ALK1-Smad1/5 signaling pathway in fibrosis development, friend or foe?

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Abstract

Fibrosis is a common phenomenon with several pathologies, characterized by an excessive extracellular matrix deposition that leads to a progressive organ dysfunction. Thus fibrosis has a relevant role in chronic diseases affecting the kidney, the liver, lung, skin (scleroderma) and joints (arthritis), among others. The pathogenesis of fibrosis in different organs share numerous similarities, with one of them being the presence of activated fibroblasts, denominated myofibroblast, which act as the main source of extracellular matrix proteins. Transforming growth factor beta-1 (TGF-β1) is a profibrotic cytokine that plays a pivotal role in fibrosis. The TGF-β1 /ALK5/Smad3 signaling pathway has been studied in fibrosis extensively. However, an increasing number of studies involving the ALK1/Smad1 pathway in the fibrotic process exist. In this review we offer a perspective of the function of ALK1/Smad1 pathway in renal fibrosis, liver fibrosis, scleroderma and osteoarthritis, suggesting this pathway as a powerful therapeutical target. We also propose several strategies to modulate the activity of this pathway and its consequences in the fibrotic process.

1. Introduction

Fibrosis is a common end-point for several pathologies in different organs, such as kidneys, the liver, lungs, the heart and skin. An extreme buildup of extracellular matrix (ECM) causing organ dysfunction characterizes Fibrosis.

Transforming growth factor beta-1 (TGF-β1) is a cytokine involved in numerous fibrotic disorders in different organs, such as kidney (1, 2), liver (3, 4), lung (5), skin (6-9), heart (10, 11), or vessels (12) among others. The canonical signaling pathway for TGF-β1 attaches a TGF-β type II receptor (TβRII) that recruits a TGF-β type I receptor (TβRI) with serine/threonine kinase activity. It phosphorylates cytosolic proteins called Smads, which act as transcription factors activating or inhibiting selective genes. Among the different TβRI, ALK1 and ALK5 are the most studied.

The involvement of TGF-β1 through its TβRI ALK5 in fibrosis development is well known (1, 13). However, the role of ALK1 in fibrotic disorders is still unknown and its role seems to be different depending on the cell and the tissue. In several cell types such as endothelial cells (ECs), rat myoblasts, hepatocytes and human chondrocytes, ALK1

seems to counteract TGF-β/ALK5 induced ECM protein expression. In hepatic stellate cells (HSCs), TGF-β promotes the expression of inductor of differentiation-1 (Id1) through ALK1/Smad1/5 pathway and potentiates HSCs transdifferentiation into myofibroblasts (4). The abundance of myofibroblasts contributes to liver fibrosis.

On the other hand, it has been described that ALK1/Smad1/5 pathway promotes fibrotic phenotype in scleroderma fibroblasts (SSc) (7, 9). Additionally, other authors have demonstrated the role of Smad1 in diabetic nephropathy and mesangial matrix expansion (14).

As the role of ALK1 in fibrosis is unclear and controversial, our purpose is to review the recent literature about its involvement in organ fibrosis and check the possible function of this receptor in several fibrotic disorders, to assess if ALK1 could be a possible therapeutic target.

Before going deep into ALK1 structure and function, we will review briefly the most important characteristic of the cytokines members of the TGF-β family.

2. The TGF-β Superfamily

The TGF-β superfamily comprises several peptides that regulate development and tissue differentiation through their influences on cell proliferation, differentiation and migration. More than 40 members are known, and have been clustered in several subfamilies: TGF-β subfamily comprises five members with only TGF-β1, TGF-β2 and TGF-β3 isoforms expressed in mammals. TGF-β4 was found in birds (15) and TGF-β5 in amphibian (16); BMPs (BMP-2/BMP-4 group, BMP-5-BMP-8 group, BMP-9/BMP-10 group, OP (osteogenic protein)-1 group, and Nodal group); GDFs (GDF1-GDF3, GDF5-GDF11 and GDF15); MIF (anti-Müllerian hormone or AMH/MIS); Activins and Inhibins (17) (see Table 1).

By binding two transmembrane receptor kinases (type I and II receptors), which form an active heterotetramer with kinase activity, the TGF-β superfamily members transduce their signals. Type I receptors are seven and include ALK1, ALK2, ALK3, ALK4, ALK5, ALK6 and ALK7), while type II receptors are four and include TβRII, BMPRII, ActRIIA/B.

The functional receptor compound controls the stimulation of downstream Smad as schematized in Figure 1, and non-Smad pathway.

2.1 Smad-dependent signal transduction

The TGF-*β* signaling can be mediated by the Smad family of proteins. In vertebrates, there are 8 R-Smads (Smad1 to Smad8). Smad2 and Smad3 are substrates for receptors triggered by TGF-βs and Activins, although Samd1, Smad5 and Smad8 mediate the pathways stimulated by BMPs, GDFs and MIFs. The phosphorylated type I receptor recruits and phosphorylates the R-Smads (receptor-regulated Smads). The TGFβRII-ALK5 complex activates Smad2 and Smad3, but the TGFβRII-ALK1 complex activates Smad1, Smad5 and Smad8 (18). Activated R-Smad form heteromeric complexes with the common partner Smad (co-Smad, Smad4 in mammals) and translocate into the nucleus. The recruitment of R-Smads to the receptor complex is mediated by SARA (Smad anchor for receptor activation). Once in the nucleus, Smad complexes remain associated, through the MH1 domain, with other DNA-binding transcription elements (SBEs) to regulate expression. They attain great selectivity and affinity for the target promoters with the appropriate binding elements (19). The different Smad pathways activation through the functional receptor complex is schematized in Figure 1. Different types of cells, or cells subjected to dissimilar conditions express discrete collection of transcriptional factors partners for Smad, thus explaining the reason why the cellular response to TGF-β is linked to the cellular context. Apart from this, it was described that TGF-β induces Smad1 phosphorylation independently of the BMP type I receptors (ALK1/2/3/6) mainly in endothelial cells and myoblasts (20). TGF-β requires the kinase activity and the L45 loop motif of the type I TGF-β receptor, ALK5 to stimulate the phosphorylation of Smad1 and Smad5, being this phosphorylation an essential event to the initiation and promotion of TGF-β-stimulated migration in mammary epithelial cells (21).

2.2. Smad-independent signal transduction

Besides Smad-mediated transcription, TGF-β triggers other cascades that in turn regulate Smad activation, or prmpt responses unrelated to transcription. TGF-β can regulate non-Smads pathways, such as Erk, p38MAPK, NFκB, Jun N-terminal kinase (JNK), HIF-1(hypoxia-inducible factor-1), PI3K-Akt and Small GTPases (rhoA and Cdc42) (22). These signaling cascades regulate basic cell functions, such as proliferation and migration. Recently, ubiquitin ligase tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) and TGFβ-associated kinase 1 (TAK1) have been indicated to be vital for the activation of the JNK MAPK and p38pathways (22), and even organ fibrosis (23).

2.3. Regulation of the TGF-β superfamily members

While BMPs are secreted in their active form and regulated by different BMP antagonists, such as, Dan, Chordin, twisted gastrulation or Noggin (24, 25), TGF-*β*s are secreted in an inactive form, as precursor proteins containing LAP (Latency-Associated Peptide). This way, they are maintained in a multiprotein complex, interacting with ECM proteins and integrins (26).

The stability and availability of both TGF-β receptors and Smads are tightly regulated. They undergo post-translational modifications such as phosphorylation, ubiquitylation or sumoylation. For a more precise regulation, Smad signals are negatively regulated. For this reason, an auto-inhibitory feedback process for ligand-induced signaling in which the I-Smads (or Inhibitory Smads), Sma6 and Smad7, are activated by BMP and TGF-β occurs. Smad6 preferentially prevents BMP signaling by forming an inactive Smad1-Smad6 complex. While Smad7 binds TGF-β, Activin and BMP type I receptors and inhibits R-Smad phosphorylation (27).

As a minimum, there are two internalization pathways that determine if receptors will stimulate a signaling response or they will undergo degradation (28). The first route comprises the clathrin-dependent internalization. In this route, the activated receptors can form heteromeric complexes with Smad4 and travel to the nucleus, or back to the cell surface. The second route involves the lipid-raft-caveolae-1 vesicles. In this case the

receptor is a target for polyubiquitination and stands predestined for degradation. The I-Smads (Smad6 and Smad7) recruit the Smurfs proteins with E3 ubiquitin ligase activity and aim the activated receptor complex for proteasomal degradation. Smurf1 (Smad-ubiquitination-regulatory factor 1) interacts with Smad1 and Smad5, influencing BMP responses, while Smurf2 relates with diverse R-Smads, interfering with both BMP and TGFβ/activin signaling. Smad4 is not subjected to ubiquitin-mediated downgrading, and sumoylation enhances stability. The proteasomal downgrading also adjusts the R-Smads levels after translocation inside the nucleus. Several reviews describe many other systems regulating TGF-β family signaling (27, 29).

3. Activin A receptor, type I-like kinase 1 (ALK1): Structure and function

Activin Receptor-Like kinase-1 (ALK1) is a serine-threonine kinase that acts as a type I cell surface receptor for the Transforming Growth Factor-β (TGF-β)/Bone Morphogenetic Protein (BMPs) superfamily of ligands. ALK1 interacts with four ligands: TGF-β1 and TGF-β3, in a complex with the receptor type II (TβRII); and with BMP-9 and BMP-10. ALK-1 is predominantly expressed in endothelial cells, although it was also found in other types of cells. ALK1 has a critical role in the regulation of physiological and pathological angiogenesis. Its function in angiogenesis is the most reviewed and controversial point until now. ALK1 mutations are also implicated in the pathogenesis of the Rendu-Osler Weber disease or Hereditary Hemorrhagic Telangiectasia (HHT) type 2, an autosomal dominant vascular dysplasia linked to the loss-of- function mutations of ALK1.

