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BMP-7 counteracting TGF-beta$_1$ activities in organ fibrosis

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1. ABSTRACT

Chronic organ injuries are accompanied by a dysregulated scarring process called "Fibrosis" that is characterized by hyperactivity of TGF-beta resulting in an imbalance of extracellular matrix homeostasis and accumulation of fibrosis-associated proteins. These changes are due to a specialized matrix-expressing cell type, i.e. the myofibroblast, which is derived from independent cellular sources. Beside resident quiescent fibroblasts that become activated, circulating bone-marrow-derived fibrocytes are attracted by the injured organ. Additionally, epithelial cells transit into mesenchymal cells in a process termed epithelial-to-mesenchymal transition. Furthermore, mesothelial cells leave their peripheral location and acquire a fibrogenic phenotype *via* mesothelial-to-mesenchymal transition. Numerous independent studies have consistently demonstrated that BMP-7 interferes with TGF-beta signaling and a diverse set of matricellular proteins (e.g. CCN proteins), Endoglin, Betaglycan, BAMBI and the members of the repulsive guidance molecule family that modulate cellular proliferation, migration, adhesion and extracellular matrix production. This protein network might therefore depict novel targets for treatment of fibrotic lesions. We here summarize recent knowledge of BMP-7 function and discuss attempts to use this cytokine as a drug to reverse TGF-beta-induced fibrogenesis.

2. INTRODUCTION

Since the first identification of human bone morphogenetic protein-7 (BMP-7), formerly known as osteogenic protein-1 or OP-1, as a factor involved in osteoblast differentiation and bone formation in 1990 (1), this cytokine has addressed the interest of many scientists. Physiologically it acts as a major and essential morphogen and survival factor in renal, bone and eye development. In agreement with this notion respective homozygous null mice are affected by arrested renal development and dysplastic kidneys resulting in death soon after birth (2, 3). Although, the precise physiological function in kidney and other organs is not fully understood, work of the last decade has shown that BMP-7 is an endogenous regulator of organ homeostasis and regeneration in kidney and liver (4, 5). Based on these findings and on the fact that recombinant BMP-7 (rBMP-7) reduces the severity of injury in acute and chronic organ failure by counteracting profibrogenic transforming growth factor-beta$_1$ (TGF-beta$_1$) activities (6), BMP-7 and its downstream signaling pathways have become potential mew drug targets for therapeutic modulation of various acute and chronic diseases. It is now generally assumed that in normal tissue a balance of biological active TGF-beta$_1$ and BMP-7 exists that shifts toward TGF-beta$_1$ during inflammation and fibrogenesis in various organs. This strict steadiness can be

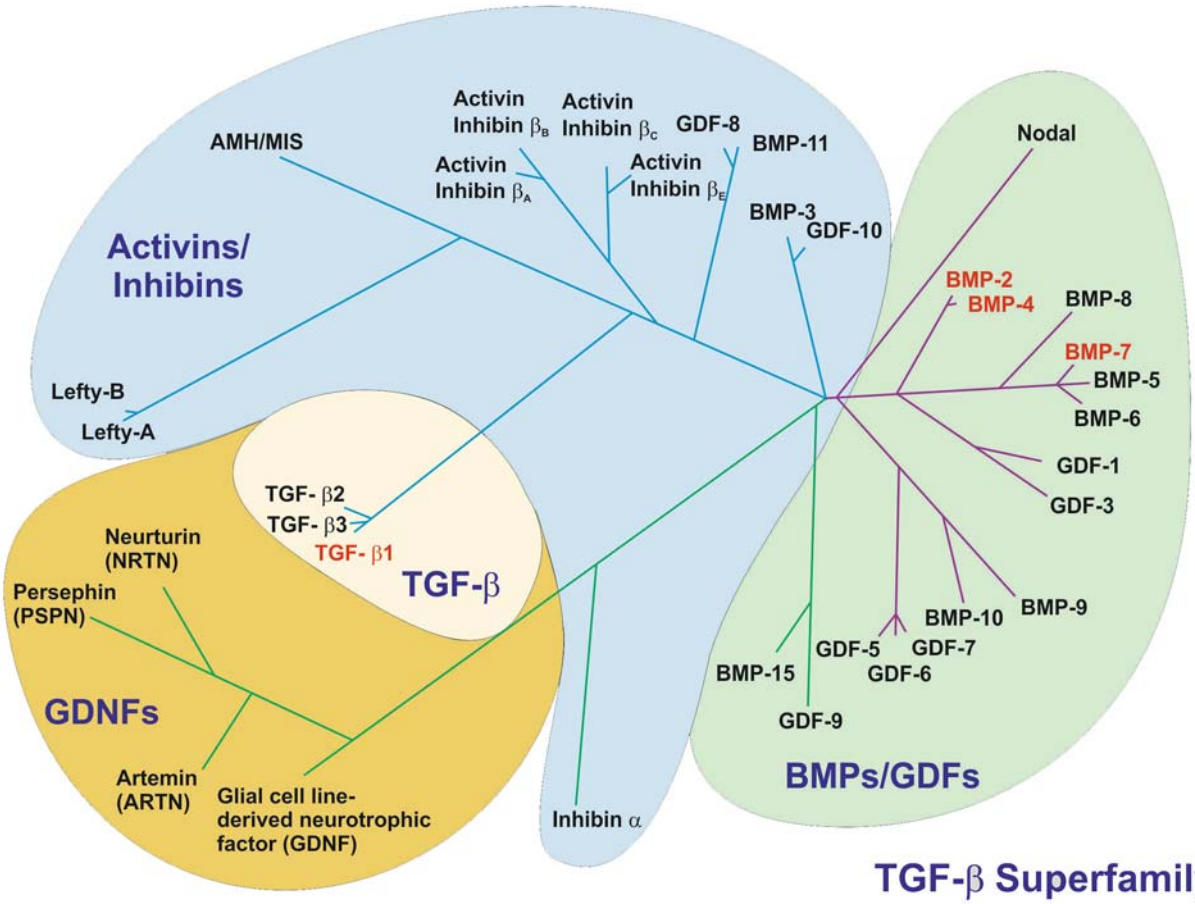

Figure 1. Groups within the TGF-beta superfamily. Based on their primary sequence and structural features, the 35 mammalian members of the TGF-beta superfamily are subdivided into the following four subgroups: (i) TGF-betas, (ii) activins/inhibins, (iii) bone morphogenetic proteins (BMPs)/growth and differentiation factors (GDFs), and (iv) the more distantly related group of GDNF ligands. The affiliation to one of these subgroups is ambiguous and handled somewhat irregular. TGF-beta$_1$, BMP-7, BMP-2 and BMP-4 (all marked in red) are those cytokines with outstanding importance in control of fibrogenesis.

further altered by several extracellular proteinogenic modifiers that bind to one of these cytokines and modulate their overall biological activity. Several of these modifiers or trapping proteins were already identified and mechanisms and pathways that are involved in this regulatory network are currently matter of numerous ongoing investigations. In particular, key questions addressed are (i) the impact of BMP-7 for embryogenesis and development, (ii) its extra- and intracellular-signaling pathways, (iii) its target genes, (iv) mechanisms by which it counteracts TGF-beta pathways, (v) modulation of its biological activity by trapping proteins, and (vi) its therapeutic applicability in experimental models of fibrogenesis and various human diseases.

The concept that BMP-7 counteracts TGF-beta$_1$ activity during ongoing fibrogenesis in diverse organs was already confirmed in many independent studies. Moreover, it was found that BMP-7 is effective in inhibition of epithelial-to-mesenchymal transition (EMT) which triggers the fibrogenic response by increasing the number of activated fibroblasts that are capable to synthesize and deposit extracellular matrix (ECM) components. It is therefore assumed that these two activities of BMP-7 are mainly responsible for the anti-fibrotic attributes of BMP-7.

EMT is a cellular (de)redifferentiation process that leads to loss of cell-cell adhesion and E-cadherin expression, elevated and *de novo* expression of specific mesenchymal markers (e. g. beta-catenin, SNAIL, fibroblast specific protein 1 = FSP1), and production of typical ECM molecules characteristic for fibrogenic processes (e.g. collagen type I and III, fibronectin) and intermediate filament proteins (e. g. alpha-smooth muscle actin = alpha-SMA, desmin).

Undoubtedly, recent advances and emerging insights in TGF-beta-/BMP-signaling and the identification of trapping proteins that are capable to modulate the biological activity of any of these cytokines by either regulating their cellular secretion or interfering with receptor binding have afforded attractive novel targets for treatment of fibrotic lesions.

Table 1: Sub-grouping of TGF-beta superfamily members

Subgroup	Gene symbol	Family member	Alternative names	Cytogenetic location	OMIM
TGF-betas	*TGFB1*	TGF-beta$_1$	TGF-BETA, TGFB	19.q13.2	190180
	TGFB2	TGF-beta$_2$		1q41	190220
	TGFB3	TGF-beta$_3$		14q24.3	190230
BMPs/ GDFs	*BMP2*	BMP-2	Bone morphogenetic protein-2A (BMP-2A)	20p12.3	112261
	BMP4	BMP-4	Bone morphogenetic protein-2B (BMP-2B); BMP-2B1	14q22.2	112262
	BMP5	BMP-5		6p12.1	112265
	BMP6	BMP-6	VG1-related sequence (VGR1)	6p24.3	112266
	BMP7	BMP-7	Osteogenic protein 1 (OP-1)	20q13.31	112267
	BMP8B	BMP-8	Osteogenic protein 2 (OP-2)	1p34.2	602284
	GDF2	BMP-9	Growth/differentiation factor 2 (GDF2), Bone morphogenetic protein-9 (BMP-9)	10q11.22	605120
	BMP10	BMP-10		2p13.3	608748
	BMP15	BMP-15	Growth/differentiation factor 9B (GDF9B), Bone morphogenetic protein-15 (BMP-15)	Xp11.22	300247
	GDF1	GDF-1		19p13.11	602880
	GDF3	GDF-3		12p13.31	606522
	GDF5	GDF-5	Cartilage-derived morphogenetic protein 1 (CDMP1), Lipopolysaccharide-associated protein 4 (LAP4), LPS-associated protein 4, BMP-14	20q11.22	601146
	GDF6	GDF-6	Cartilage-derived morphogenetic protein 2 (CDMP2), BMP-13	8q22.1	601147
	GDF7	GDF-7	BMP-12	2p24.1	604651
	GDF9	GDF-9		5q31.1*	601918
	NODAL	Nodal (mouse)	HTX5	10q22.1	601265
GDNFs	*NRTN*	Neurturin	NTN	19p13.3	602018
	PSPN	Persephin		19p13.3	602921
	ARTN	Artemin	Neublastin, Enovin	1p33-p32*	603886
	GDNF	GDNF	Glial cell line-derived neurotrophic factor	5p13.2	600837
Activins/ Inhibins	*INHBA*	Activin Inhibin beta$_A$	Inhibin beta$_1$, Follicle-stimulating hormone-releasing protein (FRP), FSH-releasing protein, FSH-releasing factor, erythroid differentiation factor (EDF)	7p14.1	147290
	INHBB	Activin Inhibin beta$_B$	Inhibin beta$_2$	2q14.2	147390
	INHBC	Activin Inhibin beta$_C$	Inhibin beta$_C$	12q13.1	601233
	INHBE	Activin Inhibin beta$_E$	Inhibin beta$_E$	12q13.3	612031
	MSTN	Myostatin, GDF-8	Growth/differentiation factor 8 (GDF8)	2q32.2	601788
	GDF10	GDF-10	Bone morphogenetic protein 3B (BMP3B)	10q11.22*	601361
	BMP3	BMP-3	Osteogenin	4q21.21	112263
	GDF11	GDF-11, BMP-11	Growth/differentiation factor 11 (GDF11), Bone morphogenetic protein 11 (BMP11)	12q13.2	603936
	LEFTY2	Lefty-A	Left-right determination factor 2 (LEFTY2), Endometrial bleeding-associated factor (EBAF), Left-right determination factor A (LEFTA), LEFTY2 transforming growth factor-beta$_4$ (TGFB4)	1q42.12	601877
	LEFTY1	Lefty-B	Left-right determination factor 1 (LEFTY1), Left-right determination factor B (LEFTB)	1q42.12	603037
	AMH	MIS	Anti-Muellerian hormone (AMH), Mullerian-inhibiting substance (MIS), Mullerian-inhibiting factor (MIF)	19p13.3	600957
	INHA	Inhibin alpha		2q35	147380

Note: The cytogenetic locations and OMIM reference numbers were extracted from the Online Mendelian Inheritance of Man (OMIM) Database that can be found at http://www.ncbi.nlm.nih.gov/omim. The three chromosomal localizations of members that are marked by asterisks were taken from the Atlas of Genetics and Cytogenetics in Oncology and Haematology that can be found at: http://atlasgeneticsoncology.org/index.html. Please note that most of the proteins have multiple names.

