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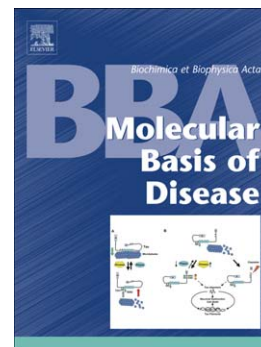
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1 **TGF- $\beta$  and fibrosis in different organs – molecular pathway imprints**

2

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24 Key words: TGF- $\beta$ , fibrosis, arthritis, nephritis, inflammatory bowel disease, Crohn’s disease,

25 wound-healing, myocarditis

26 **ABSTRACT**

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

39

## 40 **1. INTRODUCTION**

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

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

59

## 60 **2. TGF- $\beta$ SIGNALING PATHWAY**

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

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

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

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

93 Notably, that signal transduction pathways have their own intracellular regulators. An  
94 inhibitory Smad (Smad7) blocks TGF- $\beta$ 1 signaling by physical interaction with the activated  
95 TGFBR1 receptor and prevents the docking and phosphorylation of Smad2/3 [28,29].

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

100

### 101 **3. TGF- $\beta$ -RELATED MOLECULES IN RHEUMATOID ARTHRITIS**

102 The importance of TGF- $\beta$ 1 in rheumatoid arthritis (RA) ranges from an association with  
103 certain vascularization patterns in the synovial membrane (SM) [30], and an association of  
104 TGF- $\beta$  polymorphisms with the radiological signs of joint destruction [31] to an induction of  
105 pro-inflammatory cytokines, MMP [32], aggrecanase [33] and urokinase-type plasminogen  
106 activator [34]. In addition, TGF- $\beta$  plays an important role for the function of regulatory T-  
107 cells [5] in the suppression of autoimmunity. Indeed, increased levels of TGF- $\beta$ 1 have been  
108 found in the synovial membrane of patients with RA by northern blot [35],  
109 immunohistochemistry [36,37], western blot [38] and in synovial effusions [39]. Also  
110 TGFBR2 was detected at higher levels than in normal synovial tissue [35].

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

113 arrays, gene expression of TGF- $\beta$ 1, TGF- $\beta$ 3, LTBP1/2, THBS1, TGFBR1, SARA, CBP, SkiL  
114 – belonging to the TGF- $\beta$ -pathway (Figure 2) - was elevated in RA [40]. After validating  
115 array data at the mRNA and protein levels using quantitative PCR and western  
116 blot/immunohistochemistry, we confirmed an upregulated TGF- $\beta$  pathway in RA-SFB. The  
117 presence of TGF- $\beta$ 1, in conjunction with increased amounts of TGF- $\beta$ -releasing THBS1 and a  
118 higher expression of TGFBR1, may thus lead to an amplified response of RA-SFB to TGF- $\beta$ .  
119 This RA-specific response has been confirmed by increased expression of MMP-11 following  
120 TGF- $\beta$  stimulation, implying a pathogenetic relevance of the TGF- $\beta$ -pathway for MMP-  
121 induced degradation or remodeling processes in RA [40].

122 The importance of TGF- $\beta$  for the pathogenesis of arthritis is emphasized by a number  
123 of animal models. The abundant expression of TGF- $\beta$ 1, 2, and 3 as well as the TGFBR1 and -  
124 2 in rat synovium was increased after onset of the collagen-induced arthritis (CIA) [41], and  
125 direct intra-articular injection of TGF- $\beta$ 1 or TGF- $\beta$ 2 induced synovial erythema, swelling,  
126 and cellular infiltration resulting in synovial inflammation and hyperplasia [42]. Conversely,  
127 neutralization of TGF- $\beta$  inhibited acute and chronic arthritis induced by streptococcal cell  
128 wall (SCW) [43]. In line with these observations, an adenovirus-mediated overexpression of  
129 TGF- $\beta$ 1 in rabbit knees led to an increased glycosaminoglycan release, nitric oxide  
130 production and, most notably, to fibrosis and muscle edema [44]. The prevention of CIA by  
131 administration of a TGFBR1 inhibitor (HTS466284), which concomitantly reduced the  
132 expression of vascular endothelial growth factor (VEGF), platelet-derived growth factor  
133 (PDGF)-AA, TNF- $\alpha$ , and cellular proliferation [45], further underlines the pathogenetic role  
134 of TGF- $\beta$  in arthritis.