3.1. ALK1 structure

The ALK1 structure consists of four different regions. The first region is the signal peptide (SP), which is removed during processing to generate the mature protein. The second is the cysteine rich EC domain, which is important for ligand binding. The third is the intracellular domain (IC), with the glycine/serine-rich domain (GS) (172-201) at the juxtamembrane position. The IC is in charge of the kinase activity regulation; and the fourth is the kinase responsible for phosphorylation of Smad1/5/8 (202-492), and the single transmembrane domain. ALK1 misses the residue F85, involved in the

hydrophobic interactions between BMPs and their type I receptors, giving the specific structural basis of binding (30, 31). ALK1 structure is schematized in Figure 2.

3.2. ALK-1 expression

Early ALK1 expression was detected in mouse embryos at 6.5 days post conception, reaching its highest expression at day 7.5-8.5 at sites of vasculogenesis (32). The expression was shown to be more prominent in endothelial, besides to a lesser degree in vascular smooth muscle cells (33). In mice, it has been detected in bone marrow stromal cells and the mesenchymal-epithelial border and in the spleen, kidney and brain (32, 33). In the adult rat, ALK1 is most abundantly expressed in the lungs, although it has also been detected in the kidney, the brain, the heart, intestine, thymus, the stomach, the spleen, and the urogenital ridge of rat embryos (33). In humans, ALK1 was first described in the placenta, adipose tissue, and skeletal muscle (34). In adult humans, ALK1 exhibited major expression in the blood vessels of the lung, predominantly in capillaries and arterioles (35), and in the lymphatic endothelial cells. ALK1 expression is up-regulated during periods of active angiogenesis, during wound healing, heart repair or tumor angiogenesis (36, 37) (38). ALK1 has also been reported in monocytes (39), microglia (40), skin fibroblasts (41), hepatic stellate cells and chondrocytes (4, 42), neural crest stem cells (43), scleroderma fibroblast (7), and myoblasts (44).

In some pathology such as osteoarthritis ALK1 is overexpressed (45). In other fibrotic pathologies, such as scleroderma and liver fibrosis, ALK1 expression has not been analyzed but the presence of ALK1-activated proteins have been described. Thus, phosphor-Smad1 is overexpressed in the skin of patients with scleroderma (7). Moreover, Id1 (inhibitor of differentiation 1) is overexpressed in experimental models of liver fibrosis (4).

3.3. ALK-1 function

ALK1 was first believed to be an orphan receptor, but now it is known that ALK1 relates with four ligands: TGF-β1 and TGF-β3, in a complex with the receptor type II (TβRII); and with BMP-9 and BMP-10, in a complex with the Activin Receptor type

IIA (ActRIIA) and IIB (ActRIIB) or the BMP receptor type II (BMPRII) (46-52). The ALK1 specificity is because of a unique receptor orientation and a special set of interfacial contacts (53).

Ligand-dependent ALK1 activation stimulates phosphorylation of Smad1/5/8 (54), and the receptor-activated Smads form a heteromeric complex with a joint partner molecule, Smad4. The complexes are translocated inside the nucleus, where cell type-specific transcriptional factors activate or repress transcription of target genes. ALK1 was described not to bind TGF-β ligands directly, but as we indicated above, it forms a high affinity heteromeric receptor complex with TβRII in the presence of ligand (46). After ligand binding, TβRII phosphorylates the juxtamembrane GS domain of ALK1, which activates the serine/threonine kinase activity of TβRII (54, 55). However, the promoter of the gene which codifies for ALK1 protein (*ACVRL1*) was recently shown not containing any TGFβ-responsive element, suggesting that its promoter activity is not controlled by TGF-β1 as it was indicated before. Nevertheless, it contained a high amount of GC-rich Sp1consensus sites in endothelial cells (56).

It should be noted that the type III receptor (or co-receptor) Endoglin has been reported to increase TGF-β1 signaling through the TGF-β1/ALK1/Smad1 pathway (44, 57-60).

4. Is there a role for ALK1 in ECM protein expression and synthesis?

4.1. TGF-β1 in ECM synthesis

TGF-β1 induces an increase in transcription, synthesis and secretion of ECM proteins, including collagen I, III, IV, fibronectin, lamimin and glycoproteins such as osteopontin, osteonectin, tenascin, biglycan and decorin (61). On the other hand, TGF-β1 decreases ECM degradation through two main mechanisms. In the first process, it inhibits matrix metalloproteinases (MMPs), responsible for ECM degradation and in the second process promotes tissular inhibitors of metalloproteinases (TIMPs) (62-64). TGF-β also has important effects in fibroblasts, the main source of ECM production observed in organ fibrosis. TGF-β behaves as a chemotactic factor, but additionally regulates transformation of fibroblasts and other cellular types into myofibroblasts (65, 66) as well as EMT (66).

Smad3 has been considered as an essential mediator of TGF-β signaling (67-69). Moreover, the promoter areas of COL1A2, COL3A1, COL5A1, COL6A1, COL6A3 and COL2A1 have Smad3 binding sequences (67, 70-73).

4.2 ALK1 and Extracellular matrix synthesis

4.2.1 ECM synthesis in endothelial cells and blood vessels

In endothelial cells, ECM protein synthesis has been linked to the resolution stage of angiogenesis. In this way, TGF-β signaling over ALK1 receptor and Smad1/5/8 pathways would promote endothelial proliferation and migration (58, 59). On the other hand, TGF-β signaling through ALK5 receptor would promote ECM protein synthesis such as PAI-1 and fibronectin (20). ALK1/Smad1/5 pathway is an antagonistic mediator of ALK5/Smad2/3 signaling in ECs (54) and, due to this antagonism, it regulates negatively ECM protein expression in these cells.

Whereas ALK1 is mainly expressed in the endothelium, it has been demonstrated that ALK5 has a major expression in internal layers of vessels, being important in vascular smooth muscle cells, suggesting a pivotal role of ALK5 receptor in vascular fibrosis (74). ALK5 plays an important role in ECM protein expression in numerous cell types (75). A recent paper also demonstrated the expression of ALK1 in smooth muscle cells. In the paper, the authors did not attribute ALK1 to a role in smooth muscle cell differentiation (76). We suggest that the expression of ALK1 in these cells may be related with the regulation of ECM protein expression. ALK1 would regulate negatively TGF-β/ALK5 induced ECM protein expression because of its lateral antagonism between ALK1 and ALK5 demonstrated in other cells (54).

L6E9 myoblasts are a cellular model where TGF-β signaling has been described in a very exhaustive way. Scherner et al. demonstrated that in these cells, BMP-7 induces Smad1 and Smad5 phosphorylation through ALK1 receptor and differentiation inhibitor 1 (*Id1*), while Smad2 and Smad3 phosphorylation is impaired, leading to a lower expression of ECM proteins (60). These authors also observed that the overexpression of endoglin, enhanced the BMP-7/Smad1/Smad5 pathway. As a result, endoglin may play a pivotal role in this TGFβ/BMP-7 balance. On the other hand, Velasco et al (2008)

demonstrated that in L6E9 myoblasts ALK1/Smad1/Smad5 pathway promotes cellular proliferation and inhibits ECM protein synthesis, while ALK5/Smad2/Smad3 leads to an increase in ECM protein synthesis (Fibronectin, collagen type I and CTGF) and decreasing cellular proliferation (44).

4.2.2 ALK1/ALK5 ratio in human chondrocytes

ALK1 and ALK5 are expressed in cultured human chondrocytes. ALK5 was essential for TGF-β signaling trough Smad2 and Smad3, although ALK1 was necessary for TGF-β signaling through Smad1 and Smad5. ALK5 was responsible for ECM protein synthesis (collagen, fibronectin, PAI-1), though ALK1 inhibited the process (42). The same authors demonstrated that Endoglin was the molecule involved in modulating the balance between ALK1 and ALK5 pathways. Inhibition of endoglin activity led to an activation of Smad2/3 pathway that could be reverted with ALK1 overexpression. Moreover, Endoglin was overexpressed in cultured chondrocytes with osteoarthritis (OA) regarding chondrocytes from normal cartilage (77).

4.2.3 ALK1 in liver cells

Weng et al. demonstrated first that TGF-β induced connective tissue growth factor (CTGF, an ECM-related cytokine) expression through ALK5 pathway in hepatocytes. ALK5 inhibition with SB-431552 reduced CTGF expression in hepatocytes in basal conditions and after treatment with TGF-β. Moreover, overexpression of ALK5 strongly induced CTGF expression (78). Smad2 seems to hold the key in CTGF expression induced by TGF-β (79). Concersely, overexpression of ALK1 increased significantly CTGF expression in hepatocytes. Besides, this overexpression increased Smad phosphorylation following TGF-β treatment in hepatocytes (78). The studies indicate that ratio ALK1/ALK5 modulates CTGF in hepatocytes. It should be noted that ALK1 involvement in ECM protein expression in hepatocytes is not similar to other hepatic cells such as hepatic stellate cells, and thereby contributes differently to hepatic fibrosis.

The role of ALK1/Smad1/Id1 pathway in hepatic stellate cells (HSCs) transdifferentiation into myofibroblasts and its consequences in hepatic fibrosis have

been strongly studied (4, 80). Nevertheless, ALK1 role in ECM protein expression in these cells remains poorly studied. In HSCs it was demonstrated that compound 861 (Cpd861) inhibits ALK1 expression and consequent Id1 expression leading to a decrease in α-SMA expression (81). The Cpd 861 has anti-fibrotic properties, as it has been demonstrated in HSCs, downregulating mRNA levels of collagen type III and TGF-β1, and increasing MMP-1 expression (82). Thus, it could be suggested that the inhibition of ALK1 and collagen synthesis by Cpd861 in HSCs may be related mechanisms that should be studied.

