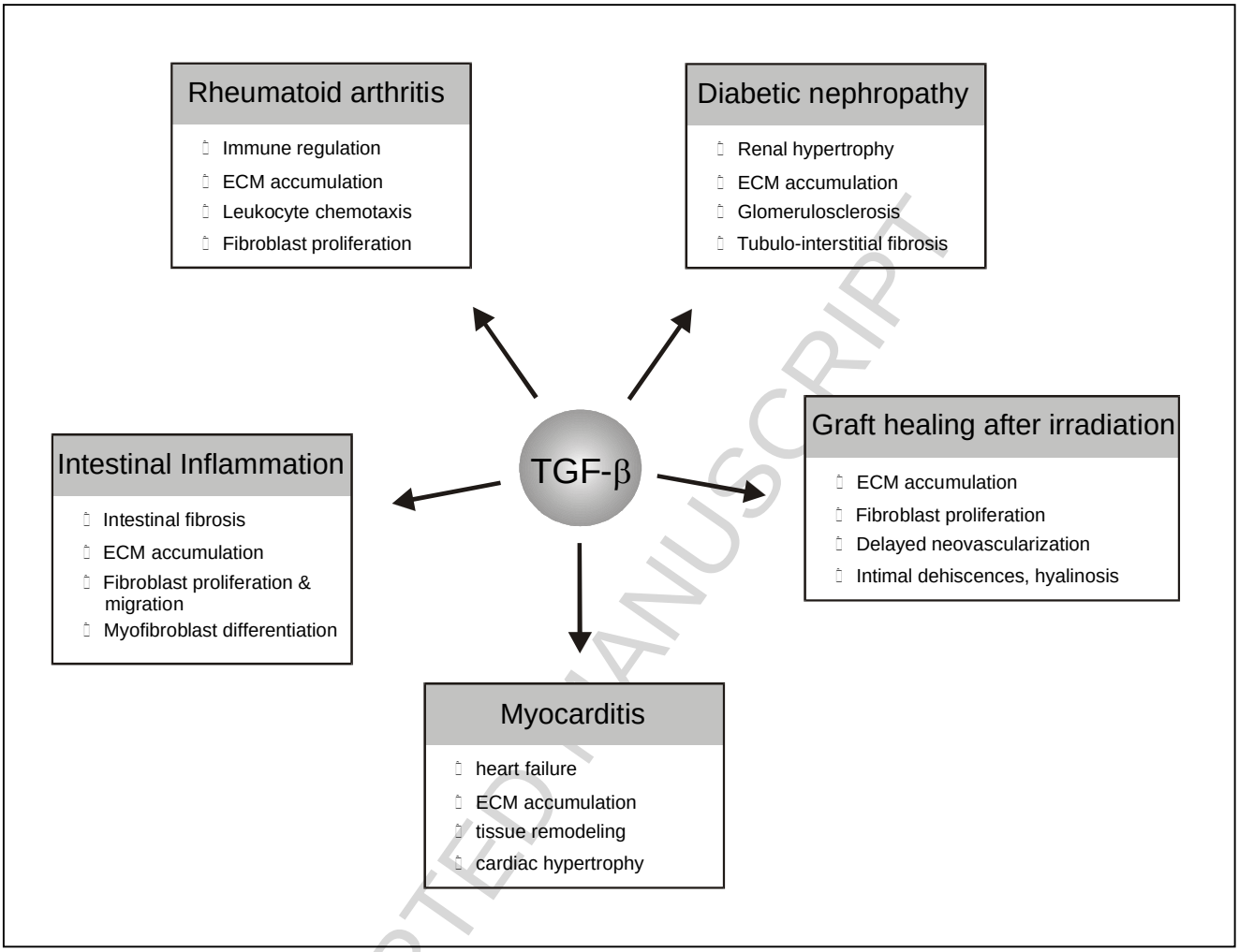


Figure 1