135 In contrast to the above-mentioned studies, numerous observations show beneficial  
136 effects of TGF- $\beta$  in arthritis. If administered systemically, TGF- $\beta$ 1 suppressed SCW-induced



137 arthritis, as measured by cellular infiltration and joint erosion [46]. In addition, investigation  
138 of the cytokine expression during CIA demonstrated a strong upregulation of TGF- $\beta$ 1/2 in the  
139 remission state of disease, possibly reflecting the anti-inflammatory regulation of T-cells by  
140 TGF- $\beta$  in arthritis. If TGF- $\beta$  signaling was inhibited by expression of dominant-negative  
141 TGFBR2 in T-cells [47] the susceptibility and the clinical severity of CIA was strongly  
142 increased. Likewise, if TGF- $\beta$ 1 was retrovirally overexpressed in arthritogenic splenocytes,  
143 CIA could not be transferred to SCID mice and established disease was ameliorated [48].  
144 Therefore, it may be important to inhibit TGF- $\beta$  only at the site of inflammation without  
145 targeting regulatory lymphocytes at extra-articular sites.

146

#### 147 **4. TGF- $\beta$ -RELATED MOLECULES IN DIABETIC NEPHROPATHY**

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

155 The TGF- $\beta$  level is elevated in the kidneys of insulin-dependent diabetic animals  
156 during both early and late stages of disease [53,54]. Treatment of the streptozotocin (STZ)-  
157 diabetic rat with sufficient insulin to reduce hyperglycemia suppressed the enhanced  
158 expression of TGF- $\beta$  and matrix components in the glomeruli. In the STZ-diabetic rat and  
159 mouse, increased TGF- $\beta$ 1 expression in the renal cortex and glomeruli as well as up-  
160 regulation of the TGFBR2 mRNA and protein was noted early after the onset of diabetes

161 [53].. The *db/db* mouse, a model of type 2 diabetes, characterized by hyperglycemia, obesity,  
162 and insulin resistance, develops increased amounts of TGF- $\beta$ 1 localized in the glomerular  
163 compartments [55]. In contrast, the mRNA and protein levels of the TGFBR2 are  
164 significantly up-regulated in both the glomerular and the tubulointerstitial compartments [55].

165         The development of diabetic renal hypertrophy and glomerulosclerosis is likely caused  
166 by heightened activity of the TGF- $\beta$  system [56-63]. Short-term treatment of the STZ-diabetic  
167 mouse with a neutralizing monoclonal antibody against all three isoforms of TGF- $\beta$  prevented  
168 glomerular hypertrophy, reduced the increment in kidney weight by 50%, and significantly  
169 attenuated the increase in TGF- $\beta$ 1,  $\alpha$ 1(IV) collagen, and fibronectin mRNAs without  
170 affecting glycemic control [56]. The results of this study suggested a cause-and-effect  
171 relationship between the renal TGF- $\beta$  system and the development of early structural changes  
172 in diabetic nephropathy. Systemic anti-TGF- $\beta$  therapy for eight weeks prevented the  
173 mesangial matrix expansion of diabetic glomerulosclerosis and, most importantly, preserved  
174 kidney function, showing for the first time that neutralization of TGF- $\beta$  activity prevents the  
175 progression of renal failure in diabetes [57]. However, the anti-TGF- $\beta$  antibody did not reduce  
176 albuminuria, which itself may promote the progression of renal insufficiency [57]. The  
177 paradox of preserved renal function in view of persistent albuminuria may be explained by  
178 postulating that the deleterious effects of proteinuria are themselves mediated by the TGF- $\beta$   
179 system [63-65].

180         In mouse mesangial and tubular cells, high glucose stimulates the transcription of  
181 fibronectin and, in addition, potentiates the transcriptional activation of fibronectin by TGF-  
182  $\beta$ 1 [58,59]. This particular effect of TGF- $\beta$ 1 appears to be mediated by Smad3, because over-  
183 expression of Smad3 alone was able to induce fibronectin promoter activity [66]. In  
184 conjunction with exogenous TGF- $\beta$ 1, Smad3 over-expression synergistically increased

185 fibronectin expression, as if the extra Smad3 had increased the efficiency of TGF- $\beta$  signaling  
186 [66]. Finally, transfection of a Smad3-dominant-negative construct inhibited TGF- $\beta$ 1  
187 stimulated fibronectin promoter activity [67,68]. However, part of the TGF- $\beta$ 1-induced  
188 fibronectin expression may be mediated in parallel by the p38MAPK pathway [68,69].  
189 Finally, there is evidence that Smad3 is a central mediator in the TGF- $\beta$ 1-induced increase of  
190 mRNA expression for  $\alpha$ 1(I) collagen [68,70]. TGF- $\beta$ -induced MAPK activation also leads to  
191 N-terminal phosphorylation of p53 that enables its interaction with TGF- $\beta$ -activated Smads  
192 [64], an indication for cross-talk between the various TGF- $\beta$  signaling pathways.

193 TGF- $\beta$ 1 may have additional effects besides the stimulation of extracellular matrix  
194 production. In podocytes, TGF- $\beta$ 1 induces apoptosis through Smad7 by inhibiting nuclear  
195 translocation of the cell survival factor NF- $\kappa$ B [70]. TGF- $\beta$ 1-mediated activation of Smad7 is  
196 specific for podocytes and is not found in mesangial cells in a limited series of biopsies from  
197 patients with diabetic nephropathy [64]. Since podocytes are terminally differentiated cells  
198 unable to undergo cell division due to the up-regulation of the cell cycle inhibitory proteins  
199 p57 and p27<sup>Kip1</sup>, apoptotic loss of cells was not replaced.