3.1.2.4. Role of ALK1 and ALK5 in ECM synthesis in fibroblasts

Mouse embryonic fibroblasts (MEFs) knock out (KO) for Smad3 have an impaired ECM protein synthesis (fibronectin and PAI-1) (83). Moreover, Piek et al. characterized KO MEFs for Smad2 and Smad3 and observed a relevant but different role of Smad2 and Smad3 in ECM protein synthesis such as PAI-1 and fibronectin (84). On the other hand, Smad3 inhibition with SIS3, a specific inhibitor, leads to a decrease in ECM protein synthesis in fibroblasts (85).

In scleroderma fibroblasts (SSc) however, it has been demonstrated that ECM protein synthesis, such as collagen I and CTGF, is promoted by Smad1 and Erk1/2 pathways (7). Moreover, CTGF has been shown as an important effector of Smad1 pathway in scleroderma fibroblasts (7-9). A recent paper proposes that Endoglin and ALK1 are responsible for Smad1 phosphorylation and consequent fibrotic response in SSc fibroblasts (9).

We have recently demonstrated that $ALK1^{+/-}$ renal fibroblasts in primary culture express more collagen I and fibronectin because of a higher Smad3 and Smad2 phosphorylation in basal conditions (86).

In conclusion, although TβRI ALK5 transduces the main TGF-β signals through Smad3 to express these proteins in several cell types such as endothelial cells, myoblasts, fibroblasts, chondrocytes and vSMCs, levels of TβRI ALK1 may regulate ALK5 signaling and its effects in these cellular types. In this way, higher levels of ALK1 impair TGFβ/ALK5/Smad2/3 induced ECM protein expression, or else lower levels of

ALK1 may promote TGFβ/Smad2/3 activity. Nevertheless, in other cells types such as SSc fibroblasts and hepatocytes, ALK1 signaling has an important role in TGF-β-dependent ECM protein synthesis. Thus, ALK1 role in ECM regulation is cell-dependent and most of the studies suggest that TβRI ALK1 counteracts ALK5 effects in ECM homeostasis. A scheme of the possible role of ALK1 in ECM production is shown in figure 3.

4.3 Possible role of ALK1 in epithelial to mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a phenomenon in which epithelial cells lose epithelial characteristics, disassembly cell-to-cell binding structures and acquire motile as well as invasive properties (87), and acquire a mesenchymal phenotype (88). EMT is necessary for embryonic development, but it also participated in tumor development and organ fibrosis (89, 90) (91, 92); However, the involvement of EMT in kidney fibrosis is a conflictive point and some authors do not consider the phenomenon relevant (93). Epithelial cells closely link with each other through laterally positioned, specific cell-cell contact structures, such as tight junctions, adherents junctions and desmosomes. The initiation and subsequent completion of EMT occurs through the precise coordination of several molecular events, including activation of EMT-inducing transcription factors, altered expression of cell-surface proteins, reorganization of the actin cytoskeleton and enhanced invasive properties (66)

Tight junctions are membrane fusions at the apical surface formed by proteins such as occludin, claudin, zonula occludens (ZO)-1,-2,-3 and p120 (66). Disassembly of tight junctions is one of the early events in EMT (66, 94). *Adherens junctions* (adjacent to tight junctions) are characterized by presence of E-cadherin. During EMT, loss of E-cadherin takes place and the adherens junction complexes disassemble leading to a reorganization of actin cytoskeleton (66, 94). *Desmosomes* are structures similar to adherens junctions, in this case characterized by the presence of proteins, such as desmocollin and desmoglein. These proteins are organized as individual patches (95). Plakophilin and plakoglobin form the “Armadillo” proteins and interact with desmoplakins. EMT is associated with loss of desmoplakin expression (66, 96). The start and subsequent end of EMT occurs through a detailed coordination of numerous

molecular events, including stimulation of EMT-inducing transcription factors, altered expression of cell-surface proteins, restructuring of the actin cytoskeleton and improved intrusive properties.

TGF-β can promote EMT by transcriptional and posttranscriptional control of an array of transcription factors that subdue epithelial features (expression of components of cell junctions and polarity complexes), and enhance mesenchymal features (assembly of matrix molecules, stimulation of cell migration) (97, 98).

4.3.1 TGF-β and EMT

As above mentioned, induction of EMT by TGF-β has been demonstrated in several epithelial cultured cells. In numerous studies, treatment with TGF-β1 leaded to an increase of mesenchymal markers (vimentin, fibronectin…etc) and a decrease of epithelial markers (ZO-1, E-cadherin) (99-102).

Activation of TβRII and the TβRI ALK5 is vital for TGF-β induced EMT. TβRII involvement in this process has been demonstrated in colon cancer cells, in skin, and mammary cancer (103). *In vitro* studies with epithelial cells, such as NMuMG cells have demonstrated the involvement of ALK5 in EMT (100, 101).

Several studies have demonstrated the function of Smads (Smad2, Smad3 and Smad4) in TGFβ induced EMT. *In vitro* studies with NMuMG cells have shown that increased expression of Smad2 and Smad3 induced EMT (100, 101). Smad3 has been considered pivotal in EMT in renal tubular cells. Treatment with TGF-β1 in Smad3 KO renal tubular cells does not induce loss of E-cadherin expression, and consequent EMT. For this reason, Smad3 KO mice do not develop renal fibrosis following unilateral ureteral obstruction (104). On the other hand, keratinocytes resulting from Smad3 KO mice have a diminished migration induced by TGF-β (105).

Many non-Smad pathways are also involved in TGF-β-mediated EMT, It was described that TGF-β can trigger JNK via MKK4 and p38 MAPK through MKK3/6 in many cell types (106-110), regulating the TGF-β-induced EMT (111) and the TGF-β/BMP-induced apoptosis (112, 113). RhoA has also an important role in TGF-β-induced EMT (109). The PI3K/Akt pathway is another non-Smad pathway implicated in TGF-β-

induced EMT. The activated PI3K/Akt controls translational responses through mTOR (mammalian target of rapamycin) and S6K, which works together with the Smad-dependent transcriptional responses during EMT (114).

4.3.2. ALK1 and EMT

The first evidence of ALK1 involvement in EMT was the detection of ALK1 in pancreatic carcinoma cell lines (115). Later, it was described the in antioncogenic role of ALK1 in pancreatic cancer cells through an inhibition of ALK5-induced EMT and ALK5-independent mechanism of growth inhibition (116). However, until currently, no enough evidences of ALK1 expression and involvement in EMT in epithelial cells exist. For example, Valcourt et al. did not detect ALK1 expression in NMuMG cells using PCR (101), and no enough evidence about the expression of ALK1 in renal tubular cells exists. Nevertheless, the corresponding Smads activated by ALK1 in other cellular types are expressed in epithelial cells and play important roles in EMT (117-119) suggesting the activation of this pathway by other receptors. Recently, it has been demonstrated the involvement of BMP-9/ALK1/Smad1 signaling in EMT in hepatocellular carcinoma. In hepatocellular carcinoma cell lines HepG2 and HLE, treatment with BMP-9 induces phospho-Smad1 activation and decreases E-Cadherin expression. In cryosections from hepatocellular carcinoma in mice, BMP-9 colocalizates with tubular cells with decreased E-Cadherin. Overexpression of ALK1 induces an increase of mesenchymal markers such as vimentin and a decrease of E-Cadherin, suggesting a role of BMP-9/ALK1/Smad1 in the induction of EMT (120).

On the other hand, as best described in section 3.2.5., Id1, a target gene of ALK1 signaling pathway, has an important role in process, such as the transdifferenciation of HSCs into myofibroblasts (4), and epithelial-to-mesenchymal transition in renal cells (121).

BMP-7 does not bind ALK1, but binds ALK3 and ALK6 (122). In some cell types, however, BMP-7 activates its receptors and promotes Smad1 and Smad5 phosphorylation (60), similar to what occurs after ALK1 activation in endothelial cells (54). For this reason, we will try to analyze in the following lines the involvement of

BMP-7 and ALK3 in EMT, mainly related to renal fibrosis (main source of information and studies about role of BMP-7 in EMT).

4.3.3 BMP-7 and EMT in renal fibrosis context

BMP-7 is endogenously expressed in distal tubules and collecting ducts in the adult kidneys (123). Its expression however, decreases in the experimental models of renal fibrosis. Moreover, overexpression of BMP-7 protects against kidney injury (124). The main beneficiaries of BMP-7 activity are tubular epithelial cells, but BMP-7 also has effect in fibroblasts, mesangial cells and podocytes (125, 126). In these cellular types, BMP-7 antagonizes TGFβ activity (127).

In NP1, a line of distal tubular cells, it has been demonstrated that treatment with TGF-β1 for 48 hours, induces a decrease in E-cadherin and ZO-1 expression, and treatment with BMP-7 for 48 hours reverts this effect and enhances E-cadherin and ZO-1 expression. Besides, treatment with human recombinant BMP-7 reverts renal fibrosis in an experimental model of fibrosis based on the administration of nephrotoxic serum (NTS) for 3 months in rats (117). These authors demonstrated that epithelial cell with loss of E-cadherin express phospho-Smad2 and phospho-Smad3 whereas tubule cells with repaired tubules with re-expression of E-cadherin co-express both phospho-Smad1 and phospho-Smad2/3. The authors concluded that Smad1 phosphorylation is essential to E-cadherin expression recovery and that BMP-7 counteracts renal fibrosis induced by NTS through Smad1 phosphorylation (117). Other studies demonstrated the antifibrotic potential of BMP-7 after unilateral ureteral obstruction (UUO) (118). They showed that intraperitoneal administration of BMP-7 reverted renal fibrosis induced by UUO. The authors suggested an antagonistic role between Smad1 and Smad2/3. While Smad2/3 phosphorylation increased, Smad1 phosphorylation decreased 3 days after UUO. Treatment with BMP-7 induced a higher Smad1 phosphorylation following UUO and its consequent decrease of renal fibrosis (118).