3. INDIVIDUAL SECTIONS

3.1. Structure of TGF-beta superfamily members

The TGF-beta superfamily is a large family of at least 35 structurally related members that are subdivided in four major subfamilies, the (i) TGF-betas, (ii) activins/inhibins, (iii) bone morphogenetic proteins (BMPs)/growth and differentiation factors (GDFs), and (iv) the more distantly related group of Glial cell line-derived neurotrophic factors (GDNFs) (Figure 1). The evolution of the superfamily members can be inferred from their expression in different organisms. Homologs of BMPs are found in worm and flies, activins and inhibins only in flies and TGF-betas are exclusively found in vertebrates (7). The genes of the three classical TGF-betas (TGF-beta$_1$, TGF-beta$_2$, and TGF-beta$_3$), and the different BMPs/GDFs are widely dispersed in the human genome. The evolution of this ligand diversity facilitated the development of complex machinery by assigning tissue specific functions to the individual family members (Table 1). Common for the different ligands is the binding to two kinds of membrane receptors with serine/threonine kinase activity, which mediate their signals through Smad-dependent and -independent pathways (8). Members of the BMP subgroup were first identified and functionally characterized in 1988 from protein extracts derived from bovine bone using their ability to induce cartilage and bone formation (9). Commonly, BMPs and other TGF-beta superfamily members are synthesized as large monomeric pre-pro-peptides consisting of a short N-terminal signal sequence, a long latency-associated peptide (LAP) and the mature cytokine that shows the highest degree of sequence conservation (Figure 2). After synthesis, two precursors dimerize, become processed by enzymatic cleavage at

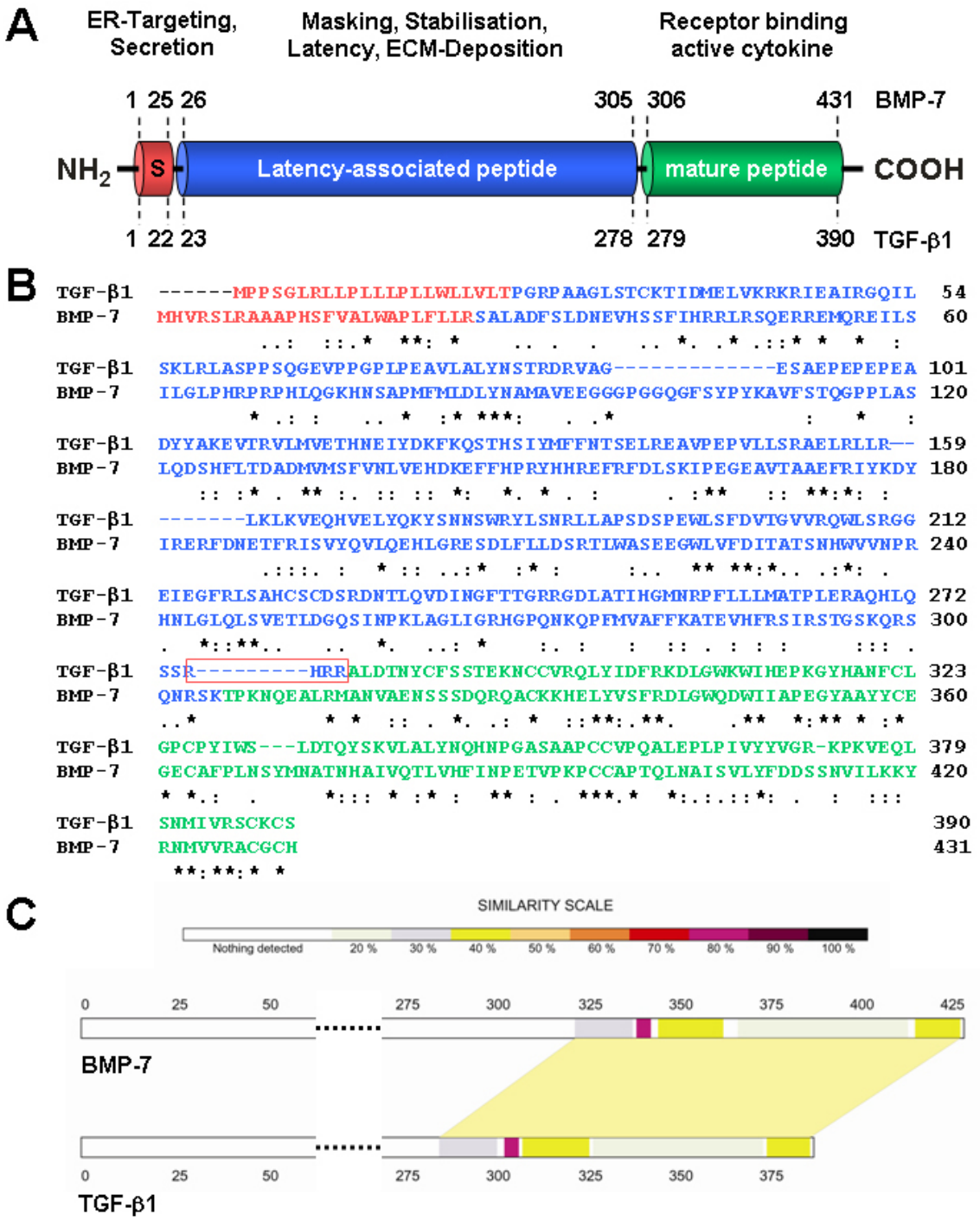

Figure 2. Structural features of human BMP-7 and TGF-beta$_1$. (A) Both, BMP-7 and TGF-beta$_1$ share the same modular structure that is characteristic for members of the TGF-beta superfamily. The N-terminal leader sequence (*in red*) is necessary for targeting to endoplasmic reticulum (ER) and subsequent cellular secretion. The latency-associated peptide (*in blue*) is required for masking (latency), stabilization and extracellular matrix deposition. The mature (biologically active) peptide (*in green*) is located at the C-terminus of the pre-propeptide. Locations of amino acid positions are given according to the human BMP-7 (Swiss-Prot P18075) and TGF-beta$_1$ (Swiss-Prot P01137) proteins. (B) Sequence alignment of human TGF-beta$_1$ and BMP-7. The regions of the leader sequence, latency-associated peptide, and mature cytokine are given in red, blue, and green, respectively (cf. part A). The RXXR cleavage site (Arg-X-X-Arg) that is necessary for proteolytic processing in TGF-beta$_1$ is marked by a red box. **(C)** Schematic overview about sequence similarities between BMP-7 and TGF-beta$_1$. The highest degree of similarity (~40-70%) of both cytokines is found at the C-terminal regions harboring the mature peptides (marked in light yellow).

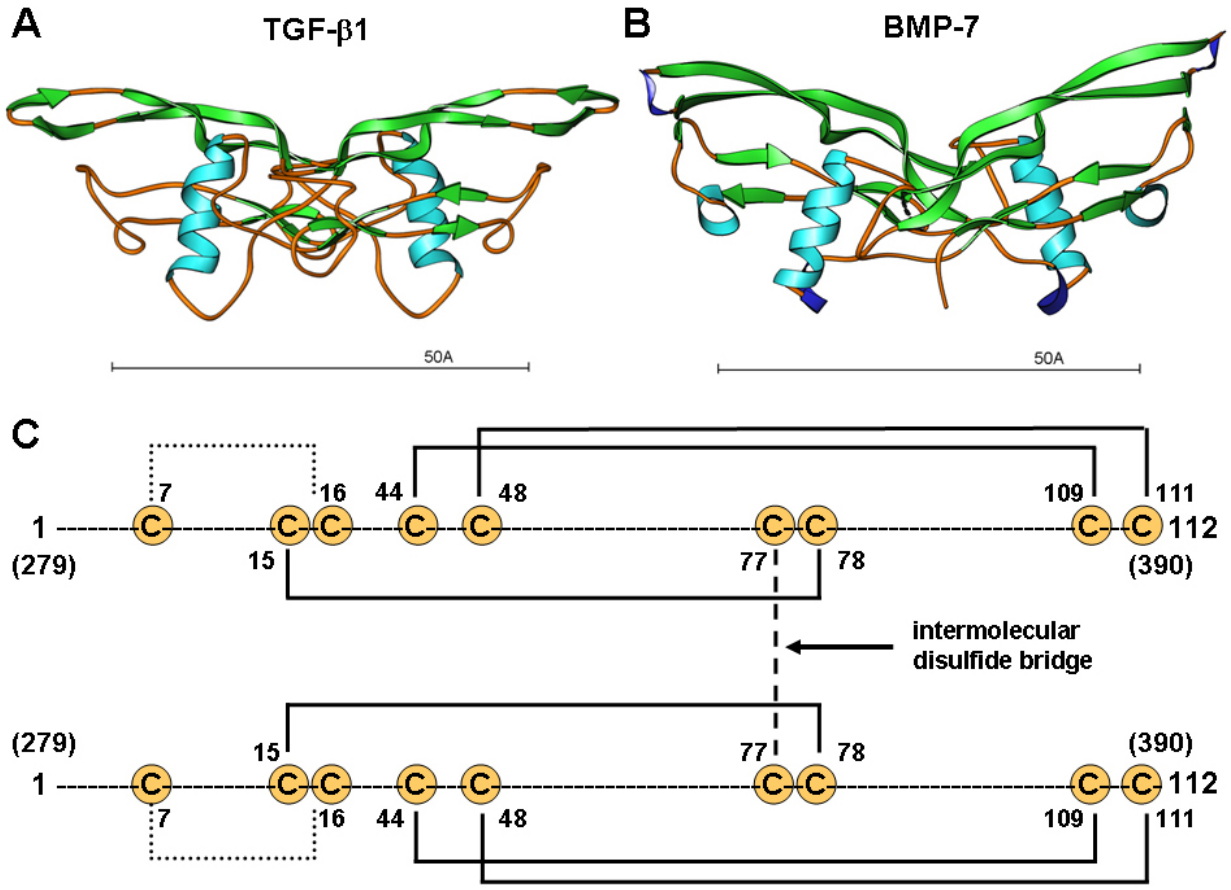

Figure 3. Two- and three-dimensional structure of mature TGF-beta$_1$ and BMP-7. (A, B) TGF-beta$_1$ (A) and BMP-7 (B) are forming similar, symmetric homodimers in which the individual monomers have an overall similar three-dimensional butterfly like fold. The monomers in each dimer contain four characteristic strands of antiparallel beta-sheet (in light green) and an alpha-helix (in light blue) that is on the opposite end of the knot. The images were generated with the Ribbon 2.0 software and the coordinates of human TGF-beta$_1$ (1KLC) and human BMP-7 (1BMP) that are deposited in the Brookhaven Protein Databank (PDB). (C) Intra- and intermolecular disulphide bond network in human TGF-beta$_1$. Each TGF-beta$_1$ monomer contains four intrachain disulphide bonds (*solid lines*) that are conserved in TGF-betas and inhibin-betas, while the other members of this family lack the first bond (*speckled line*). Both dimers are bridged by one intermolecular disulphide bond (*dotted line*). The sequence positions of cysteines (*marked in orange*) involved in disulphide bonding correlate to those counted from the beginning of human mature TGF-beta$_1$ that contains 112 amino acids and is formed by proteolytic cleavage of its 390 amino acid long pre-propetide (cf. Figure 2). For more structural details of these human TGF-beta superfamily members, refer to the original literature describing the respective three-dimensional structures (194, 195).