200 Studies performed in diabetic patients with various degrees of nephropathy also  
201 underline the importance of the renal TGF- $\beta$  system in disease development [71-73]. All three  
202 isoforms of TGF- $\beta$  are elevated in both the glomerular and the tubulointerstitial compartments  
203 of patients with established diabetic nephropathy [71,72]. Furthermore, glomerular TGF- $\beta$ 1  
204 mRNA is markedly increased in biopsy specimens from patients with proven diabetic kidney  
205 disease. These investigations suggests that increased renal TGF- $\beta$  levels closely correlate with  
206 the degree of mesangial matrix expansion, interstitial fibrosis, and renal insufficiency.

207 Another study, designed to assess renal production of TGF- $\beta$  [73] measured aortic,  
208 renal vein, and urinary levels of TGF- $\beta$  in 14 type 2 diabetic and 11 non-diabetic control  
209 patients undergoing elective coronary artery catheterization [73]. Both groups were roughly  
210 matched with regard to the range of renal function and the presence of hypertension and  
211 proteinuria. Renal blood flow was measured to calculate the net mass balance across the  
212 kidney. The gradient of TGF- $\beta$ 1 concentration across the renal vascular bed was negative in  
213 the non-diabetic patients indicating net renal extraction of TGF- $\beta$ 1, whereas the gradient was  
214 positive in the diabetic patients indicating net renal production of TGF- $\beta$ 1. When the renal  
215 TGF- $\beta$ 1 mass balance was calculated, a similar pattern was observed with the non-diabetic  
216 kidney removing approximately 3500 ng/min of TGF- $\beta$ 1 from the circulation, and the diabetic  
217 kidney adding approximately 1000 ng/min of TGF- $\beta$ 1 [73]. In addition, the level of  
218 bioassayable TGF- $\beta$  was increased four-fold in the urine of diabetic *versus* non-diabetic  
219 patients. This was not simply a function of enhanced glomerular permeability to protein since  
220 diabetic patients both with and without microalbuminuria displayed similarly high rates of  
221 urinary TGF- $\beta$  excretion [73]. These results demonstrated that the kidneys of diabetic patients  
222 overproduce TGF- $\beta$ 1 protein; further details, e. g., the exact contribution of the different renal  
223 cell types, need to be investigated.

224 An interesting *post-hoc* study assessed whether treatment with the angiotensin  
225 converting enzyme inhibitor captopril would lower serum TGF- $\beta$ 1 levels in a small subset of  
226 patients with diabetic nephropathy who had been enrolled in the Collaborative Study Group  
227 [74,75]. After six months, the serum TGF- $\beta$ 1 level decreased significantly by 21% in the  
228 captopril-treated group, whereas it increased slightly by 11% in the placebo-treated group  
229 [75]. Interestingly, the captopril-treated patients with decreased serum TGF- $\beta$ 1 levels tended  
230 to have better preserved renal function over the ensuing two-year period [75]. This association

231 was even more pronounced in the subset of patients with an initial glomerular filtration rate of  
232 less than 75 ml/min. These results suggest that TGF- $\beta$ 1 plays a pivotal role in the progression  
233 of diabetic nephropathy and that angiotensin converting enzyme inhibitor therapy may protect  
234 the kidney by lowering TGF- $\beta$ 1 production.

235

## 236 **5. TGF- $\beta$ -RELATED MOLECULES IN INTESTINAL INFLAMMATION – A DOUBLE-** 237 **SIDED SWORD**

238 TGF- $\beta$  is constitutively expressed by epithelial cells, fibroblasts, and mononuclear cells in the  
239 gastrointestinal tract [76]. Its critical role in intestinal homeostasis as a negative master  
240 regulator of inflammation is well-established [77,78] and indisputable. However, translation  
241 of elegant mouse experiments into therapeutic interventions in humans requires a clearer  
242 understanding of TGF- $\beta$  activity in the human gut. Mice with global TGF- $\beta$  defects, such as  
243 TGF- $\beta$ -null mice or transgenic mice expressing a dominant negative TGFBR2 chain, are  
244 unresponsive to TGF- $\beta$ 1 signaling. The former die soon after birth due to systemic  
245 inflammation, and the latter develop severe colonic and pulmonary inflammation [79,80].  
246 These manifestations of global TGF- $\beta$ 1 defects are mirrored in mouse models of colitis in that  
247 the secretion of TGF- $\beta$ 1 is consistently associated with either the protection from colitis or a  
248 greatly diminished severity of colitis. This is seen both in the T<sub>H</sub>1 model of colitis induced by  
249 the haptening reagent trinitrobenzene sulfonic acid (TNBS), which mimics Crohn's disease  
250 (CD), or the T<sub>H</sub>2 model of colitis induced by the haptening agent oxazolone, which mimics  
251 ulcerative colitis (UC) [81,82]. In addition, it is seen in the colitis of SCID or RAG2-deficient  
252 mice receiving CD45RB<sup>high</sup> (naive) T cells in which the protective effect of the cotransfer of  
253 CD45RB<sup>low</sup> T (memory) cells is abolished by concomitant administration of a neutralizing  
254 TGF- $\beta$ 1 antibody [83]. These and other studies quite conclusively establish that TGF- $\beta$ 1 plays