The primary target of BMP-7 in its protective effect against renal fibrosis seems to be the tubular epithelial cells (122). Other studies with human renal proximal tubule epithelial cells (HKC8 and HK2) have demonstrated that treatment with BMP-7 not only induced an increase in E-cadherin expression but also induced a decrease in basal

and TGF-β-induced expression of alpha-smooth muscle actin (α-SMA). The authors suggest that BMP-7 induces Smad1/5 phosphorylation promoting E-cadherin synthesis and inhibiting Smad2/3 phosphorylation and with the consequent decrease of α-SMA expression (128). Furthermore, BMP-7 inhibits TNF-alpha stimulated expression of proinflammatory and chemoattractants genes in proximal tubule epithelial cells (129). It should be noted that inflammation plays also a major role in promoting EMT (89).

4.3.4. ALK3 and EMT

BMP-7 binds to type I receptors ALK2, ALK3 and ALK6 and the type II receptor BMPR2 in several different cell types (130). BMP type I receptors stimulate Smad1, Smad5 and Smad8 signaling (130). In the kidney epithelial cells, BMP-7 receptors stand express and might facilitate BMP-7 effects in the kidneys (131).

The involvement of ALK3 to the protective effect of BMP-7 against EMT was first demonstrated using tubular epithelial cells. In these cells, constitutive transfection with ALK3 induced BMP-7-driven pathway (Smad5) and regulated E-cadherin promoter (117).

In mice treated with nephrotoxic serum, ALK3 expression increases after 1 week but decreases until the sixth week of kidney injury. Deletion of ALK3 in mice treated with nephrotoxic serum promotes kidney injury and EMT, observed by a decrease in E-cadherin and an increase in S100A4 (specific marker for fibroblasts) (119). THR-123, a peptide agonist for ALK3 reversed EMT in tubular epithelial cells. Furthermore, in other several mouse models of acute and chronic renal injury, THR-123 prevented inflammation, apoptosis and EMT and reversed established fibrosis (119). On the other hand, removal of ALK3 in mice prevents THR-123 effects in the NTS model, thus suggesting the specificity of this drug on the ALK3 signaling pathway (119).

4.3.5. Id1 and EMT

Id1 belongs to a family of helix-loop-helix (HLH) transcriptional inhibitors (*Id1, Id2, Id3* and *Id4*) (132). *Id1* is considered a target gene of ALK1/Smad1/Smad5 pathway in

endothelial cells (54). Id1 is involved in the inhibition of myoblast and neuronal differentiation (133). As bHLH proteins control the activation of several genes required for the maintenance of cell differentiation status, Id1 may play a pivotal role in EMT phenomenon. In this way, *in vitro* studies with HKC-8 cells demonstrated that Id1 is induced by TGF-β during EMT. Overexpression of Id1 in these cells suppressed E-cadherin and ZO-1 expression (132). In addition, the authors demonstrated that the expression of Id1 was mediated through the TGF-β canonical signaling pathway. Liang et al. demonstrated through *in vitro* studies with the epithelial cell line MCF10A that Smad3 took part in the induction of Id1 by TGF-β (134). Although the involvement of Id1 in EMT and renal fibrosis seems to be clear, the involvement of ALK1activation in this process still remains unclear. Id1 induction in epithelial cells may be Smad3 (but not Smad1) dependent, at difference of endothelial cells, where Id1 induction is Smad1/5/8-dependent (134). On the other hand, as it has been previously described, Id1 is responsible for transdifferentiation of HSCs into myofibroblasts through the activation of the ALK1/Smad1 signaling pathway (4).

A scheme of the role of the several TGF-β and BMPs receptors and signaling pathways in EMT is given in figure 4.

5. ROLE OF ALK1 IN FIBROTIC DISEASES

5.1. Kidney fibrosis

Renal fibrosis is the common en of all progressive kidney disease (135). Pathological fibrosis results in glomerulosclerosis, tubulointerstitial fibrosis, tubular atrophy and dilation as well as and rarefaction of the glomerular and peritubular capillaries (135-137). Synthesis and accumulation of ECM in the interstitial space, inflammatory cell infiltration, tubular cells apoptosis, abundance and proliferation of fibroblasts/myofibroblasts, inflammatory cell intrusion, rarefaction of the microvasculature are major characteristics of tubulointerstitial fibrosis (136, 138). Interstitial fibroblasts and myofibroblasts are considered the main cells taking part in the synthesis and accretion of ECM proteins in tubulointerstitial fibrosis (139-141). The

normal renal interstitium contains fibroblasts and dendritic cells. However, mechanical stress, cytokines and various other factors induce fibroblasts to acquire a myofibroblast phenotype. Myofibroblasts contain stress fibers, and express alpha-smooth muscle actin, considered as a marker for this cell type (135, 142-144). Although not unanimously admitted, myofibroblast can also derive from epithelial (and perhaps endothelial) cells by EMT, as above described in section 4.3.

Glomeruloesclerosis is caused by a buildup of ECM in the renal glomeruli, mainly in the glomerular mesangium resulting in the narrowing of glomerular capillary (145), excessive protein ultrafiltration and a reduced glomerular filtration rate (GFR) (1).

5.1.1. TGF-β and renal fibrosis

From the first studies published in 1990 demonstrating the fibrogenic effects of TGF-β, a vast body of evidence has been accumulated demonstrating that overexpression of TGF-β is a key mediator of fibrotic disease. Its fibrogenic actions include stimulation of matrix synthesis, inhibition of matrix degradation and modulation of matrix receptor expression to facilitate cell-matrix interactions (1).

The effect of increased TGF-β levels in renal fibrosis has been studied in several animal models. Initially, studies in transgenic mice that overexpressed TGF-β in plasma showed ECM accumulation, interstitial fibrosis and glomerulonephritis in the kidney (146). Alternatively, blocking TGF-β with neutralizing antibodies impairs the advancement of renal fibrosis in different animal models (147). Moreover, treatment with anti-TGF-β antibodies prevented both tubular apoptosis and atrophy, but increased the tubular proliferation in the UUO experimental model of tubulointerstitial fibrosis (148). Relevance of TGF-β in the origin and maintenance of glomerulosclerosis (149) and tubulointerstitial fibrosis (150) has been clearly demonstrated. These initial data have been later confirmed by multiple studies (1). These effects of TGFβ are a consequence of its controlling role in cell proliferation (151, 152) and in renal ECM synthesis and degradation (149, 153).

TβRI ALK5 has been considered traditionally the main receptor taking part in the phenomenon of TGF-β induced renal fibrosis (13). Smad3 Knock-out (KO) mice are protected against renal fibrosis after 14 days UUO (104). The authors propose that the

key of Smad3 in renal fibrosis following UUO is its involvement in epithelial-to-mesenchymal transition (EMT). *In vivo* experiment with cultured Smad3 deficient tubular epithelial cells demonstrated that treatment with TGF-β1 did not decrease E-cadherin (epithelial marker) expression whereas did not increase α-SMA (mesenchymal marker) (104). Other authors have demonstrated that Smad3 deficient mice are protected against renal tubular apoptosis and inflammation (154).

Although Smad3, the main effector of ALK5 receptor, has a clear role in renal fibrosis development, the role of Smad2 remains unclear. Smad2 seems to be a protector molecule against TGF-β/Smad3 induced renal fibrosis. Meng et al. (2010) generated conditional knock-out (KO) Smad2 mice, observed an increase in extracellular matrix protein expression, and a higher phospho-Smad3 phosphorylation following UUO. Moreover, mouse embryonic fibroblasts (MEFs) from these mice express less ECM protein and more metalloproteinases, such as MMP-2 (155).

Smad4 (co-smad) is otherwise essential for UUO-induced renal fibrosis, as it has been demonstrated in *in vivo* experiments with conditional knock-out (KO) Smad2 mice and MEFs, although Smad7 deficiency increases renal fibrosis after 7 days of UUO (156). For this reason, Smad7 has been considered as a therapeutic target against renal fibrosis(157), (158).

5.1.2. Is there a role of ALK1/Smad1/5 pathway in renal fibrosis development?

First evidences of Smad1 involvement in renal fibrosis were found in diabetic nephropathy (DN) studies. It has been reported in a model of DN induced by streptozotozin administration that collagen type IV overexpression was mediated through Smad1, and the process was activated by cytokines such as PDGF and synergized with other pathways such as STAT3 (159, 160). *In vitro* studies with rat cultured mesangial cells demonstrated that Smad1 activation was related with alpha-SMA overexpression in these cells and contributed to mesangial matrix expansion (14, 161). In other estreptozotocin-induced experimental model of DN in rats, it was observed that Smad1 activation was Angiotensin II dependent. The inhibition of Angiotensin II receptor with Omelsartan impaired Smad1 activation and following mesangial expansion in DN (162).