specific R-X-X-R recognition sites and the carboxyl-terminal domain harboring the mature cytokine is released and secreted. In each dimer the individual monomers are linked by a single intermolecular disulphide bond, whereas the conformation of each monomer is stabilized by a tight network of three (BMPs, GDFs) or four (TGF-betas, inhibin-betas) intramolecular disulphide bonds (Figure 3A). This arrangement accounts for the typical butterfly like structure that is a general hallmark of members of the TGF-beta superfamily (Figures 3B and 3C).

The different ligands act as effective morphogens during embryonic development, organogenesis, bone formation, and are indispensable in other physiological processes. For example, BMP-7 plays a key role in the transformation of mesenchymal cells into bone and cartilage. In line, rBMP-7 enhanced bone graft incorporation and implants fixation, increased bone remodeling and ingrowth in bone grafts and substitutes (10) and is further effective in the repair of a resistant tibial non-union (11). Therefore, rBMP-7 was introduced as a novel surgically effective therapeutics. Moreover, BMP-7 was found to reduce the severity of injury after ischemic acute renal failure in rats (6). Recently, the concept that BMP-7 treatment abolishes the formation of EMT-derived fibroblasts by counteracting TGF-beta-induced Smad signaling has been established in various organs (12) and may explain part of its biological function.

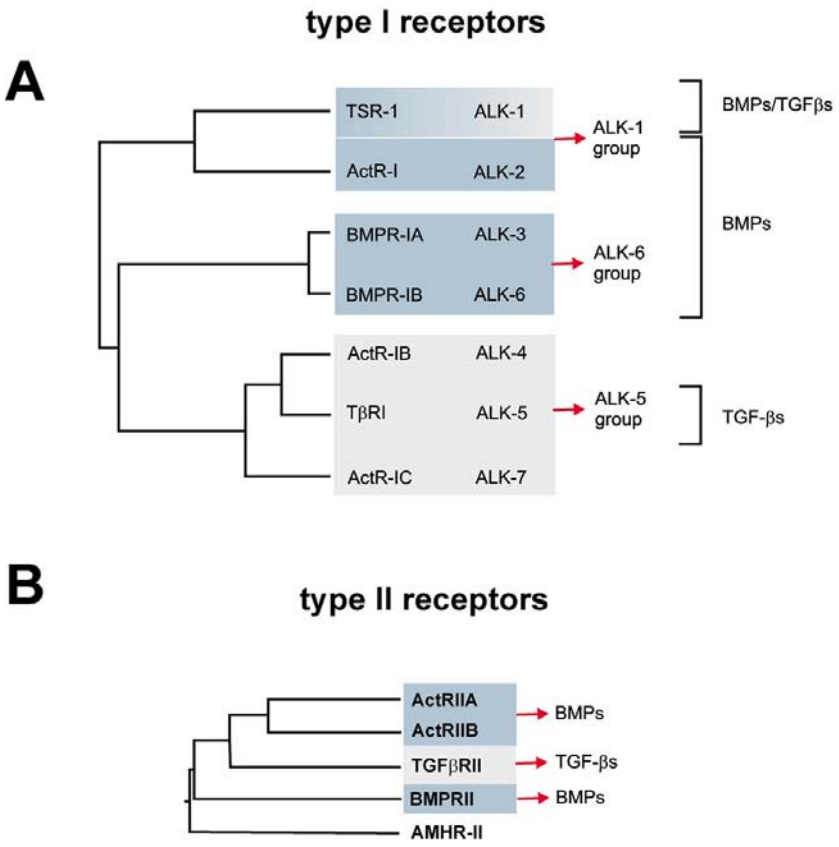

Figure 4. Type I and type II receptors of TGF-beta/BMP signaling. (A) The type I receptors are grouped into three subgroups: (i) the ALK-1 group contains ALK-1 and ALK-2, (ii) the ALK-6 group contains ALK-3 and ALK-6, (iii) while the ALK-5 group contains ALK-4, ALK-5 and ALK-7. The protein sequences of the different ALKs from rat were taken as input for this dendrogram. (B) The type II receptors BMPRII, ActRIIA and ActRIIB are specific for BMPs, while TGFbetaRII is specific for the TGF-beta isoforms. In this dendrogram the following proteins were aligned: human ActRIIA (hActRIIA), human ActRIIB (hActRIIB), human TGFbetaRII, murine BMPRII (mBMPRII), human Mast cell immunoreceptor signal transducer (hMIST-II), *Drosphophila melanogaster* receptors Wishful Thinking (dwit) and Punt (dpunt), and *Caenorhabditis elegans* Cell surface receptor of the abnormal dauer formation family member (cdaf-4).

3.2. BMP signaling: Ligand:receptor interactions and signal transduction

3.2.1. BMP ligands

The different members of the BMP subfamily can be divided into several subgroups. A first group, i.e. the BMP-2/4 group, includes BMP-2, BMP-4. The second group, i.e. the osteogenic protein-1 (OP1) group, encompasses BMP-5, BMP-6, BMP-7, and BMP-8 (OP-2). GDF-5, also termed cartilage-derived morphogenetic protein-1 (CDMP-1), GDF-6 (CDMP-2 or BMP-13), and GDF-7 (BMP-12) form the third (GDF-5 group), whereas a fourth group contains BMP-9 and BMP-10 (13, 7).

Expression of members of the BMP-2/4, the OP-1 group and BMP-9 has been reported in kidney, lung and liver, all representing tissues that are susceptible for fibrogenesis. Therefore, it is commonly suggested and in part experimentally shown that these BMPs are involved in susceptible control processes regulating organ injury and fibrogenesis.

As it has been delineated for TGF-beta signaling, BMP signal transduction is controlled by several mechanisms including availability of the ligand, receptor-binding, receptor activation, receptor-complex internalisation, Smad phosphorylation, turnover of receptors and Smad proteins and specific interaction of Smads with a variety of positive and negative transcription factors. Thereby, although BMP signaling *per se* only relies on a limited set of molecular components it is able to regulate a large variety of cellular responses in a time resolved and cell type-specific manner.

3.2.2. BMP receptors and coreceptors

A characteristic of the TGF-beta superfamily members is that they exert their activities by binding to type I and II cell surface receptors (14) that are further divided by their ligand affinities into different evolutionarily conserved subgroups (Figure 4). In contrast to TGF-beta itself, which exclusively binds to TβRI (with a few exceptions, see below) and TβRII, BMP ligands are

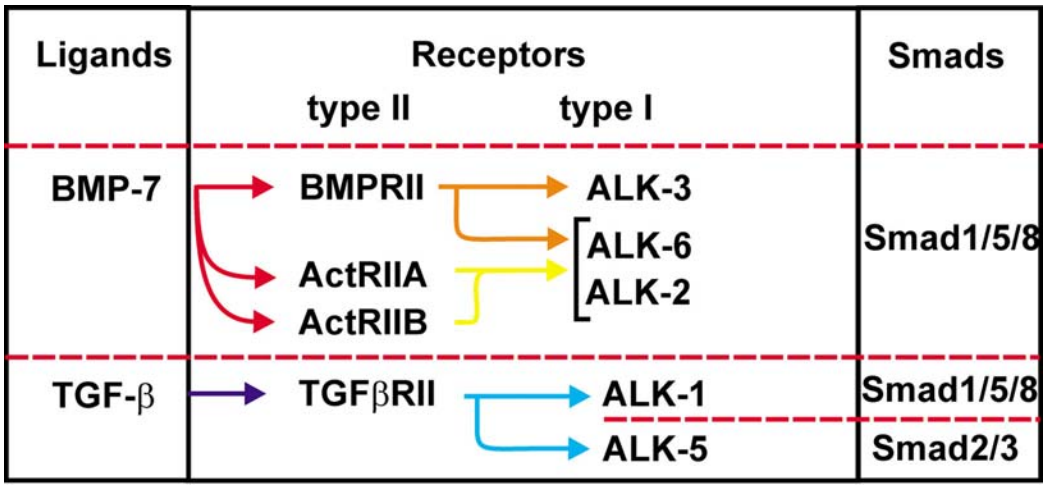

Figure 5. Receptors for BMP-7 and TGF-beta. BMP-7 binds to the type II receptors BMPRII, ActRIIA and ActRIIB that subsequently activate with different specificities the type I receptors ALK-3, ALK-6 or ALK-2. In contrast, TGF-beta binds only to TGFbetaRII which in turn transphosphorylate the type I receptors ALK-1 and ALK-5. Details on the promiscuity and specificity in BMP receptor activation can be found elsewhere (198).

somewhat promiscuous and bind to different subsets of type II receptors and type I receptors (Figure 5). In addition, binding of BMPs to the corresponding receptors is different from TGF-betas, in that BMPs first bind to the type II receptors, which are BMPRII, ActRIIA, and ActRIIB (cf. Figure 4) (15, 16, 7).The type II receptors subsequently recruits and activate the activin-like receptor-kinase (ALK)-1, ALK-2, ALK-3, and ALK-6 (17, 18). The ligands of the BMP-2/4 group have a bias to bind to ALK-3 and ALK-6, whereas proteins of the OP-1 group preferentially bind to ALK-2 and ALK-6 (19). In contrast, members of the GDF-5 group bind primarily to ALK-6, but BMP-9 and BMP-10 are ligands for the ALK-1 receptor (20, 21). However, it was recently shown that GDF-9 may also signal *via* the classical TGF-beta-receptor ALK-5 (22). On the other hand, besides the binding to ALK5, in several cells TGF-beta is able to signal *via* ALK-1, ALK-2 and ALK-3 receptor containing complexes (23). The implication of ALK-1 in BMP and TGF-beta signaling implies another level of complexity into signaling crosstalk (24), since ALK-1 mediates TGF-beta responses during angiogenesis in endothelial cells in which the TGF-beta/ALK-1 pathway counteracts TGF-beta/ALK-5 responses (25-28). The possibility that TGF-beta can signal *via* ALK-1 is of great importance for the interplay of TGF-beta *vs.* BMP-type signaling pathways. TGF-beta was shown to mediate activation of intermediates and target genes that were previously categorized as ALK-1 regulated BMP responses (26-30).