255 an essential, regulatory role in the control of colitis. On the other hand, in mice TGF- $\beta$ 1 in  
256 combination with IL-6 induces a strong pro-inflammatory T<sub>H</sub>17 cell differentiation [84-86].  
257 Notably, in humans TGF- $\beta$ 1 does not have a direct effect on the development of human T<sub>H</sub>17  
258 cells, but it can indirectly favour the development of these cells by suppressing the expression  
259 of T-bet and selectively inhibiting the expansion of IFN- $\gamma$ -producing T cells [87-89].

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

269 TGF- $\beta$  has been also implicated as a key inducer of epithelial-mesenchymal transition  
270 (EMT) [92-97]. Amongst others, EMT is an essential component of tissue remodeling and  
271 wound repair (reviewed in [98,99]) and fibrosis (reviewed in [100]). During this transition,  
272 the epithelial phenotype, characterized by strong cell-cell junctions and polarity, is replaced  
273 by a mesenchymal phenotype, with reduced cell-cell interactions, a fibroblastic morphology  
274 and increased motility. TGF- $\beta$  stimulates the proliferation of many cell types, particularly  
275 those of mesenchymal origin, and it is also a potent inhibitor of epithelial cell proliferation.  
276 EMT in response to TGF- $\beta$ 1 and in fibrosis is mediated predominantly via Smad-dependent  
277 (mainly Smad3) pathways [101,102]. A loss of Smad3 in mice blocked both morphological

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

280 CD is a chronic, progressive disease of the gastrointestinal tract with an unknown  
281 etiology. It is characterized by transmural inflammation of all layers of the bowel wall. The  
282 formation of stenoses and strictures is common in this disease, which causes abdominal pain,  
283 anorexia, and weight loss. Approximately 50% of CD patients undergo surgery for this type  
284 of complication during a 10-year course; however, the recurrence rate after surgery is high. In  
285 contrast, UC rarely causes intestinal stenosis. Cytokines released from inflammatory cells  
286 have long been implicated in the pathogenesis of intestinal fibrosis. TGF- $\beta$ /Smad signaling  
287 plays an important role in CD [103-105]. The transmural infiltrate of CD is responsible for  
288 initiating and maintaining a series of connective tissue changes not only involving the  
289 mucosa, but also the submucosa and *muscularis mucosae* and *muscularis propria*, where a  
290 marked increase of collagen type I, III, and V mRNA is observed [106,107]. TGF- $\beta$  has been  
291 identified as one of the central growth factors/cytokines that specifically induces a fibrotic  
292 response after inflammatory injury in the intestinal tract. In CD, there is a marked  
293 overexpression of TGF- $\beta$ 1 and TGFBRs in the colonic mucosa [76,108]. Fibrosis in CD can  
294 therefore be interpreted as an aberrant healing response to mucosal injury [109]. In addition,  
295 TGF- $\beta$  appears to be involved in intestinal fibrosis in other enteropathies, such as radiation  
296 enteritis, collagenous colitis, and intestinal graft-versus host disease [110-112]. Both TGF- $\beta$   
297 and its receptors are overexpressed in the intestine of patients with CD [113]. Intestinal  
298 fibroblast expression of TGF- $\beta$  isoforms varies according to the nature of tissue. Fibroblasts  
299 from normal and inflamed mucosa both express the TGF- $\beta$ 1 and TGF- $\beta$ 3 isoforms, while  
300 those from fibrotic tissue show reduced expression of TGF- $\beta$ 3, but enhanced expression of  
301 TGF- $\beta$ 2 and TGF- $\beta$ 1 [114]. This is remarkable, since the TGF- $\beta$ 1 and TGF- $\beta$ 2 isoforms have