Until today, the only study that related directly ALK1 expression with renal fibrosis development was performed in two models: First, in a model of radiation-induced renal fibrosis using ALK1 heterozygous mice ($ALK1^{+/-}$) and control mice ($ALK1^{+/+}$). This study demonstrated that $ALK1^{+/-}$ mice develop less renal fibrosis and inflammation that $ALK1^{+/+}$ mice 20 weeks after irradiation. However, an increase in pro-inflammatory and pro-fibrotic gene expression 30 weeks was found in $ALK1^{+/-}$ compared with $ALK1^{+/+}$ mice 30 weeks after irradiation (163). On the other hand, we have recently demonstrated that $ALK1^{+/-}$ mice develop more renal fibrosis after UUO compared to $ALK1^{+/+}$ mice. Moreover, we have observed that ALK1 expression is up-regulated in this experimental model. The increase in ECM protein expression in obstructed kidneys from $ALK1^{+/-}$ mice is not because of an increase in myofibroblast abundance, suggesting that the role of ALK1 in renal fibrosis in this model resides in the modulation of ECM protein expression by myofibroblasts (86).

In contrast to these studies, it has been reported that a potential treatment against renal fibrosis entails an administration of BMP-7 that activates the ALK1/Smad1/Smad5 signaling pathway as described in section 4.3.3. During the last years a higher number of studies have demonstrated that BMP-7 has a protective effect against renal fibrosis in several experimental models (117, 164, 165). BMP-7 protection in the OUU model has been related with Smad1/5 activation following UUO (118). An overview of the potential role of ALK1 in renal fibrosis is shown in Figure 5.

5.2. Liver fibrosis

Liver fibrosis takes place in numerous categories of chronic liver diseases. It is exemplified by buildup of ECM proteins, causing interference of tissue function and homeostasis and in some cases leading to organ failure (166). Several factors, such as infection with hepatitis viruses, prolonged liquor abuse and aflatoxin may provoke hepatic fibrogenesis. Activation of the hepatic stellate cells (HSCs) is one of the early ocurrences in the progress of hepatic fibrosis. During this process, HSCs acquire a new phenotype (myofibroblasts) and produce ECM proteins (4).

One of the main singularities studied in liver fibrosis development is the transdifferentiation of HSCs into myofibroblasts, a phenomenon similar to EMT in

which TGF-β plays a major role (167). Other authors demonstrated that HSCs transdifferentiation into myofibroblasts was mediated by Id1, a target gene of Smad1/Smad5 signaling in endothelium (54) and other cell types (44). In cultured HSCs and cirrhotic fat storing cells (CFSCs), treatment with TGF-β1 induced Smad1 phosphorylation and Id1 expression. The increase of Id1 relates to expression of α-SMA (4). The authors suggest that the receptor involved in TGFβ signaling through Smad1 is ALK1. For this purpose they silenced and overexpressed ALK1 and observed parallel changes in Smad1 and id1 expression. Moreover, phospho-Smad1 and Id1 were overexpressed following bile duct ligature in rats, an experimental model of liver fibrosis (4).

In vitro studies with different hepatic cell types (HSCs, myofibroblasts (MFB), hepatic sinusoidal endothelial cells (LSEC) and Kupffer cells (KC) have demonstrated that treatment with TGF-β induces an increase in Endoglin expression. Overexpression of endoglin induces an increase in fibrogenic properties of TGF-β increase of α-SMA expression, and its consequent transdifferentiation from HSC into MFB and fibronectin. An overview of the potential role of ALK1 in hepatic fibrosis is shown in Figure 5.

4.3. Cartilage homeostasis and osteoarthritis

Osteoarthritis (OA) is a pathology characterized by cartilage disorganization. In normal conditions, chondrocytes are cells the responsible for cartilage homeostasis. In OA, chondrocytes overexpress MMP-13, a metalloproteinase that degrades collagen, leading to a weak structure of the cartilage (168). TGF-β, Smad2 and Smad3 are involved in chondrocyte differentiation (169). TGF-β is considered as a powerful cytokine involved in reparation in OA (170), (171). Blaney-Davidson et al. have demonstrated that TβRI receptors ALK1 and ALK5 are expressed in chondrocytes and transduce TGF-β signals. In an OA experimental model (DMM), ALK1/ALK5 ratio is increased. ALK1 overexpression is related to MMP-13 expression, whereas ALK5 is related with agrecan synthesis and cartilage structure stabilization (45). On the other hand, BMP-2, a possible ALK1 binding ligand, induces collagen II synthesis and agrecan, and degrades cartilage matrix in physiological conditions, in order to maintain tissue homeostasis (171).

Both ALK1 and ALK5 receptors are expressed in cultured human chondrocytes. ALK5 is essential for ALK1 signaling and ALK1 inhibits TGFB/ALK5/Smad2/3 signaling. TGF-β induced ECM protein such as fibronectin, collagen type II and PAI-1 in human chondrocytes whereas ALK1/Smad1 signaling inhibits this pathway (77). Endoglin is involved in this balance between ALK1 and ALK5 pathways and is overexpressed in OA (77). As a result, the ratio ALK1/ALK5 regulates cartilage degradation and osteoarthritis development. For this reason, ALK1 and ALK5 may be considered as therapeutic targets against OA because of their regulatory properties of ECM synthesis.

5.4 Systemic sclerosis

Systemic sclerosis (SSc) is a fibrotic pathology with important relevance in several organs such as heart, lung, gastrointestinal tract and skin. End stages of SSc are characterized by an excessive accumulation of ECM protein buildup leading to tissue and organ dysfunction (172, 173). In SSc, activated myofibroblasts are the main source of ECM compounds, and this process is regulated by numerous cytokines, such as TGF-β (9, 174). In SSc fibroblasts, Smad2/3 and Smad1/5 balance is altered, mainly because an overexpression of Endoglin (175) that counterbalances TGF-β signaling and lead to a Smad1 overphosphorylation in SSc fibroblasts (9). Pannu et al. demonstrated that profibrotic phenotype in SSc fibroblasts is regulated mainly by Smad1 and Erk1/2 pathways. A higher phosphorylation of Smad1 and Erk1/2 leads to an increase in ECM protein synthesis, such as Collagen I and CTGF (7), (176). A recent paper (9) demonstrated that both endoglin and ALK1 are important molecules in Smad1 activation and consequent fibrotic phenotype in SSc fibroblasts.

In conclusion, several papers suggest that ALK1/Smad1/Smad5 pathway plays a fundamental purpose in the origin of systemic sclerosis. Many evidences that the ALK1/Smad1/Smad5 signaling pathway takes part in the regulation of organ fibrosis in a way that depends of the type of cells implicated and other circumstances exist. A scheme of the role of ALK1 in several fibrotic diseases is given in figure 5.

6. THE ALK1/SMAD1 PATHWAY AS A TARGE FOR FIBROSIS THERAPY

As TGF-β was considered as a powerful cytokine in organ fibrosis, TβRI and TβRII have been considered as potential anti-fibrotic therapeutic targets. Several strategies to interfere with the TGF-beta signaling pathway have been used. Among them, the use of TGR inhibitors, or TGR agonists have been the most developed (Figure 6).

6.1. ALK1/Smad1 inhibitors

ALK1 inhibition has been less studied compared to ALK5. Compound 861 (Cpd861) is a conventional herbal medicine used by the Chinese to manage liver illnesses. It has been shown to possess anti-fibrotic properties and to reverse cirrhosis, particularly in the early stage. (82). The TGFβ1/ALK1/Smad1 signaling pathway has been discovered in LX-2 cells, a line of immortalized human HSCs (80). In these cells, ALK1-dependent phosphorylated Smad1 leads to Id1 gene expression (4). Treatment with Cpd861 decreased both Smad1 phosphorylation and Id1 expression in these cells (81). The mechanism of action of Cpd861 in HSCs seems to be based on the decrease of ALK1 expression in HSCs (81). Furthermore it has been demonstrated that Cpd861 can significantly inhibit LX-2 cell proliferation in a dose-dependent manner, and reduces the expression levels of alpha-SMA mRNA and type II collagen expression whereas increases MMP-1 expression n LX-2 cells (82, 177, 178).

Imatinib mesylate (Glivec ®; Novartis, Basel, Switzerland) is an inhibitor of numerous tyrosine kinases, such as the c-Abelson (c-Abl) proto-oncogene activity, and it has become the standard treatment for Philadelphia chromosome-positive chronic myeloid leukemia. Imatinib mesylate binds to the ATP-binding compartment of c-Abl and efficiently inhibits tyrosine kinase activity. Notably, c-Abl has lately been acknowledged as an important downstream molecule in TGFβ signaling (179, 180). TGF-β treatment caused an enhanced c-Abl kinase activity in several cell types, while imatinib pretreatment of cultured fibroblasts abolished c-Abl activity triggered by TGF-β, except it did not influence the secretion TGF-β secretion (179). The c-Abl signifies a new Smad2- and Smad3-independent and thus ALK5-independent target of TGF-β signaling (179).

The Smad1 signaling pathway has been reported to be an effector a c-Abl and other kinases such as c-Kit (8). Thus, it was demonstrated that Smad1 signaling pathway is triggered in cultured fibroblasts and skin samples obtained from patients with systemic sclerosis (SSc). Imatinib mesylate down-regulated TGFβ-induced mRNA for collagen1A1, collagen 1A2 and fibronectin 1, as well as protein levels in SSc fibroblasts (181). Pannu et al. had demonstrated in an experimental model of SSc that the profibrotic response is mediated by a persistent activation of Smad1 and ERK-1/2 signaling pathways but is independent of the Smad2/3 signaling pathway (7). Pannu et al. also demonstrated that Imatinib mesylate inhibited Smad1 and Erk1/2 activation in cultured SSc fibroblasts leading to a decrease of CTGF (CCN2) expression, a gene whose expression is activated by TGF-β and mediates several of its pro-fibrotic actions (182). Moreover, it was reported that c-Abl was essential to Smad1 activation and consequent expression of CTGF and collagen type I (8). Thus, SSc fibroblasts, a multiligand receptor complex comprising of TGF-beta and CCN2 receptors drives constitutive activation of the Smad1 pathway, also being CCN2 a prime effector of this pathway, therefore, establishing an autocrine loop that increases TGF-β signaling (183). This amplification loop is blocked by Imatinib mesylate. Furthermore, in vivo studies also reported that Imatinib mesylate prevented bleomycin-induced dermal fibrosis (181) and lung fibrosis (179) in mice.