Beside the two essential groups, i.e. type I and type II signaling receptors, BMP receptor binding and signal transduction is fine-tuned by several accessory (co-) receptors. These include for example the pseudoreceptor BMP and Activin Membrane-bound inhibitor (BAMBI) that is transcriptionally induced by BMPs and binds to the BMP type I receptors ALK-3 and ALK-6 thereby competing with type I/ type II receptor complex formation (31, 32). In addition, membrane-associated receptors of the repulsive guidance molecule (RGM) family are facilitators and enhancers of BMP signaling (33, 34). RGM receptors possess three paralogues (RGM_A, RGM_B, and RGM_C) in vertebrates. The RGM_C receptor is one of the critical regulators of iron balance and may cause upon mutations juvenile haemochromatosis. RGM_C is a co-receptor that is necessary for BMP-2/4-induced hepcidin expression in hepatocytes (35, 36). Beside aforementioned receptors, there is a type III TGF-beta receptor group, which comprises two receptors, i.e. Betaglycan (TbetaRIII) and Endoglin (CD105). These receptors are shared between TGF-beta- and BMP-signaling pathways. Both of these receptors possess, similar to other co-receptors, a short cytoplasmic domain without kinase activity. Betaglycan is a type I transmembrane proteoglycan that is more or less ubiquitously expressed. As an accessory receptor, it binds to BMP-2, BMP-4, and BMP-7, and promotes BMP-2-induced EMT (37). These effects are in part mediated by a modulation of trafficking routes of the TGF-beta type I and type II, as well as ALK3 and ALK6 receptors through TbetaRIII (38, 39) and Endoglin (CD105). Endoglin that is also a type I membrane glycoprotein shows a more restricted expression pattern, being highly expressed in endothelial cells, activated macrophages and hepatic stellate cells (40-42). Endoglin binds in the presence of the corresponding type II receptor to BMP-2, BMP-7, and BMP-9 (Figure 6) and increases BMP-7- and BMP-9-mediated responses (43, 29, 20, 21). Although the underlying mechanisms for this activation is not precisely unravelled yet, it had been recently shown in Endoglin deficient mice that in regard to BMP-9 this modulator activity is a central switch that influences not only the SDF1/CXCR4 chemokine axis (44) but further impacts cellular attributes by affecting integrin signalling pathways (45).

3.2.3. Soluble modulators of BMP activities

The mentioned surface receptors which are expressed in the membrane of a certain cells endow cells

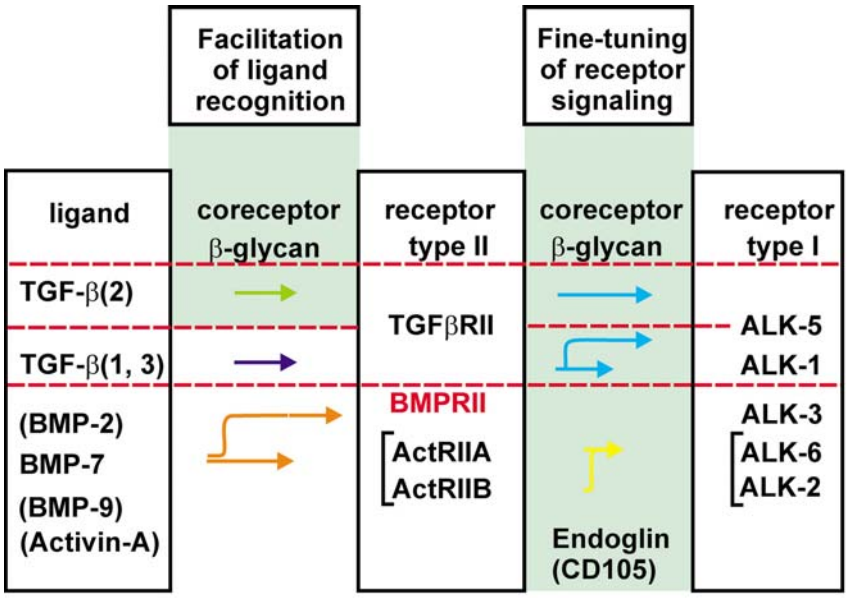

Figure 6. Modulation of BMP/TGF-beta signaling by the coreceptor Endoglin (CD105). The accessory type III receptor Endoglin binds individual members of the TGF-beta superfamily and influences thereby affinity for individual type II receptors. Typically, in the presence of Endoglin, BMP-7 signaling is increased suggesting that Endoglin is not only necessary to facilitate ligand recognition but further is an essential component necessary for fine tuning of TGF-beta/BMP signaling. For more details refer to (37).

with simple but specific machinery that is appropriate to transmit signals in a complex, combinatorial and versatile manner. Moreover, the signal transmission is further fine-tuned by the different affinities of an individual ligand for respective receptors and the occurrence of different downstream intracellular signaling molecules which mediate signaling in a positive or negative manner. On the other hand, several secreted peptides which function as BMP antagonists and belonging to different families encompassing Noggin, Chordin, Gremlin and Dan have been identified. These proteins antagonize BMP-signaling by physically interaction with BMPs thereby preventing their association with their cognate receptors. In a similar fashion, members of the CTGF/CYR61/NOV (CCN) family, that include the connective tissue growth factor (CCN2/CTGF) and the Nephroblastoma-overexpressed protein (CCN3/NOV), antagonize BMP responses by binding to their von Willebrand factor type C domain (46, 47), whereas the Kielin/Chordin-like protein (KCP) was shown to enhance BMP-7 signal transduction and target gene expression (48). The tremendous importance of these secreted modulators is evident from their regulatory functions under normal and fibrotic conditions (see 3.6) that become visible in experimental models and conditions in which individual members of these families are disrupted or mutated (49).

3.2.4. Intracellular BMP signaling pathways

As discussed above, ligand binding to the corresponding membrane receptors complexes leads finally to activation of the type I receptor kinases. This step mediates the transfer of an external signal to the inside of the cell mainly by two different pathways that are i) Smad-independent or ii) Smad dependent. In the latter case the intracellular Smad mediators are phosphorylated by the respective type I receptor (50). The BMP receptors ALK-1, -2, -3 and -6 activate the BMP receptor regulated Smads (BRSmads) subclass Smad1, Smad5 and Smad8. In contrast, TGF-beta mediates activation of TGF-beta-receptor regulated Smad subclass Smad2 and Smad3. Smad activation in response type I receptor action is common to both Smad subclasses and is achieved by phosphorylation of the most C-terminally located serine residues by the type I receptor kinase (51-53). Structurally, RSmads are composed of three functionally different modules. The MAD homology domain 1 (MH1) mediates DNA-binding and negatively regulates the MH2 domain. The MH2 domain is involved in interaction and phosphorylation by the type I receptor at the SSXS-motif (phosphorylated serines are underlined) and mediates binding to Smads and DNA-binding partners (54). The linker region that connects the two MH-domains is substrate amongst others for mitogen-activated protein (MAP) kinases, cyclin dependent kinases (CDKs) which regulate the nuclear translocation of RSmads. Smurf proteins also bind to the linker region and mediate the ubiquitination of specific residues within the linker to mark RSmads for degradation (50).

With respect to the mode of sensing a BMP ligand on the surface and sequential transfer of the signal to the intracellular pathways to finally provoke a transcriptional response, BMP-2 is the best characterized ligand. Two different modes of signalling have been described. In the first mode, binding of BMP-2 is accomplished by preformed heterooligomeric type I/type II receptor complexes. In the second mode, BMP-2 binding

must first induce the assembly of the heterooligomeric type I and type II receptors complexes. The consequences of the two modes for intracellular signaling are different. Sequential recruitment of receptors activates a Smad-independent pathway resulting in induction of the p38 MAP kinase (55). This is governed by assembly of a protein complex which includes special receptor binding proteins called X-linked inhibitor of apoptosis (XIAP), TGF-beta-activated kinase 1 (TAK1), and TAK interacting protein 1 (TAB1) at the activated receptor that induce consecutive activation of the p38 MAP kinase (56). In contrast, BMP-2 binding to preformed receptor complexes leads to phosphorylation and activation of the BRSmad-dependent pathways. In TGF-beta signaling, the RSmads Smad2 and Smad3, which are substrates for the TGF-beta-activated ALK-5 receptor, fulfil different functional roles in several cells and are therefore differentially activated by TGF-beta (57). In a similar manner Smad1 and Smad5 are differentially activated but this phenomenon has not been investigated in detail yet. This differentiation may either be achieved by the usage of alternative BMP type I receptors or arise from a differential expression of the targeted Smad proteins (58, 59).

As has been already mentioned above there are subtle exceptions from the strict paradigm that TGF-beta ligands exclusively activate Smad2 and Smad3 while on the other hand BMP-like ligands specifically and exclusively activate BRSmads by the BMP receptor kinases. It has been shown recently that there are some exceptions to this general rule. One extensively analyzed example is the signaling of TGF-beta *via* ALK-1, a receptor of the "BMP receptor group" which transmits signals to the BRSmads Smad1 and/or Smad5 (53, 60. Nevertheless, in some cells TGF-beta mediates phosphorylation of Smad1/Smad5 by binding to receptor complexes involving ALK2 and ALK3 (23). Moreover, GDF-9 has been shown to signal *via* the classical TGF-beta receptor ALK-5 to activate signaling involving Smad2/Smad3 (22). Once activated, Smad proteins interact with the common Smad4 and translocate into the nucleus to regulate transcription of target genes.

Beside the regulation of Smad transcriptional activity by phosphorylation of the C-terminal SXS motive as a direct response to type I receptor activation, Smads are subject to further phosphorylation by other pathways allowing for signal integration and conversion into a Smad-regulated transcriptional response. Negative and positive crosstalk with MAPK-pathways is further underpinned by the fact that Smad linker phosphorylation by MAP kinase modulates the transcriptional activity of Smads (61). Nevertheless, the BMPRII receptor is also able to directly interact with cytoskeleton-associated proteins, e.g. LIM domain kinase-1 (LMK1) and dynein light chain proteins (e.g. Tctex1), similar to the interaction that was reported for Endoglin and Tctex2beta (62). The binding regulates the overall function of these proteins suggesting that there is a close link of Smad activity and other signaling pathways and cellular attributes (63, 64).