302 been specifically implicated in pathogenic fibrosis, while TGF- $\beta$ 3 appears to have antifibrotic  
303 properties [115]. Monteleone and colleagues have provided insight into the failure of TGF- $\beta$   
304 down-regulation in CD. Despite the abundant expression of TGF- $\beta$  in the mucosa of patients  
305 with CD, phospho-Smad3 is diminished in the mucosa compared to control mucosal samples,  
306 as is the complex of Smad3 with Smad4. This may be due to the induction and overexpression  
307 of Smad7 in the mucosa of patients with CD and UCs [116]. However, upregulation of Smad7  
308 is not specific for inflammatory bowel disease (IBD), but also occurs in *Helicobacter pylori*-  
309 induced gastritis [117]. In addition, mucosal T cells in both whole tissue and isolated cells  
310 show defective TGF- $\beta$ 1 signaling as measured by reduced immuno-reactivity against  
311 phospho-Smad3 [117]. Specific anti-sense oligonucleotides for Smad7 reduce expression of  
312 Smad7 in cells isolated from IBD patients which then become responsive to exogenous TGF-  
313  $\beta$ 1. TGF- $\beta$ 1 cannot inhibit pro-inflammatory cytokine production in isolated lamina propria  
314 mononuclear cells from CD patients, but inhibition of Smad7 with anti-sense oligonucleotides  
315 restores TGF- $\beta$ 1 signaling and allows TGF- $\beta$ 1 to inhibit cytokine production. In inflamed  
316 mucosal tissue explants from CD patients, inhibition of Smad7 also restores phospho-Smad3  
317 and decreases pro-inflammatory cytokine production, an effect which is partially blocked by  
318 anti-TGF- $\beta$ 1. The extension of these studies examined the interactions between Smad  
319 signaling and NF- $\kappa$ B activation in inflamed gut: While TGF- $\beta$ 1 is a potent inhibitor of TNF-  
320  $\alpha$ -induced NF- $\kappa$ B activation in normal gut, it has no activity in inflamed gut. This can be  
321 attributed to over-expression of Smad7, since treatment of cells from inflamed gut with anti-  
322 sense to Smad7 allows TGF- $\beta$ 1 to rapidly down-regulate NF- $\kappa$ B activation [117].

323 If it becomes possible to specifically inhibit Smad7, endogenous TGF- $\beta$ 1 in the  
324 inflamed gut may negatively regulate pro-inflammatory cytokine production and NF- $\kappa$ B  
325 activation, the major components of the immune overactivity which drives tissue injury in



326 IBD. At the same time, however, it is important to discover the factors which control Smad7  
327 expression in the inflamed gut. Furthermore, cell-specific expression of Smad7 will be  
328 important because TGF- $\beta$ 1 has different effects on different cell types. Thus, while blocking  
329 Smad7 will allow TGF- $\beta$ 1 to reduce pro-inflammatory cytokine production by T cells and  
330 macrophages, it may allow TGF- $\beta$ 1 to increase collagen production in myofibroblasts,  
331 resulting in fibrosis. However, at the moment the relative importance of the known inducers  
332 of Smad7 in the gut, such as IFN- $\gamma$  or TNF- $\alpha$ , or even whether TGF- $\beta$ 1 itself induces Smad7  
333 in a negative regulatory loop, is still unclear.

334

## 335 **6. TGF- $\beta$ -RELATED MOLECULES IN RADIATION-INDUCED FIBROSIS**

336 TGF- $\beta$ 1 levels are increased in irradiated mouse skin [118,119] and decrease slowly after  
337 irradiation in both pig and human skin [120,121]. Following microvascular hard or soft tissue  
338 transfer, TGF- $\beta$ 1 is again upregulated in a biphasic manner. The first expression peak on day  
339 3 post operation is due to enhanced activation of latent TGF- $\beta$ 1 by extracellular enzymes  
340 while the second between day 14 and 28 after surgery is a result of *de novo* synthesis [122].  
341 Its most important signaling receptor TGFBR2 is upregulated in irradiated graft beds as well  
342 [123]. Signaling leads to increased nucleoplasmatic shuttling of active Smad2/3 and induction  
343 of TGF- $\beta$ 1 target genes in fibrotic healing, which is mainly due to decrease in cytoplasmatic  
344 levels of the inhibitory Smad7 [124].

345 As a consequence, the extracellular matrix is qualitatively and quantitatively altered  
346 [125]. Prolyl-hydroxyprolinase- $\beta$  overexpression [126] promotes synthesis of collagen I, III  
347 and IV [124], while repression of degrading enzymes, such as MMP-1 and induction of tissue  
348 inhibitors [127] suppresses the degrading pathways. Moreover, integrin surface receptors,  
349 such as  $\alpha$ 2 $\beta$ 1 integrin are up-regulated as well and modulate transmission of tensile forces

350 [128]. In the presence of such forces fibroblasts differentiate into myofibroblasts resulting in  
351 constrictive fibrosis [129].

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

356         Taking the central role of TGF- $\beta$ 1 in radiation-impaired wound healing into account,  
357 the question of whether TGF- $\beta$ 1-levels differ in the healthy tissue between different patients  
358 is of utmost importance. It has been demonstrated that patients with an increased TGF- $\beta$ 1  
359 plasma level exhibit an increased risk of developing skin fibrosis following irradiation [133].  
360 To find a particular predictor which sufficiently corresponds with the frequency to develop  
361 wound healing complications following surgery in previously irradiated graft beds would give  
362 a large clinical impact on planning individual treatment protocols. The availability of reliable  
363 markers may eventually allow the prediction of outcome prior to commencement of treatment,  
364 and thus allow modification of combined protocols to minimize late adverse effects without  
365 compromising tumor control.