Other ALK1 inhibitors such **as PF-3446962** (anti-human ALK1) and **ACE-041** (17, 184) have been studied as therapeutic drugs against tumor growth, and they may be considered as potential anti-fibrotic molecules in situations in which ALK1 behaves as a pro-fibrotic receptor, although the properties have not been assessed . However, in the pathologies in which ALK1 behaves as an anti-fibrotic receptor, ligands such as BMP-7, BMP-9 and BMP-10 that activates AKL1/Smad1 signaling pathway could be potential useful tools in order to neutralize fibrosis.

BAMBI (BMP and Activin receptor Membrane Bound Inhibitor), a pseudoreceptor that forms a complex with TβRI ALK5 and inhibits Smad3 and Smad2 phosphorylation (185), plays an crucial purspose in development (186). BAMBI also inhibits Smad1/5 and Erk1/2 signaling in endothelial cells (187)**,** suggesting a possible inhibition of ALK1. It has been demonstrated that BAMBI protects the heart from biochemical stress induced by pressure overload because of the inhibition of TGF-β signaling.

BAMBI$^{-/-}$ mice show increased cardiac fibrosis following thoracic aortic constriction (TAC) and cultured BAMBI$^{-/-}$ cardiac fibroblasts express more collagen I and fibronectin compared to BAMBI$^{+/+}$ cardiac fibroblasts (188).

6.2. ALK1/Smad1 agonists

As we mentioned before, no evidences about ALK1 expression in non-transformed epithelial cells exists. However, ligands and receptors, such as ALK3 and BMP-7, known to imitate ALK1 signaling via Smad1/5, and whose effects must be important to know if we want to design a therapeutic approach in which ALK1 is ubiquitous overexpressed in this cell type.

During the last years a higher number of studies have demonstrated that BMP-7 has a protective effect against organ fibrosis (189). In NP-1 distal tubular epithelial cells, treatment with recombinant human BMP-7 reverses TGF-β-induced EMT, observed by a restoration of E-Cadherin and ZO-1 expression. Treatment with recombinant human BMP-7 in rats with renal fibrosis induced by administration of NTS recovers renal architecture and reduces markedly renal fibrosis (117). This effect has been observed in other models of experimental renal fibrosis (118, 164). Thus, intraperitoneal administration of BMP-7 reversed renal fibrosis induced by UUO. This effect seems to be mediated by ALK3 activation, as while Smad2/3 phosphorylation increased, Smad1 phosphorylation decreased 3 days after UUO, and treatment with BMP-7 induced a higher Smad1 phosphorylation following UUO and its consequent decrease of renal fibrosis (118).

THR-123, a peptide agonist for ALK3 and ALK2, reverses EMT induced by TGF-β in tubular epithelial cells. Furthermore, in several mouse models of acute and chronic renal injury, THR-123 prevented inflammation, apoptosis and EMT, and reversed established fibrosis (119).

Figure 6 describe the different possible targets in the TGF-β/ALK1/SMADs pathway that can be modified by the described drugs in order to prevent or reverse the emergence of organ fibrosis

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FIGURE LEGENDS

Figure 1: Schematic representation of Smad-mediated signaling pathways. The different members of the TGF-β family signal via specific heteromeric complex of type I and type II serine/threonine kinase receptors. The intracellular signaling is mediated by the downstream effectors, Smads. On endothelial cells, TGFβ1 can either activate ALK-5/Smad2/3, inducing plasminogen activator inhibitor-1 (PAI-1), or ALK1/Smad1/5/8 pathways, with opposite effects; Id1 (a dominant inhibitor of basic helix-loop-helix proteins) is a specific target gene for TGFβ/ALK1/Smad1/5/8 pathway.

Figure 2: Structural representation of Activin Receptor-Like kinase 1 (ALK-1) The structure of the serine/threonine kinase type I receptor ALK1 can be divide into several parts: a short extracellular (EC) domain with the signal peptide and the cysteine rich EC domain; the transmembrane domain, and the intracellular domain, which contains the GS (glycine/serine-rich) domain (172-201) at the juxtamembrane position in charge of regulating the kinase activity, and the serine-threonine kinase domains responsible for Smad phosphorylation (The scheme is not to scale).

Figure 3. Schematic representation of ALK1 and ALK5 signaling role in ECM protein expression. ALK1 and ALK5 signal through Smad1/5 and Smad2/3 respectively, regulating ECM protein expression in different cell types. In this way, in endothelial cells, fibroblasts, myoblasts, chondrocytes, vascular smooth muscle cells and hepatic stellate cells, ALK5 promotes ECM protein expression through Smad2/3 pathway whereas ALK1 would inhibit this process. However, in scleroderma fibroblasts and hepatocytes ALK1 promotes ECM protein expression.

Figure 4. Role of TGF-β and BMP receptors in EMT. TGF-β and BMP receptors play an important and different role in EMT. ALK5 induces EMT through Smad2/3. ALK3 and BMP-7 inhibits EMT through Smad1/5. The role of ALK1 in EMT in unclear and it depends on the cell type. Until now, the role of ALK1 in EMT has only

been studied in cancer. On the other hand, Id1 induces EMT in renal tubular cells and it may be induced by Smad3.

Figure 5: Role of ALK1 in fibrotic disorders o Schematic representation of ALK1 and ALK5 signaling role in fibrotic diseases. TGF-β induces fibrosis in different organs through ALK5 and ALK1signalling pathways. In kidney, activation of the ALK5/Smad2/3 pathway induces fibrosis but the role of ALK1/Smad1/5 pathway in this phenomenon in unclear. The ratio ALK1/ALK5 regulates cartilage homeostasis and osteoarthritis. Elevated ALK1/ALK5 ratio is related with cartilage degradation. On the other hand, activation of the ALK1/Smad1 pathway promotes scleroderma and liver fibrosis.

Figure 6. Pharmacological approach to ALK1 inhibition and its effect in fibrosis. There are different mechanisms of inhibition of ALK1 that may be used as therapeutic targets against fibrosis. BAMBI inhibits ALK5 and possibly ALK1 as well, and protects against cardiac fibrosis. Imatinib mesylate inhibits Smad1/5 phosphorylation and decreases scleroderma. On the other hand, BMP-7 activates Smad1/5 and reverses EMT and renal fibrosis. THR-123, an ALK3 agonist, inhibits renal fibrosis. Compound 861 inhibits ALK1 expression. Other ALK1 inhibitors such as ACE-041 and PF-3446962 could be used to inhibit the effect of ALK1 in the tissues where ALK1 behaves as a fibrotic mediator.

TABLE LEGENDS

Table 1: Chart of the mammalian TGF-β superfamily

Figure 1.

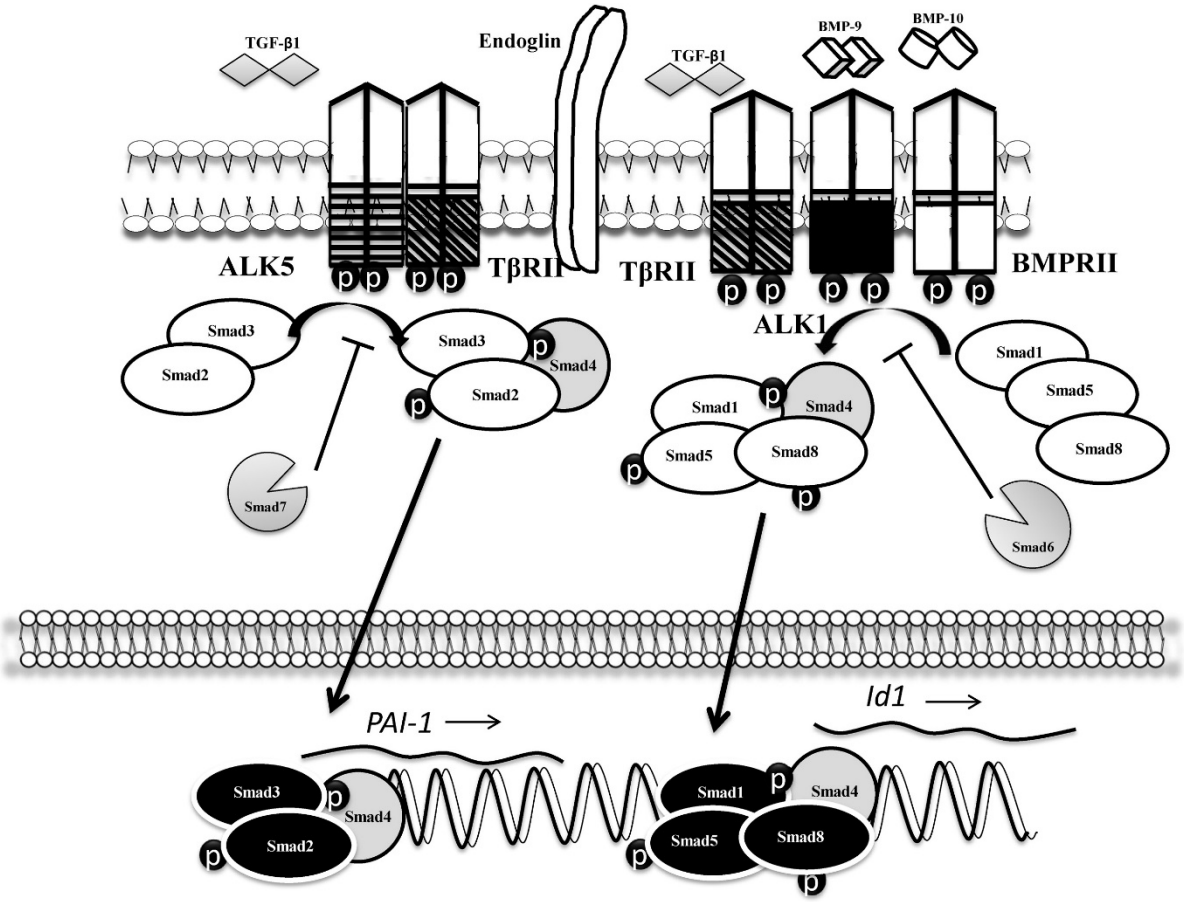

TGF-β1
Endoglin
TGF-β1
BMP-9
BMP-10
ALK5
TβRII
TβRII
ALK1
BMPRII
Smad3
Smad2
Smad3
Smad4
Smad2
Smad7
Smad1
Smad4
Smad5
Smad8
Smad1
Smad5
Smad8
Smad6
PAI-1
Id1
Smad3
Smad4
Smad2
Smad1
Smad4
Smad5
Smad8

Figure 2.