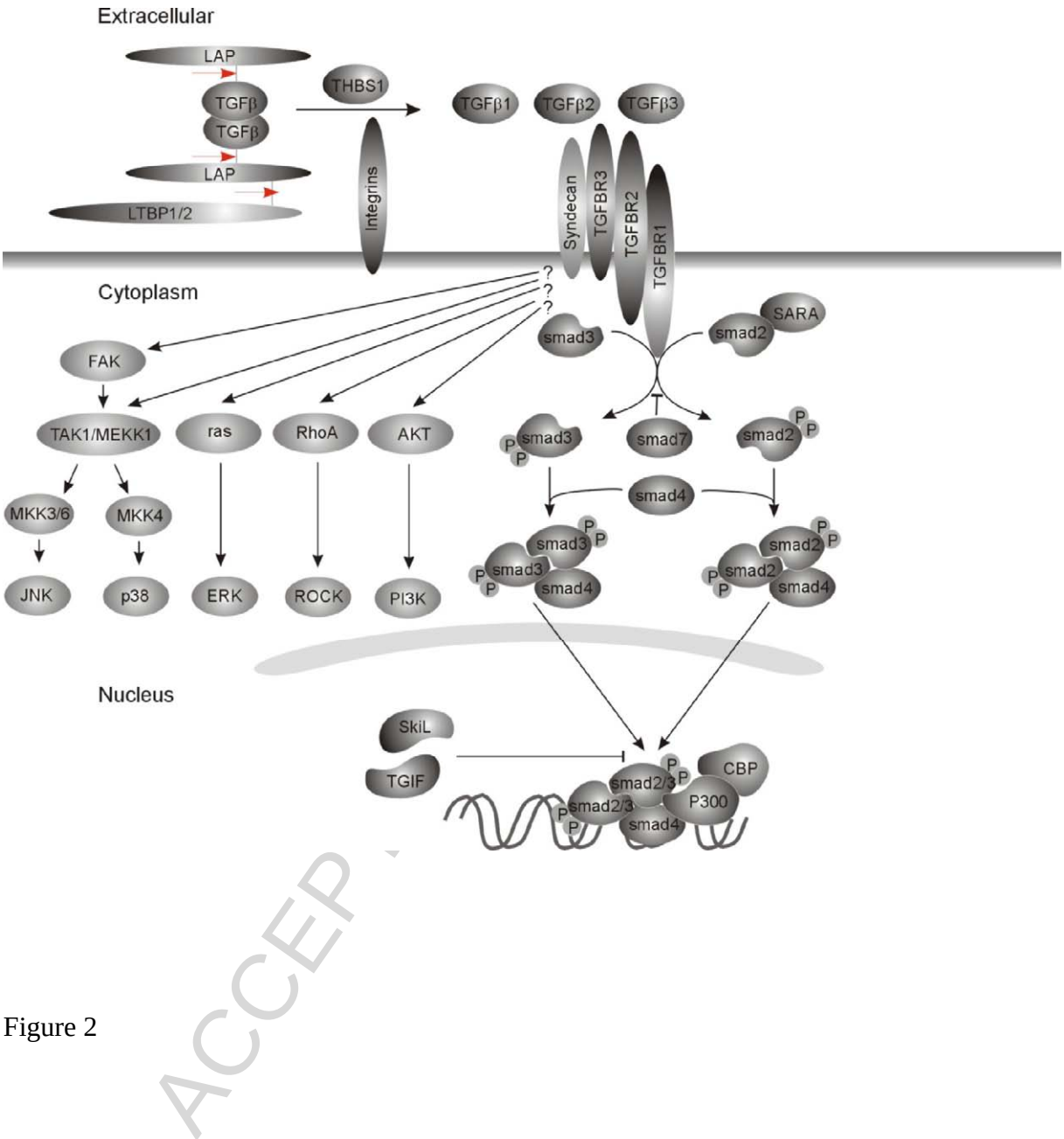


Figure 2