3.2.5. Regulation of gene expression

Once the phosphorylated Smads are translocated into the nucleus, they regulate the transcription of target genes (Figure 7). Because BMPs regulate a broad array of transcriptional responses *via* Smad proteins there is a need for switches to specify these responses in time and space. To facilitate this and to overcome the low intrinsic DNA binding affinity of Smads (65), they associate with co-factors to execute the integration of different signaling inputs resulting in the generation of positive and negative gene responses. This interaction is therefore an important mechanism that accounts for the high diversity and cell specific responses of gene expression that is controlled by the few Smad proteins. Using different methods for the identification of proteins that interact with each other and other molecular techniques, a growing number of proteins were identified during the last decades that physically interact with the Smad proteins to regulate their transcriptional responses (66). One of the groups of transcription factors interacting with BRSmads is the family of Runt-related transcription factors (Runx) that comprises the three isoforms Runx1, Runx2, and Runx3. These TFs are involved in various biological processes, including haematopoiesis and bone formation. Runx2 (*Cbaf1*, *PEBP2αA*) is transcriptionally induced by BMP-2, involving the homeobox transcription factor Dlx5 (67, 68). Stimulation with BMP causes physical interaction of Runx2 and BRSmads, and this complex is translocated subsequently into the nucleus, where it co-operatively regulates transcription of target genes (69). Among the co-activators of transcription are the p300 and CBP (CREB binding protein) proteins. Both proteins (p300/CBP) comprise a histone acetyl transferase (HAT) domain which leads to modification of the chromatin structure (opening) thereby enhancing the accessibility of transcriptional initiation sites for the general transcription machinery. Hence, the cooperation of phosphorylated Smad1 with p300/CBP results in increased Smad1-dependent transcription (70).

The antiproliferative protein Tob (transducer of ErbB2) has been described as a specific negative regulator of BMP-2-mediated Smad1, Smad5 and Smad8 responses (71). Tob is rapidly induced by rhBMP-2, associates with BRSmads and the common Smad4 (co-Smad4), translocates into the nucleus and inhibits the transcriptional activity of BRSmads (71). In addition, Tob inhibits BMP signaling by facilitating Smad6 function (72). c-*Ski* and the Ski-related protein SnoN are inhibitors of Smad2 and Smad3 signaling (73). Although interaction of c-Ski with Smad1 and Smad5 is only weak this complex formation leads to complete suppression of BMP signaling (74). The inhibitory effect of c-Ski is mediated by binding to co-Smad4 and recruitment of a histone deacetylase 1 (HDAC1) to this complex. The ability to suppress Smad1/Smad5 signaling is lost upon modification of c-*Ski* (ARPG mutation), which abolishes binding of c-Ski to co-Smad4, whereas the inhibitory action on Smad3-dependent responses is preserved (75). More recent work has demonstrated that c-Ski can further directly interact with activated TGF-beta type I receptors suggesting that several

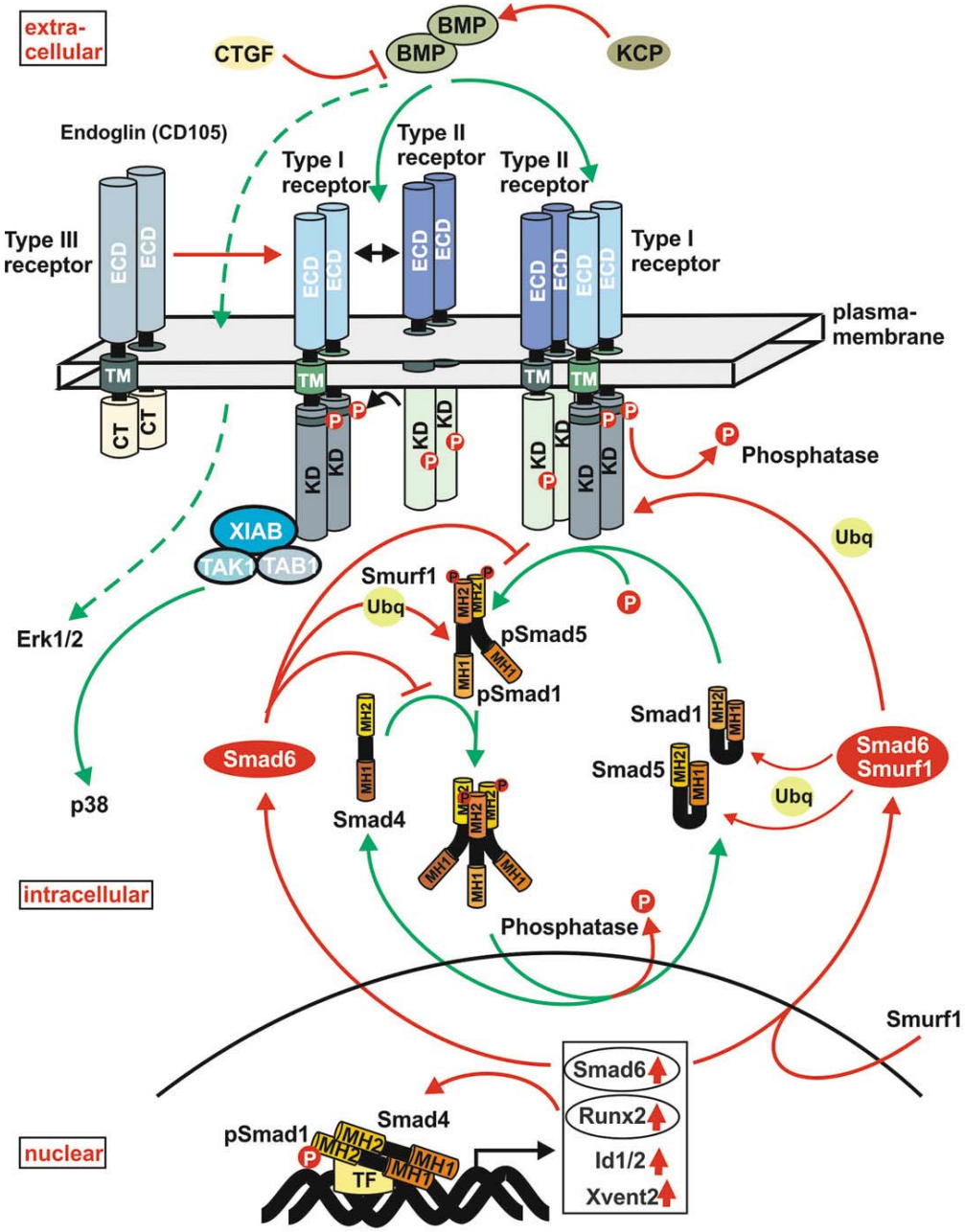

Figure 7. BMP signal transduction. Signaling by members of the BMP-subfamily of ligands is initiated by binding to a heteromeric complex of type I receptors, e.g. ALK-2, ALK-3 and ALK-6, as well as type II receptors, e.g. BMPRII, ActRII and ActRIIB (see Figs. 4 and 5). The access of ligands to the receptors is regulated by several secreted proteins including the CCN protein CTGF (negatively) or KCP (positively). In addition, type III receptors like Betaglycan or Endoglin modulate the signal transmission. Subsequently, intracellular mediators belonging to the family of MAP kinases (p38, Erk1/2; *left*) that form a network with diverse other kinases (e. g. XIAB, TAK1, TAB1) become activated. Alternatively, intracellular BMP responsive Smads (*right*) become activated by phosphorylation, associate with the co-Smad4 and translocate into the nucleus to regulate transcription of target genes in conjunction with co-repressors and co-activators. The signaling circuit (*green arrows*) is controlled at several steps by the inhibitory Smad (i.e. Smad6). The activated forms of type I receptors and Smads are deactivated through dephosphorylation by specific phosphatases and marked for proteolytic degradation with ubiquitin by the ubiquitin ligase Smurf1. Abbreviations used are: CTGF: connective tissue growth factor; KCP: Kielin/Chordin-like protein; BMP: bone morphogenetic protein; ECD: extracellular domain; TM: transmembranal domain; KD: kinase domain; P: phosphate; Ubq: ubiquitin; MH1/MH2: MAD homology domain 1/2; TAB1: TAK1 binding protein;TAK1: TGF-beta activated kinase-1; TF: transcription factor; XIAB: X-linked inhibitor of apoptosis protein.

of the identified Smad binding partners have additional cellular targets impacting TGF-beta/BMP responses (76).

3.2.6. BMP target genes and opposing factors

There are several genes identified which are directly induced by BMPs, i.e. the family of inhibitors of DNA binding 1-4 (Id1-4), GATA2, Dlx5 and Tob (see above) (77). The best studied of those target genes of BMP signaling are the *Xenopus Vent2* gene and the Id genes (78-82). The respective promoters of these genes contain specific Smad binding elements (SBEs) conferring binding of Smad1/Smad5 and Smad4 that are necessary and sufficient for BMP-responsiveness (83, 84, 79). Respective binding elements have been cloned into different reporter systems allowing monitoring and quantitating BMP activity (85, 86). These gene reporters were also taken to identify novel BMP target genes (77). The demonstation that *Xvent2, Id* and other proteins that belong to the group of immediate early genes and become activated after stimulation with BMPs further points to the fact that BMPs are relevant for several independent gene networks in various experimental conditions (87-89, 29). Another family of target genes of the BMP signaling cascade are the Runx transcription factors (see also 3.2.5) that are essential for the commitment of the osteogenic program and are in turn pivotal regulators of Smad-signaling itself (see 3.2.7). As a member of the inhibitory Smads (ISmads), Smad6 has been identified to be an essential feed-back regulator of BMP signaling (90, 91). Smad6 belongs to the inhibitory Smads, e.g. Smad6 and Smad7, and is upregulated as an immediate early gene in response to BMP stimulation and this is transcriptionally facilitated by a cooperation of Smad1 and RunX2 transcription factors (92, 93). As described above their target genes which are induced by BMPs are common. Nevertheless, some genes are differentially induced by individual members of the BMP subgroups. With respect to EMT it is worth to note that BMP-4 is able to enhance expression of typical EMT markers including SNAIL and SLUG that reduce E-cadherin expression (94) and which are components of the so called “EMT proteome” (see 3.5.). Thus, BMP-4 and TGF-beta act in the same direction on EMT marker proteins (95). Interestingly, these proteins are inversely regulated by BMP-7, demonstrating i) a heterogeneity in the BMP subgroups and ii) the opposing activities between TGF-beta and BMP-7. Other potential downstream target genes of the BMP network that were reported recently are the death inducer-obliterator 1 (Dido1) (96) and cochlin (97). However, the precise impact on BMP and TGF-beta signaling of these two proteins is still elusive.