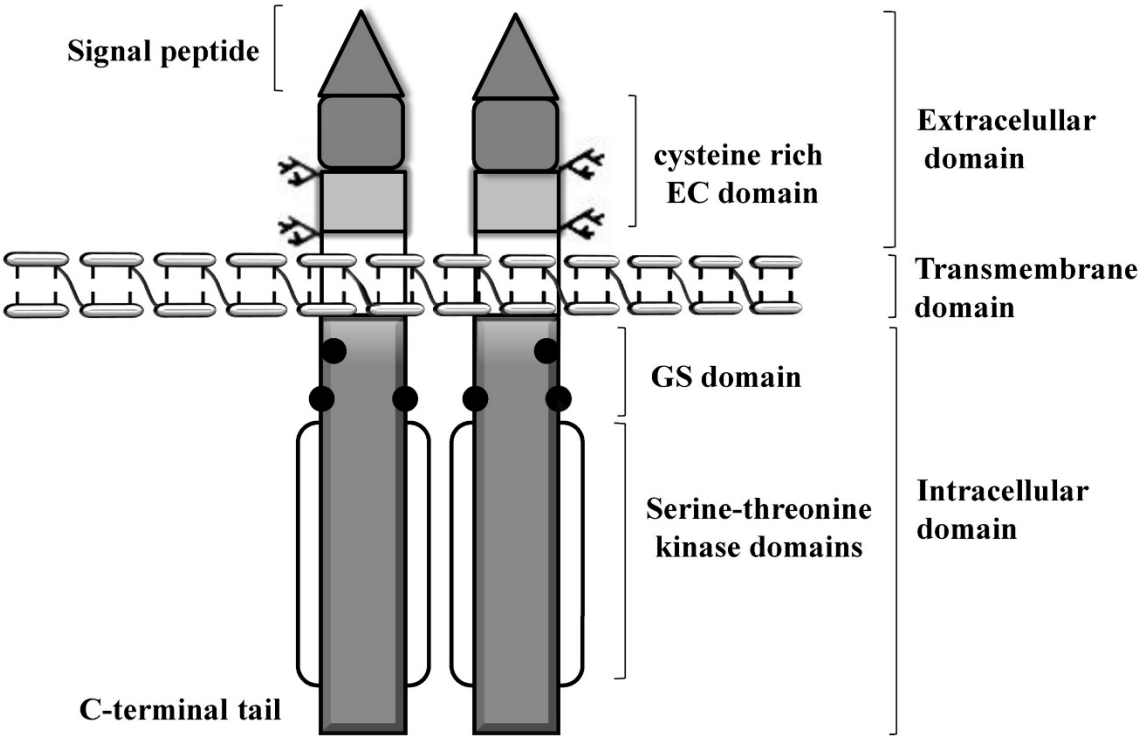

Signal peptide
cysteine rich
EC domain
Extracelullar
domain
Transmembrane
domain
GS domain
Serine-threonine
kinase domains
Intracellular
domain
C-terminal tail

Figure 3.

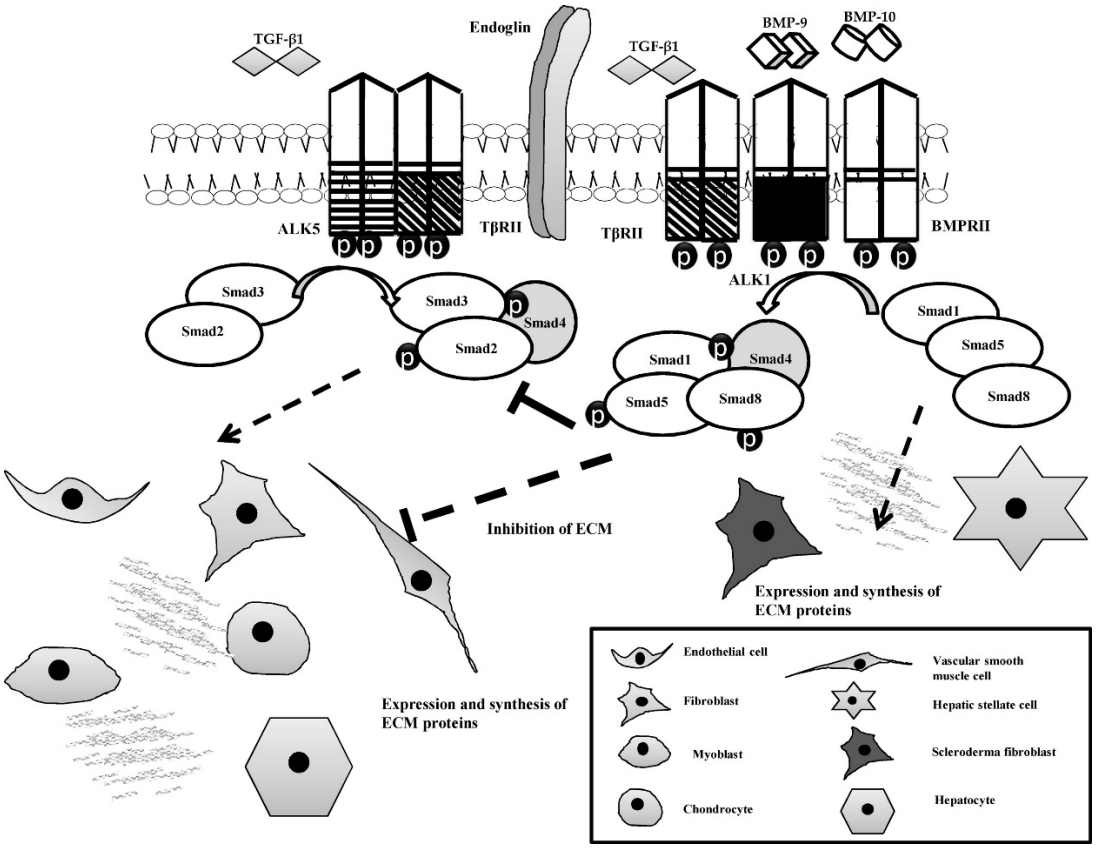

TGF-β1
Endoglin
TGF-β1
BMP-9
BMP-10
ALK5
TβRII
TβRII
BMPRII
ALK1
Smad3
Smad2
Smad3
Smad4
Smad2
Smad1
Smad4
Smad5
Smad8
Smad1
Smad5
Smad8
Inhibition of ECM
Expression and synthesis of ECM proteins
Expression and synthesis of ECM proteins
Endothelial cell
Vascular smooth muscle cell
Fibroblast
Hepatic stellate cell
Myoblast
Scleroderma fibroblast
Chondrocyte
Hepatocyte

Figure 4.