366

## 367 **7. TGF- $\beta$ -RELATED MOLECULES IN MYOCARDITIS**

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

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

376 Like humans, mice develop a marked age-related susceptibility to CVB infections  
377 [137]. The myocardial lesions in mice closely resemble those seen in human disease [138];  
378 therefore, experimental murine models of coxsackievirus-induced myocarditis have been  
379 developed to investigate the pathogenesis of this disease. Although chronic inflammation is  
380 characteristic for the human disease as well as for our mouse model, fibrosis is more prevalent  
381 in the latter.

382 Excessive fibrosis, as it occurs under conditions of chronic myocarditis, can be  
383 classified as either replacement fibrosis, when functional tissue is replaced by connective  
384 tissue, or as reactive fibrosis, which is part of an adaptive process [139]. In addition to  
385 collagen, tenascin C, and fibronectin, splice variants of these molecules are often part of the  
386 fibrotic tissue [140,141]. Various cytokines and growth factors are believed to contribute to  
387 the induction of fibrosis, including TGF- $\beta$  [142], IL-1, [143], TNF- $\alpha$  [144,145], and PDGFs  
388 [146,147]. For example, persistent expression of cytokines in the chronic stage of CVB3-  
389 induced myocarditis has been described for various mouse models [148-150], as well as for  
390 human dilatative cardiomyopathy [151]. New observations suggest that sustained pro-  
391 inflammatory signaling is associated with a pro-fibrotic phenotype based on TGF- $\beta$ -mediated  
392 signaling [152].

393 For fibrogenesis in the heart, members of the PDGF family are apparently important  
394 mediators. Transgenic overexpression of PDGF-C and PDGF-D, two more recently  
395 discovered PDGF isoforms [153], in the heart leads to massive cardiac fibrosis [146,147].  
396 Therefore, the importance of PDGF for the pathology of chronic myocarditis was investigated  
397 in mice with CVB3-induced myocarditis. Interestingly, all analysed isoforms of PDGF-A, -B,  
398 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- $\beta$ -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- $\beta$  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- $\beta$  signalling in the heart is reviewed in [165]. But the  
417 relative contribution of the different TGF- $\beta$ -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- $\beta$ 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).

424 TGF- $\beta$ 1 is elevated in most pathologies in its active or latent form and shows  
425 similarities with the isoform TGF- $\beta$ 2 in most cases. In contrast, the expression of TGF- $\beta$ 3  
426 varies from down-regulation during wound healing and CD, abundance without regulation in  
427 rheumatoid arthritis and UC, to up-regulation during diabetic nephropathy. If determined, the  
428 expression of TGFBR2 is often enhanced during disease. The widespread appearance of the  
429 term “n.d.” points out the urgent need for further investigation of the expression and the  
430 effects of TGF- $\beta$ -related molecules in human diseases associated with fibrosis, particularly at  
431 the stage of signaling and transcriptional regulation.

432

### 433 **9. THERAPEUTIC STRATEGIES**

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

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

442 Regarding TGF- $\beta$  inhibition, some clinical trials have been performed with  
443 neutralizing antibodies, especially in fibrotic diseases, including a TGF- $\beta$ 2 neutralizing  
444 antibody (lerdelimumab) which effectively decreased the amount of scarring after glaucoma  
445 surgery [167]. A TGF- $\beta$ 1 neutralizing antibody (CAT-192, metelimumab) has been  
446 administered intravenously to patients with systemic sclerosis, which causes scarring in skin  
447 and internal organs, however, without any evidence of efficacy and with more adverse events

448 and serious adverse events in *verum* than in placebo patients [168]. This raises the question of  
449 whether broader or even complete blockade of the TGF- $\beta$  axis will be safe or rather  
450 associated with substantial toxicity [168]. Despite these concerns, a pan-TGF- $\beta$  (GC-1008)  
451 neutralizing antibody has recently been administered to patients with idiopathic pulmonary  
452 fibrosis in a phase I clinical trial for safety evaluation [169] and is being administered to  
453 patients with focal segmental glomerulosclerosis [170], renal cell carcinoma, and malignant  
454 melanoma [171]; however, safety and efficacy data from these studies are not yet available.  
455 Similar to neutralizing antibodies, soluble TGF- $\beta$  superfamily receptors abrogate signaling at  
456 the ligand level by binding the ligand and preventing it from binding to its cell surface  
457 receptors. Due to the lack of studies in humans, only data from animal models are available.  
458 For example, soluble TGFBR3 has demonstrated efficacy against renal damage progression  
459 associated with diabetes in mice [172]. Similarly, soluble TGFBR2 has anti-cancer effects in  
460 mice, as it suppresses the growth and metastasis of pancreatic cancer cells [173] and also  
461 inhibits breast cancer cell growth, migration, invasion, and metastasis [174].  
462 Using anti-sense oligonucleotides to reduce the expression of TGF- $\beta$  superfamily members is  
463 a relatively new therapeutic tool that has been already successfully applied in clinical trials for  
464 cancer treatment [175,176]. Also, small molecule inhibitors have been introduced to block  
465 TGF- $\beta$  signaling. The TGFBR1-inhibitor Ly573636, which blocks intrinsic receptor-kinase  
466 activity, is currently being assessed in patients with certain cancers (see [176]), whereas other  
467 TGFBR1-inhibitors have shown to be efficient in mouse tumor models [177].