3.2.7. Regulation of intracellular signaling

To achieve a tight regulation of signaling in time there are several regulatory mechanisms which are either common throughout the TGF-beta superfamily or are specific for the individual TGF-beta or BMP branches. As mentioned above, among the diverse BMP target genes Smad6 acts as a switch that deactivates BMP signaling. In contrast to Smad7, which blocks TGF-beta- and BMP signaling (98, 99), the inhibitory function of Smad6 is primarily restricted to BMP signaling (99, 100). There are multiple steps by which Smad6 interferes and switch off respective signaling cascade (66*)*. It binds to Smad4 and prevents the formation of BR-Smad-Smad4 complexes (101). In addition, Smad6 directly interacts with the activated type I receptors thereby inhibiting further phosphorylation of BRSmads by the receptor (102). It also has affinity for BRSmads (Smad1) and abrogates their interaction by acting as a Smad4 decoy (91). Moreover, beside this competitive binding of Smad6 to receptors and BRSmads it engages the WW and HECT domain E3-ubiquitin ligases Smurf1 and Smurf2 that both interact with type I receptors and BRSmads to induce post-translational polyubiquitination of the corresponding signaling components (103). In particular, the binding of Smurfs to the activated type I receptor is enhanced by the binding of Smad6 and leads to ubiquitination of the receptors, which marks them for degradation (103, 104). A similar mechanism targets activated BRSmads that are also bound cooperatively by Smad6/Smurf1 and labeled with ubiquitin for degradation (105, 103). The above mentioned activation and deactivation steps are intimately linked to receptor trafficking. In general there are two different routes by which receptor complexes are handled by the cell. If partitioned to clathrin-coated pits, active signaling takes place from early endosomes. These are enriched in the protein Smad anchor for receptor activation (SARA) which governs Smad2 and Smad3 activation by the receptor. The endosome-associated FYVE domain protein endofin is endowed with a similar function in the BMP axis. It preferentially binds to Smad1 and promotes its phosphorylation and nuclear localization. Beside Smad1, endofin binds to the protein phosphatase PP1c and facilitates thereby the dephosphorylation of the type I receptors ALK3 and ALK6 (106). Finally, receptors can travel back to the cell surface. If endocytosis occurs through lipid-rafts/caveolae, receptors are complexed with I-Smads and Smurfs, get ubiquitinated and degraded (104). Dullard is a protein which specifically regulates BMP receptor signaling by mediating ubiquitin-dependent degradation of the BMP receptor complex *via* the lipid-raft pathway. In addition, this protein represses the BMP-dependent phosphorylation of the type I receptor (107). Since phosphorylation is an essential step in the activation of type I receptors and BRSmads, mechanisms that lead to a block of phosphorylation or to dephosphorylation are another important regulatory step. The removal of phosphate groups within respective proteins by specific phosphatases consequently results in deactivation of these components (108). It has already been mentioned before that Smads are composed of three structural modules which serve to perform the different functional aspects of Smads. Especially the linker region is important for post-translational modification, e.g. ubiquitination and phosphorylation (see above). Thereby, the Smad proteins function as integrators for signals of different sources, which modulate their activity. In order to specifically regulate these different inputs individually Smads are subject to dephosphorylation by a versatile set of phosphatases. The two protein phosphatases PDP1 (pyruvate dehydrogenase phosphatase1) and PPM1A remove phosphorylation of the C-terminal serines in Smad1 thereby blocking BMP-induced responses (108-110). Smad phosphorylation by type I receptors occurs at the C-

terminal domain of Smads, whereas the phosphorylation of BRSmads by MAP kinases typically effects serine and threonine residues in the linker regions (111). Erk1/2 phosphorylation of the Smad1 linker region leads to inhibition of nuclear accumulation of Smad1 (112). Smurf1 binds to this phosphorylated linker region and causes cytoplasmic retention and poly-ubiquitination of Smad1 (111). Another group of phosphatases, e.g. small C-terminal domain phosphatases (SCP1, 2 and 3) were shown to remove Smad1 linker and C-terminal phosphorylations resetting Smad1 phosphorylation status (113).

3.3. Physiological functions of BMP-7 in normal and fibrotic organs

It is now known for over two decades that BMPs have a variety of functions during embryonic development (1). They typically function as morphogens acting as graded positional cues to dictate cell fate specification, tissue patterning, and have further important roles in the regulation of other genes (e.g. Nodal) that are necessary in the development of vertebrate embryo and prediction of left-right asymmetry (114). As mentioned above (see 3.1.), the BMPs were first purified from bone and thought to play essential functions in chondrogenesis and osteogenesis. Subsequently, several lines of evidence indicate that the BMPs are influencing a wide range of tissues during development and have essential roles in keeping organ homeostasis. Beside several other experimental BMP knock-out models, unambiguous demonstration of their multifunctional role in morphogenesis came particularly from mice that were deficient for BMP-7 (105). These mice die shortly after birth because of poor kidney development. Moreover, the lack of BMP-7 resulted in skeletal patterning defects and induced severe impairment of eye development demonstrating that BMP-7 is not only an early inducer of glomeruli formation but is also involved in the formation of other organs. The absence of endogenous BMP-7 led to small dysgenic kidneys with less glomeruli combined with hydroureters. Most interestingly, mice lacking BMP-7 showed severe defects in the "*nephrogenic process"* in which metanephric mesenchyme undergoes an epithelial transition to form glomeruli and tubules of the nephron (115). This process is an important mechanism for cellular reorganization during kidney development and it is reasonable that BMP-7 is the driving force involved in controlling the ratio of mesenchymal to epithelial cells in morphogenesis. Following its inductive action in kidney development, BMP-7 in normal kidney continues to be heavily expressed specifically in podocytes, distal tubules and collecting ducts (58). In line, it has been demonstrated that the inhibition of endogenous BMPs in transgenic mice ectopically expressing the BMP antagonist Noggin in the glomerular podocytes resulted in a severe phenotype that is characterized by mesangial matrix expansion. These results further strengthen the notion that BMPs have an important role in regulating glomerular structural homeostasis and that the lack of BMP-7 leads to extensive mesangial matrix expansion. (116). The finding that BMP-7 expression is downregulated in diseased kidney and the demonstration that the balanced administration of recombinant BMP-7 reduces the progression of renal fibrosis in animals with experimental renal diseases further indicate that BMP-7 has therapeutic (antifibrotic) properties (117-120).

Beside the important function of BMP-7 in renal development and homeostasis, it has been shown that liver regeneration is also massively affected by this multifunctional cytokine. In particular, it was demonstrated that the systemic application of neutralizing antibodies targeting endogenous BMP-7 after hepatectomy in mice resulted in impaired hepatic regeneration, whereas administration of rBMP-7 led to an enhanced regeneration suggesting that BMP-7 is a physiological regulator of hepatocyte proliferation and liver homeostasis (5, 121). More recent findings have demonstrated that the either the oral administration of a recombinant virus that directs the synthesis of BMP-7 or the systemic application of BMP-7 suppresses carbon tetrachloride-induced hepatic fibrosis in rodents (122, 123) suggesting that BMP-7 has therapeutic potential for treatment of liver lesions.

In more mechanistically studies, it became evident that some of the antifibrotic effects are mediated by the inhibition of profibrogenic TGF-beta activities (120). This mechanisms of balancing the "Good (i.e. BMP-7) against the Bad (i. e. TGF-beta) guy" has nowadays attracted many scientists and clinicians and was transferred to different organs (29, 124-127), in which the final common pathways by which TGF-beta establishes fibrosis are similar or the same. Based on these findings many independent studies were initiated with the aim to clarify some of the basic aspects of the beneficial molecular and cellular mechanisms of this potential therapeutic in different organs (see below).

3.4. Efficacy of BMP-7 as a therapeutic drug targeting fibrosis

The therapeutic efficacy of BMP-7 to act as an opposing factor for TGF-beta was tested in several experimental models of organ fibrosis in liver, heart, kidney, lung, and eye (Table 2). In kidney it was already demonstrated in 2003 that the applications of BMP-7 in rodents have *in vivo* a high therapeutic potency without significant side effects or toxicity (128). Thus, renal fibrogenesis associated with ureter obstruction in mice was prevented by systemic application of rBMP-7, and fibrotic symptoms, e.g. interstitial accumulation of type IV collagen or tubular atrophy, were significantly reduced (117, 119). Remarkably, these studies showed BMP-7 effectiveness superior to enalapril, a drug used to treat kidney disease related to diabetes. Another mouse model reflecting human diabetic nephropathy confirmed the benefit from BMP-7 in this pathological context (129). In CD1 mice that were made diabetic by single injection of streptozotocin (STZ) and developed glomerular hypertrophy combined with tubulointerstitial fibrosis, BMP-7 effectively prevented progression of diabetic nephropathy, inhibited glomerular hypertrophy and tubular damage, decreased interstitial type III collagen, and reduced the quantities of serum creatinine reflecting overall recovery of renal function (129). In line, transgenic mice that overexpressed human BMP-7 under transcriptional control of a rat phosphoenolpyruvate carboxykinase promoter fragment showed reduced

Table 2. Therapeutic effects of BMP-7 in experimental models of fibrosis

Organ	Injury model	Treatment	Antifibrotic effects	References
Kidney	STZ[1]-treated CD1 mice	rBMP-7	inhibition of glomerular hypertrophy, reduced tubular damage and relative interstitial volume, decreased type III collagen accumulation, reduced serum creatinine	(129)
	STZ-treated mice	transgenic	decreased expression of type I collagen and fibronectin, reduced glomerular and interstitial fibrosis level, elevated activity of renal MMP-2 and MMP-4	(130)
	MRL/MpJ$^{lpr/lpr}$ lupus mice	rBMP-7	reduced glomerular hypertrophy and relative interstitial volume, reduced serum creatinine, decreased interstitial type I collagen	(133)
	Col4A3$^{-/-}$ mice	rBMP-7	reduced tubular atrophy and relative cortical interstitial volume, decreased renal pathology-related mortality rate, reduced serum creatinine, blood urea nitrogen, and urine protein	(133)
	unilateral ureteral ligated rats	rBMP-7	reduced tubular atrophy and relative cortical interstitial volume, decreased interstitial type IV collagen, recovery of glomerular filtration rate	(119)
	cold ischemia in rats	rhBMP-7	BMP-7 decreased cellular damage in rat kidney	(132)
	Reversible urinary tract obstruction	rBMP-7	BMP-7 promotes the formation of Smad1/5/8–Smad4 transcription factor complexes and reduces the formation of Smad2/3–Smad4 transcription factor complexes in the injured kidney resulting in resolution of fibrotic lesions in the kidney	(134)
Liver	TAA-treated rats	adenoviral expressed BMP-7	decreased expression of alpha-SMA and type I collagen, decreased hydroxyproline content	(135)
	CCl_4-treated mice	rBMP-7	decreased type III collagen accumulation, reduced number of FSP1$^+$ and FSP1$^+$/Alb$^+$ fibroblasts, increased serum albumin	(136)
	CCl_4-treated mice	rBMP-7	Downregulated expression of Col-1, alpha-SMA, TIMP-2; upregulation of MMP-2	(137)
Heart	pressure overload in mice	rBMP-7	reduced accumulation of extracellular matrix and fibroblasts, increased microvascular density, elevated left-ventricular end-diastolic pressure	(138)
	chronic heart rejection in mice	rBMP-7	reduced accumulation of extracellular matrix and fibroblasts, increased microvascular density, decrease in FSP1$^+$CD31$^+$- and alpha-SMA$^+$CD31$^+$-positive cells	(138)
	Vascular calcification induced by high levels of vitamin D and phosphate	rhBMP-7	Reduction of vascular calcification; attenuation of increased expression levels of osteopontin and osteocalcin	(139)
Lung*	asbestos exposure in mice	rBMP-7	reduced hydroxyproline contents	(140)
Eye	capsular injury with hypodermic needle	adenoviral expression of rBMP-7	suppression of injury-induced EMT in lens, increased expression of Id2 and Id3	(141, 196)

[1]Abbreviations used are: Alb$^+$: albumin positive; Col4A3$^{-/-}$: deficient for the alpha3-chain of type IV collagen; MMP: matrix metalloproteinase; rBMP-7: recombinant BMP-7; STZ: streptozotocin; TAA: thioacetamide. * Note: In one study, the systemic treatment with rBMP-7 was not suitable to inhibit intratracheal or subcutaneous bleomycin-induced expression of collagen in lung (197).

glomerular fibrosis and expression of ECM components after STZ treatment (130). Prevention of glomerular sclerosis by BMP-7 treatment, superior to enalapril therapy, was also observed in a diabetic rat model (131) and a general reversion of impaired tubular architecture in a rat model of ischemic acute renal injury or in mice with nephrotoxic serum induced nephritis by BMP-7 is described (6, 128, 132). The antifibrotic efficiency of BMP-7 has also been demonstrated in two genetic models of renal diseases. In both, MRL/MpJ$^{lpr/lpr}$ mice, which develop a lupus-like disease with progressive renal fibrosis, or mice lacking the type IV collagen-alpha3 gene, which develop progressive renal disease, the systemic administration of rBMP-7 resulted in reversion to glomerular and tubular homeostasis and reduction of serum creatinine (133). At the molecular level it was demonstrated in a reversible urinary tract obstruction model that BMP-7 reduces the formation of Smad2, Smad3, and Smad4-containing transcription factor complexes (134).