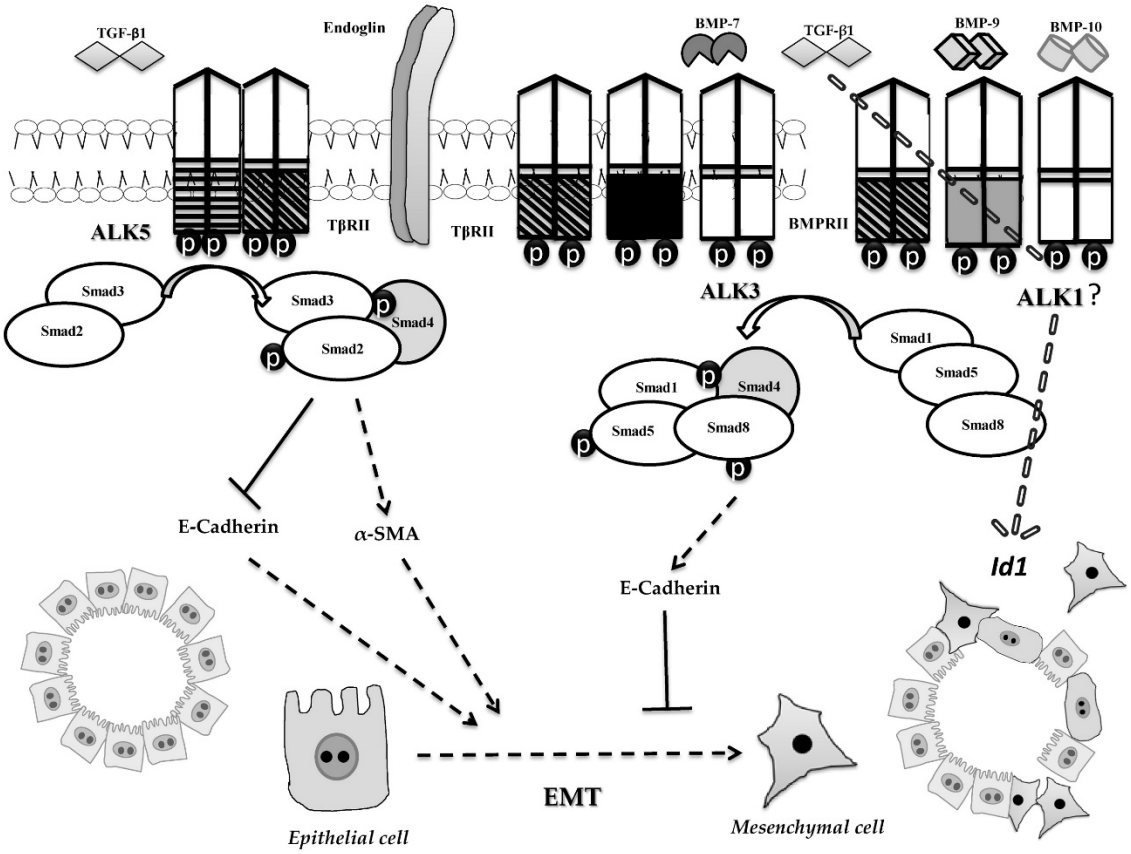

TGF-β1
Endoglin
BMP-7
TGF-β1
BMP-9
BMP-10
ALK5
TβRII
TβRII
BMPRII
Smad3
Smad2
Smad3
Smad4
Smad2
ALK3
ALK1?
Smad1
Smad5
Smad8
Smad1
Smad4
Smad5
Smad8
E-Cadherin
α-SMA
E-Cadherin
Id1
EMT
Epithelial cell
Mesenchymal cell

Figure 5.

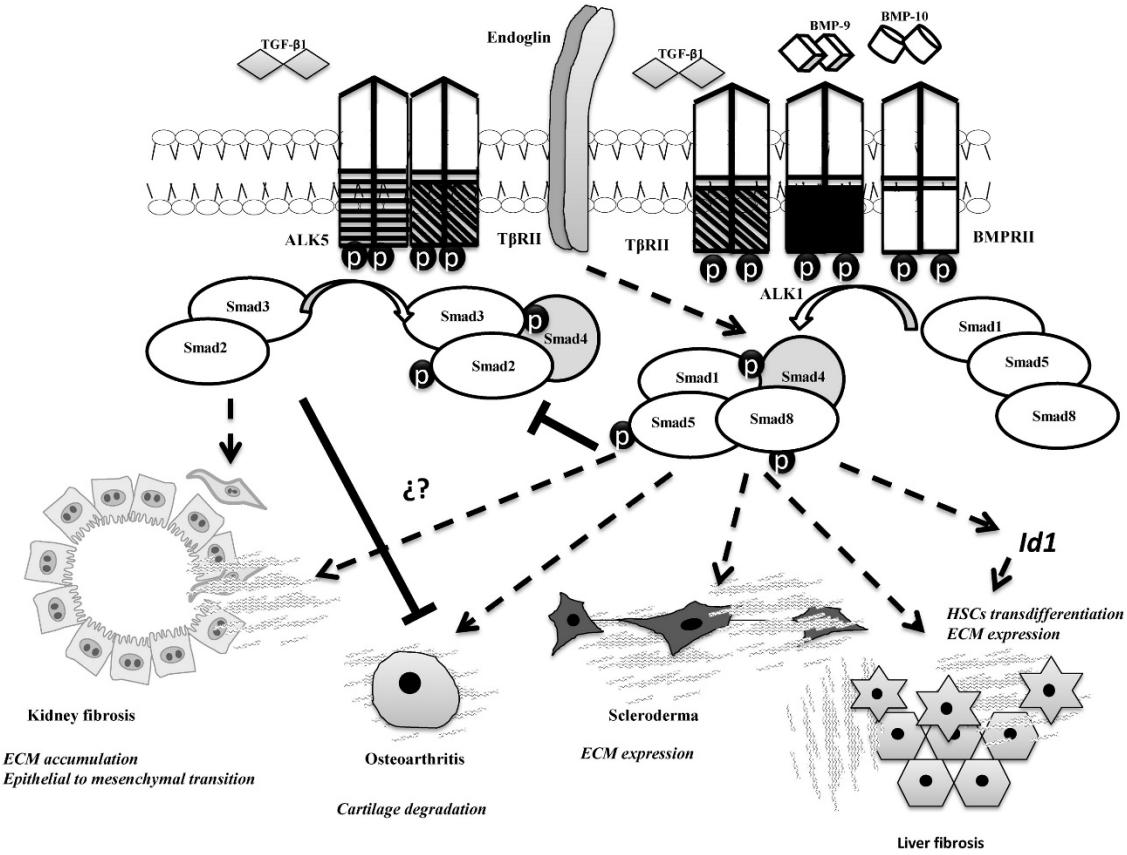
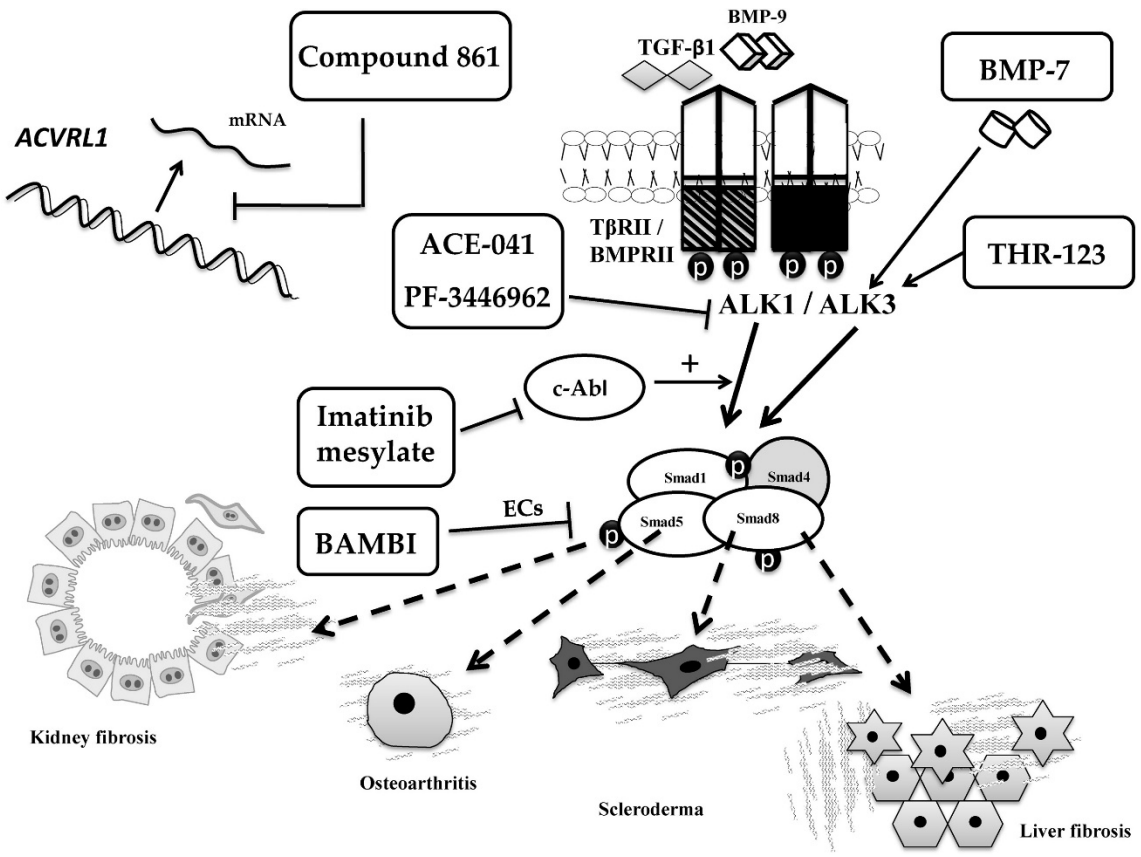

TGF-β1
Endoglin
TGF-β1
BMP-9
BMP-10
ALK5
TβRII
TβRII
BMPRII
ALK1
Smad3
Smad2
Smad3
Smad4
Smad2
Smad1
Smad4
Smad5
Smad8
Smad1
Smad5
Smad8
¿?
Id1
HSCs transdifferentiation
ECM expression
Kidney fibrosis
ECM accumulation
Epithelial to mesenchymal transition
Osteoarthritis
Cartilage degradation
Scleroderma
ECM expression
Liver fibrosis

Figure 6.


Compound 861
BMP-9
TGF-β1
BMP-7
ACVRL1
mRNA
TβRII /
BMPRII
ACE-041
PF-3446962
THR-123
ALK1 / ALK3
+
c-Abl
Imatinib
mesylate
Smad1
Smad4
Smad5
Smad8
ECs
BAMBI
Kidney fibrosis
Osteoarthritis
Scleroderma
Liver fibrosis

TGF-β superfamily

TGF-β subfamily

TGF-β1
TGF-β2
TGF-β3

Activins

Activin A
Activin B
Activin AB
Activin C
Activin E

Inhibins

Inhibin A
Inhibin B
Inhibin C

BMP-2
BMP-3b (GDF10)
BMP-4
BMP-5
BMP-6
BMP-7 (OP-1)
BMP-8a (OP-2)
BMP-9 (GDF-2)
BMP-10
BMP-11 (GDF-11)
BMP-12 (GDF-7)
BMP-12 (GDF-6)
BMP-14 (GDF-5)
BMP-15 (GDF-9b)
BMP-16 (Nodal)
GDF-1
GDF-3
GDF-8 (Myostatin)
GDF-9
GDF-15

MIF (AMS/MIS)

Müllerian inhibitory substance