468

## 469 **10. CONCLUSIONS**

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

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

482

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**Table 1. Expression of TGF- $\beta$  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	<b>n.d.</b>	↑[178,179]	↑[40,180]	↓[181]	<b>n.d.</b>	↔[181]	↑[182]
Latent-TGF- $\beta$	↑[122]	<b>n.d.</b>	↔[183], ↑[39]	<b>n.d.</b>	<b>n.d.</b>	<b>n.d.</b>	↑[182]
TGF- $\beta$ 1	↑[122]	↑[184,185]	↔[35,36,40,183], ↑[37,38]	↑[76,108,114]	↑[108,114]	↑[76,108,114]	↔[186], ↑[53,72]
TGF- $\beta$ 2	↑[122]	↔[185]	↔[35,183]	↑[114]	↑[114]	↔[114]	↑[72]
TGF- $\beta$ 3	↓[187]	↑[185]	↔[35,183]	↓[114]	↓[114]	↔[114]	↔[186], ↑[72]
TGFBR1	<b>n.d.</b>	↓[184]	↑[40]	↔[113]	<b>n.d.</b>	<b>n.d.</b>	<b>n.d.</b>
TGFBR2	↑[188]	↔[184]	↑[35,183]	↑[113]	<b>n.d.</b>	<b>n.d.</b>	<b>n.d.</b>

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

**FIGURE LEGENDS**

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

**Figure 2:** Molecules of the TGF- $\beta$ -pathway ranging from the release of TGF- $\beta$ , 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]).