The therapeutic effects of BMP-7 were not only examined in renal fibrosis but also found in hepatic, cardiac and pulmonary models of fibrosis. Adenoviral delivery of a construct that constitutively expressed murine BMP-7 in thioacetamide-treated rats, which develop hepatic fibrosis, resulted in a reduced expression of alpha-SMA and type I collagen that was accompanied by a decrease in liver hydroxyproline contents reflecting the antifibrotic potential of BMP-7 in this organ (135). In the same study, these effects were shown to be mediated by antagonism of TGF-beta signaling in hepatic stellate cells that represent the key effector cell during hepatic fibrogenesis. Another chemotoxic animal model of liver fibrosis in which carbon tetrachloride (CCl_4) was utilized as a hepatoxin revealed that rBMP-7 inhibited progression of liver fibrosis in mice by counteracting the TGF-beta-induced EMT of hepatocytes in the injured organ (136). Likewise, rBMP-7 downregulated expression of collagen, alpha-SMA, and TIMP-2 and increased expression of matrix metalloproteinase-2 (MMP-2) in livers of animals subjected to CCl_4 (137). This study further demonstrated that BMP-7 suppressed expression of TGF-beta and increased expression of gremlin representing a known endogenous antagonist for BMPs.

Three different mouse models of cardiac and vascular fibrogenesis were used to assess the efficiency of systemic administered rBMP-7 including pressure overload-induced fibrosis by aortic banding, allograft rejection by heart transplantation of MHC class II-

incompatible donors and recipients, and vascular calcification induced by high levels of vitamin D and phosphate (138, 139). In all three models, BMP-7 therapy resulted in reduced accumulation of ECM and fibroblasts and increased microvascular density. Additionally, the chronic heart rejection model revealed a decreased number of fibroblast specific protein 1 (FSP1)- or alpha-SMA-positive cells, indicating reversal of TGF-beta-induced EMT. In lung, it was recently demonstrated that rBMP-7 reduces the hydroxyproline content in mice that were exposed to asbestos (140).

In vitro experiments revealed that the underlying mechanisms that regulate the interrelation between TGF-beta and BMP-7 signaling are in the cell transmitted by Id2, Id3 and Smad6 (135, 141, 142). These studies have already shown that BMP-7 increases the expression of Smad6 and Id proteins in several cellular systems, which directly lead to blockage of collagen expression. Moreover, transient expression of Id proteins had similar effects like overexpression of BMP-7 (143).

In summary, a number of *in vivo* models in different organs congruently demonstrated the high efficiency of BMP-7 as a therapeutic effective agent in fibrotic diseases. Nevertheless, no clinical approach has been made so far to translate these promising data from animal models into human therapy and clinics.

3.5. The complex regulatory network of BMP-7 and TGF-beta in epithelial-to-mesenchymal transition and mesothelial-mesenchymal transition

EMT is the phenomenon whereby fully differentiated epithelial cells transit into a mesenchymal phenotype giving rise to fibroblasts and myofibroblasts that play an important role in tissue repair and fibrosis following epithelial injury. TGF-beta$_1$ has been shown to be a prominent trigger of EMT in normal mammary epithelial cells (144) and to mediate EMT *in vitro* in a large variety of epithelial cells including renal proximal tubular, lens, alveolar epithelial, cardiac endothelial, biliary endothelial cells, skin, hepatocytes and other cells (145-151). EMT response to TGF-beta$_1$ in fibrosis is predominantly mediated *via* Smad-dependent signal cascades, mainly by Smad3-driven pathways (152). As mentioned above, TGF-beta$_1$ signals are transduced by transmembrane serine/threonine kinase type II and type I receptors resulting in subsequent phosphorylation of Smads. Upon TGF-beta$_1$ stimulation, the receptors are internalized *via* clathrin-coated pits into early endosomes where the Smad anchor for receptor activation (SARA) is localized and modulates the formation of ALK-5 complexes with Smad2 or Smad3. Both, Smad2 and Smad3 are phosphorylated at C-terminal serine residues by the type I receptor and associated with Smad4 and further translocated as a multimeric complex to the nucleus where they interact with other transcription factors to regulate transcription of TGF-beta-responsive genes such as CTGF, alpha-SMA, collagen 1 type alpha 2 and plasminogen activator inhibitor-1 (PAI-1) (153) (Figure 8). Non-Smad-dependent pathways implicated in TGF-beta-dependent EMT include the RhoA, Ras, MAPK, PI3K, Notch, and Wnt signaling pathways. Stimulation of these cooperative pathways usually provides the context for induction and specification of EMT within a particular tissue, with Smads representing the dominant pathway (154). In addition, the integrin-linked kinase (ILK), an intracellular serine/threonine kinase that interacts with the cytoplasmic domains of beta-integrins and cytoskeletal proteins, has been identified as a potential downstream mediator of Smad-mediated TGF-beta$_1$ signaling in the setting of EMT (155). Modulation of the TGF-beta$_1$-dependent Smad pathway in animal models has provided strong evidence for a role of TGF-beta in fibrotic EMT *in vivo*. In particular, EMT was ameliorated in Smad3 knockout mice (156, 146), and in cultured hepatocytes overexpressing Smad7, an antagonist of TGF-beta signaling (157, 158). Retro-EMT (or mesenchymal-to-epithelial transition, MET) in which mesenchymal cells with a motile, multipolar or spindle-shaped structure transit into planar layers of polarized cells has also been brought in context with TGF-beta/BMP signaling. In particular it was demonstrated that MET is a highly orchestrated process in which pro-EMT signals and genes including Snail, TGF-beta$_1$ and TGFbetaRII are suppressed, while mesenchymal genes that initiate a morphological reprogramming of the cells become activated (159).

However, the final relevance of EMT and MET for the generation of hepatic lesions is presently intensively debated among researchers (160, 161). While this exciting discussion of EMT/MET is still ongoing, pioneering work has shown that mesothelial cells have also potential to transit in a TGF-beta-dependent manner into mesenchymal fibrogenic cells *via* a mechanism that is called Mesothelial-mesenchymal transition (MMT) (162). This novel concept is extremely challenging and adds another good explanation for the presence and formation of large profibrogenic cell quantities during ongoing hepatic fibrogenesis and further might explain the occurrence of cellular heterogeneity of the fibrogenic cell populations in liver. Similar experimental findings were also reported for pleural mesothelial cell that were shown to differentiate TGF-beta-dependent into myofibroblasts (163) (Figure 8). However, future work is necessary to confirm this mechanism and to clarify if MMT is specific for liver and lung or occurs also in other organs under fibrotic conditions. Moreover, it will be interesting to see if this new cellular transition can be blocked by BMP-7.

There is now a multitude of independent reports demonstrating that BMP-7 blunts TGF-beta$_1$-induced EMT in adult organ fibrosis by directly counteracting TGF-beta-induced Smad3-dependent signals as evidenced by the observed reduction of fibrosis occurring *via* EMT *in vivo* (164, 140, 133). Similarly, drugs (e.g. alendronate sodium, vitamin E) that increase endogenous expression of BMP-7 and reduce expression of endogenous TGF-beta can effective arrest the progression of hepatic and renal fibrosis and further modulate expression of genes involved in EMT (165, 166). In association with Smad2 downregulation, BMP-7 delays EMT in lens epithelium, whereas overexpression of inhibitory Smad7 blocked EMT and decreases nuclear translocation of Smad2 and Smad3 (157).