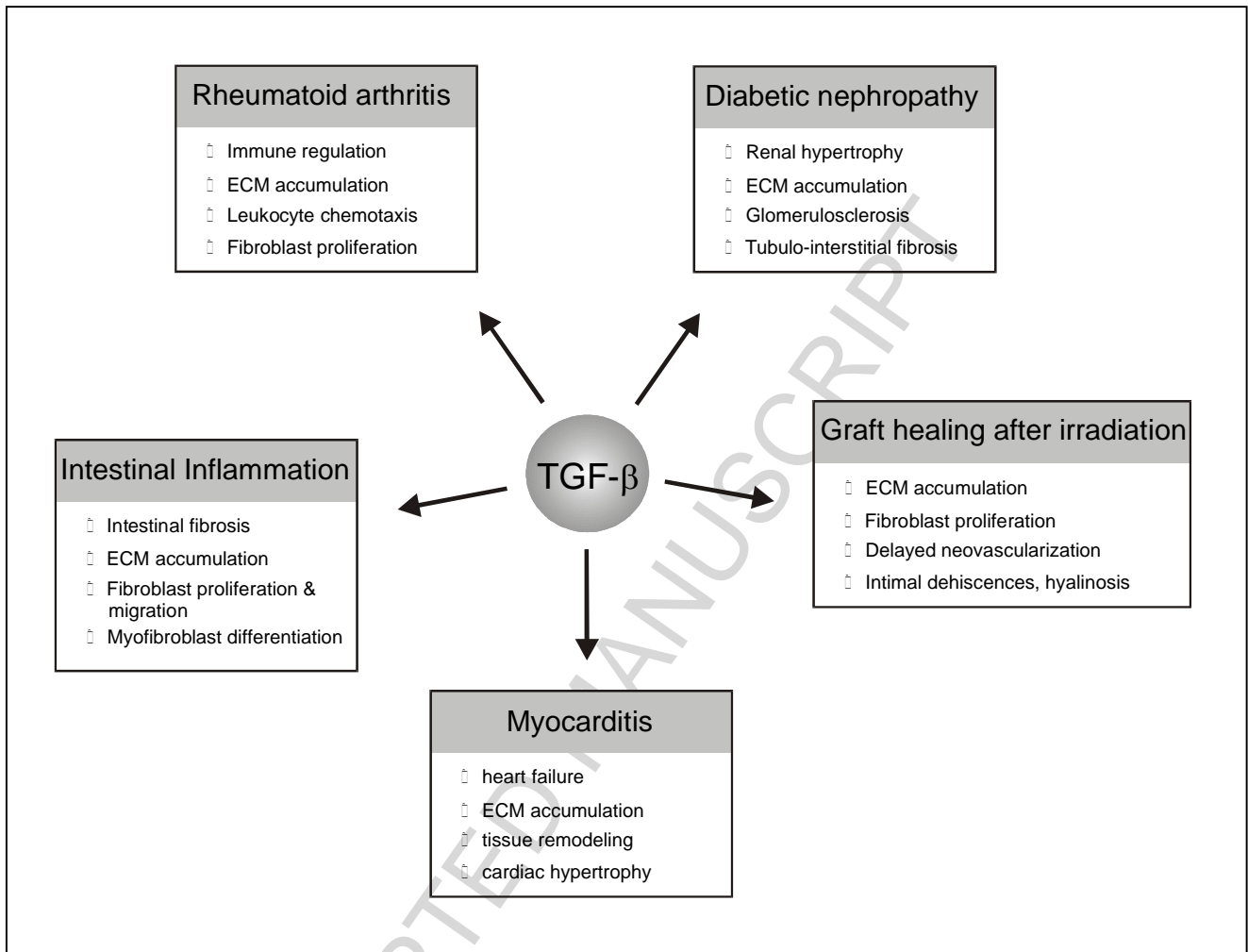


Figure 1

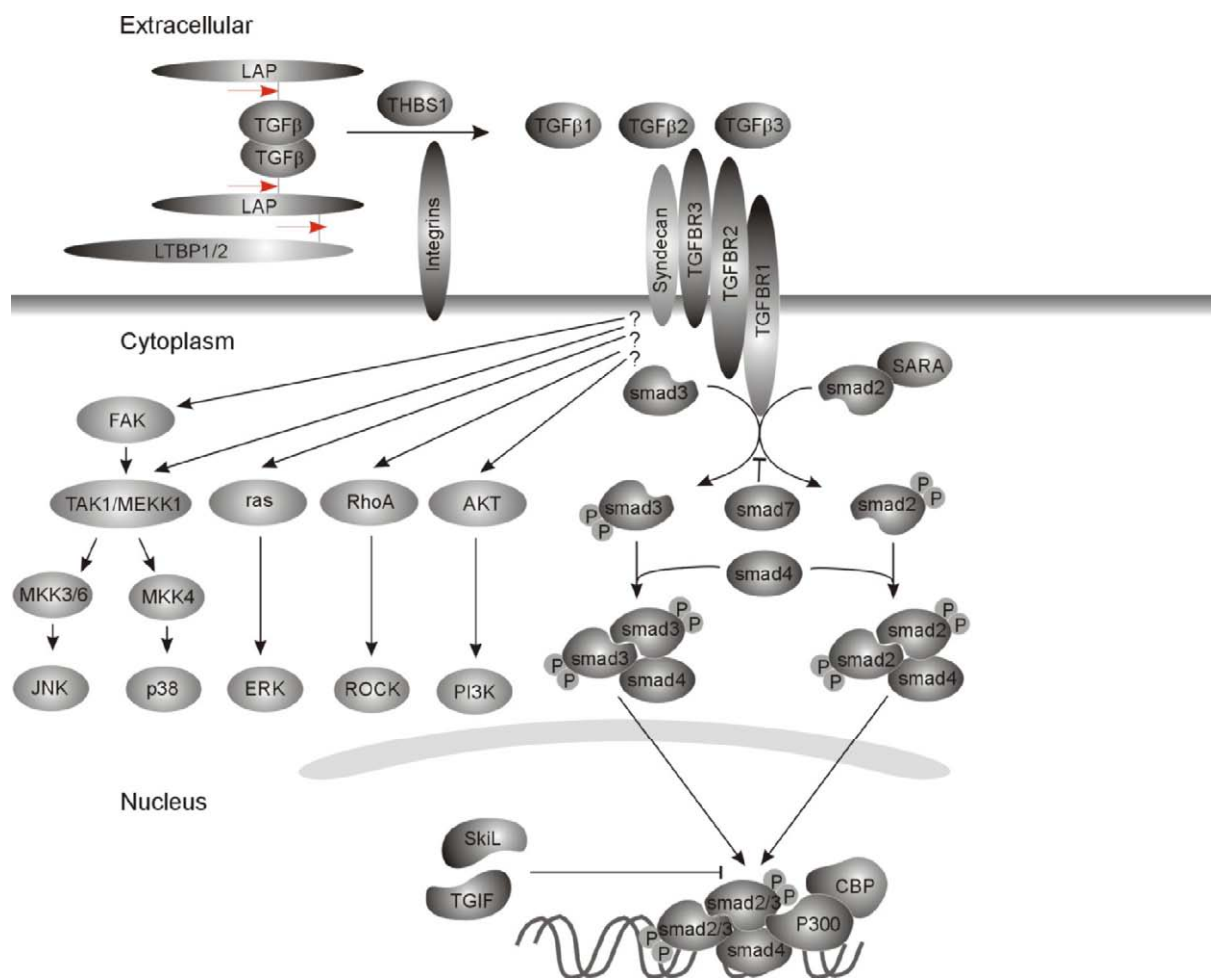


Figure 2