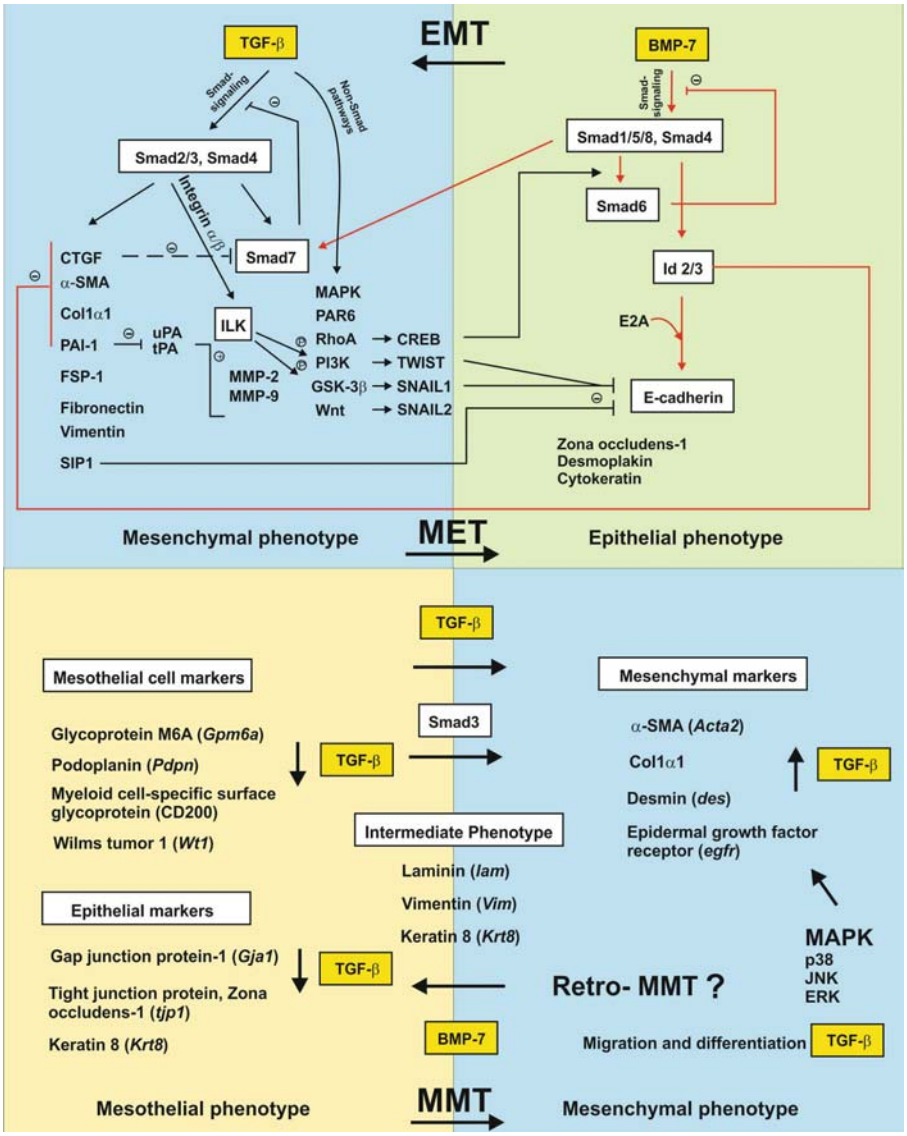

Figure 8. Complex pattern of interaction between TGF-beta and BMP-7 in regulating EMT, MET and transition MMT. BMP-7 supports the epithelial phenotype by inducing the expression of Smad7 through the Smad1/4/GATA complex that inhibits TGF-beta signaling, and Id2/3 which inactivate the repressor E2A to permit expression of E-cadherin (*upper part*). Ids also inhibit several TGF-beta responsive genes including CTGF and alpha-SMA, Col 1 and PAI-1. On the other hand, TGF-beta supports the mesenchymal phenotype by the rapid induction of CTGF. CTGF binds BMP-7 and inhibits BMP-7 signaling as evidenced by lower levels of pSmad1/5 and Id1 mRNA (183). CTGF activates several receptor systems that lead to the induction of transcription repressors and inhibition of E-cadherin. CTGF further inhibits the expression level of Smad7, thus enhancing the transcription of TGF-beta-responsive genes. Moreover CREB, activated *via* CTGF, associates itself with the BMP/Smad complex, activating expression of Smad6 that not only inhibits the BMP-7 signaling pathway but also Id2/3 activity that leads to repression of E-cadherin. TGF-beta-induced PAI-1 in turn inhibits the activities of uPA and tPA that can activate the MMPs. Integrin-linked kinase (ILK), an intracellular serine/threonine kinase, associates with beta-integrin and regulates E-cadherin at the transcriptional level *via* the transcriptional repressor SNAIL-1. In addition, ILK phosphorylates Akt (PI3K) and glycogen synthase kinase (GSK), phosphorylation of GSK-3 resulting in nuclear translocation of beta-catenin and activation of the Wnt signaling pathway, which has also been strongly implicated in EMT. In MET, the transition runs back from mesenchymal cells to cells with epithelial phenotype. In a novel conceptional study, it was recently shown that mesenchymal cells can also result from mesothelial cells that usually express mesothelial (Glycoprotein M6A, Podoplanin, CD200, WT1), intermediate (Laminin, Vimentin), and epithelial (Gap junction protein-1, Zona occludens-1, Keratin 8) markers. Driven by the TGF-beta$_1$/Smad3 pathway these cells acquire a high migration and differentiation capacity and transit into a mesenchymal phenotype that express alpha-SMA, collagen type I, desmin and the epidermal growth factor receptor. This target genes expression is further enhanced by the activity of TGF-beta$_1$ and diverse MAPKs. Presently, it is not known if Retro-MMT in which mesenchymal cells transit back into a mesothelial cell type can occur and which impact BMP-7 has on MMT.

The underlying mechanism is thought to involve the BMP-7 dependent induction of Id proteins (167), which then in turn inhibit TGF-beta the driving force in EMT. Id proteins lack a basic DNA binding region, but they possess a basic helix-loop-helix (bHLH) dimerization motif that allows them to interact with and inactivate other HLH transcription factors that inhibit or activate transcription. CTGF, PAI-1 and thrombospondin-1 are among those TGF-beta responsive genes directly downregulated by BMP-7 (168). Blocking of TGF-beta-dependent upregulation of PAI-1 by BMP-7 also results in induced expression of active MMP-2 that promotes degradation and breakdown of the fibrotic matrix. BMP-7 counteracts TGF-$beta_1$-induced EMT, reversing chronic renal injury through induction of E-cadherin, a key epithelial cell adhesion molecule, through direct antagonism involving Smad signaling pathways as evidenced by co-localization of phospho-Smad2/3 and Smad1 in nuclei (133). Furthermore, BMP-7 regulates the expression of target genes that are characterized by BMP responsive elements (BRE) in their promoters. The human and murine Id1 promoters contain a 29-bp GC-rich sequence element that is fully conserved. This sequence comprises a consensus motive of early growth response-1 (Egr-1), which has been identified as a regulatory element for the expression of Id1 in response to serum (84).

Other genes can comprise different BRE sequences which are bound by alternative Smad1 complexes. In case of the Smad7 gene, binding of the Smad1/4/GATA complex to the I-BRE element confers a high affinity switch in the presence of GATA transcription factors. This regulation may enhance Smad7 induction leading to a blockage of TGF-beta signaling and allowing BMP to signal, even at low BMP concentrations (169) suggesting that the observed effects at a given concentration of BMPs are strongly dependent on TGF-beta concentrations and *vice versa*.

3.6. Modulation of BMP-7 function by members of the CCN protein family and other modifiers of organ fibrogenesis

Our and other laboratories have previously shown that liver parenchymal cells (i.e. hepatocytes) substantially synthesize CTGF during prolonged culture and during experimental liver injury, and further that this cell type is the major source of CTGF in the liver (170-173, 49). CTGF, a designation introduced in 1991 for connective tissue growth factor (174) is a 36-38 kD, cysteine-rich, heparin-binding and secreted protein, which was initially identified in the culture supernatant of vascular endothelial cells. It is now classified as the second of six distinct members of the CCN gene family containing CTGF itself, Cysteine-rich protein 61 (CYR61), NOV, and three WNT-induced secreted proteins (WISP) (175, 49). These proteins share approximately 40 to 60% sequence similarity and are characterized as mosaic proteins that comprise four conserved structural modules including an insulin-like growth factor binding domain (module I), a von Willebrand Factor type C motif (module II), a thrombospondin type I module (module III), and a carboxyl-terminal cystine knot (module IV). All these proteins are known to interfere with the BMP/TGF-beta signaling network during ongoing fibrogenesis (176, 49).

CTGF as a prototype of this protein family is an important downstream modulator protein of the profibrogenic master cytokine TGF-beta, amplifying its pro-fibrogenic action in a variety of tissues (176). Based on this endogenous attribute, CTGF has reached considerable pathophysiological relevance because of its involvement in the pathogenesis of fibrotic diseases, atherosclerosis, skin scarring, and other conditions with excess production of connective tissue (177). The strong expression of CTGF in fibrotic tissue occurs on the level of transcription and is stimulated by specific growth factors such as TGF-beta and endothelin-1, but also by environmental influences such as biomechanical stress and hypoxia (177). CTGF gene activation by TGF-beta is mediated by a functional SBE, which resides within the CTGF promoter (178).

The CTGF protein contains a proteinase-sensitive hinge region between modules II and III and might form different variants (179). Although the molecular mechanism of action of the different variants is still not known, its crucial role in fibrogenesis is well documented in fibrotic liver tissue (180, 181, 175). In line with this assmumption recent studies have shown that knock-down of CTGF by siRNA leads to substantial attenuation of experimental liver fibrosis (182, 183). Another investigation has shown that the upregulation of CTGF inhibits BMP-7 signal transduction in the diabetic kidney (184). Abreu and coworkers furthermore presented data that describe CTGF as extracellular trapping protein for BMP and TGF-beta thus modulating the activity of these cytokines (46).

According to functional studies in *Xenopus laevis*, CTGF directly binds BMP and TGF-beta through their cysteine-rich (CR) domain, thus antagonizing BMP activity by preventing its binding to BMP receptors. Of note, the opposite effect, enhancement of receptor binding, was observed for TGF-beta. These results suggest that CTGF inhibits BMP and activates TGF-beta signals by direct binding in the extracellular space. From this, CTGF that is mainly synthesized and secreted in hepatocytes within the liver would act profibrogenic by shifting the balance toward mesenchymal activity during hepatocellular EMT and during the course of MMT (Figure 9). However, clarification is still pending. Moreover, previous other reports have postulated that the von Willebrand factor type C motif within chordin, chordin-like 2, and crossveinless 2 have capacity to bind members of the BMP family (185) demonstrating that the occurrence of different proteins modules may even sequester BMP with different affinities and that their concentrations may be relevant for overall activity of BMPs.

Comparable to CTGF, there are several other proteinogenic modifiers that interfere with the activity of BMP-7. BMP antagonists identified so far include those of the Dan/Cerberus group (e.g. Gremlin), Noggin, Chordin and Follistatin. Although there is presently only limited information about the affinity and specificity of these

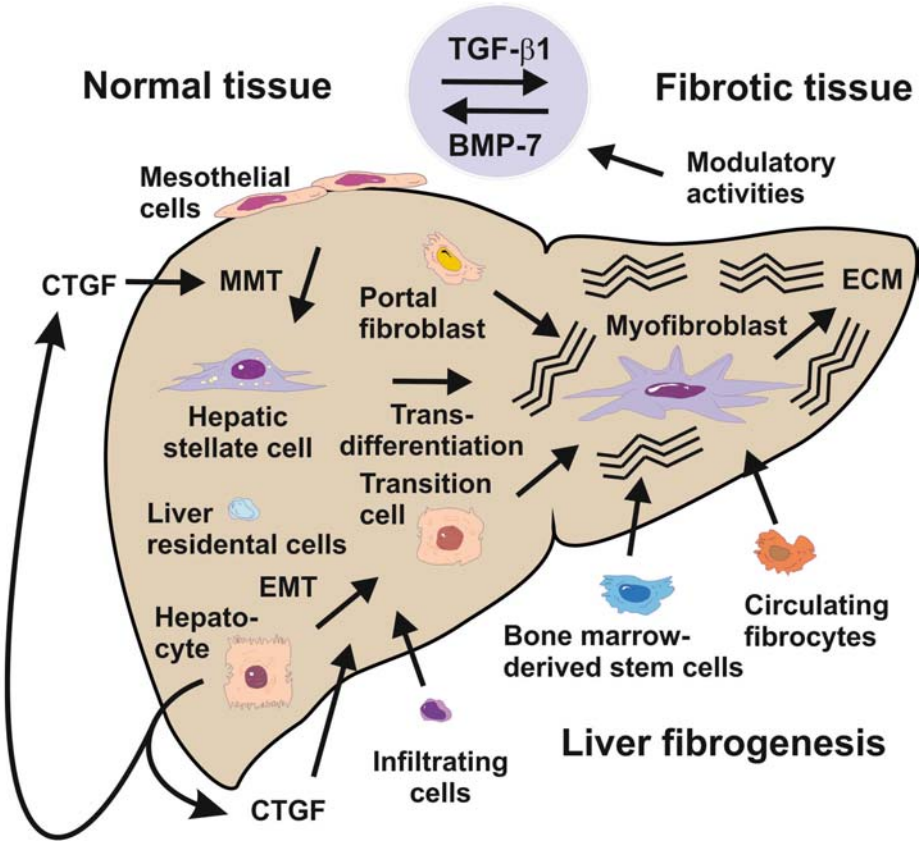

Figure 9. Novel pathogenetic concepts for recruitment of extracellular matrix (ECM)-producing myofibroblasts during ongoing hepatic fibrogenesis. In the inflamed liver tissue, hepatocytes undergo necrosis and release several profibrogenic mitogens (i. e. TGF-beta) that subsequently activate hepatic stellate cells (HSC). These cells transdifferentiate into myofibroblasts (MFB) capable to produce large amount of proteoglycans, collagens, glycoproteins, and hyaluronic acid. Hepatocytes are further induced to undergo epithelial-to-mesenchymal transition (EMT) in which they transit *via* a transitional phenotype into MFB. The cellular fraction of MFB is further increased by circulating fibrocytes and bone marrow-derived stem cells that infiltrate into the liver and change their phenotype. Moreover, in a recent concept it was proposed that mesothelial cells that usually form the outer organ lining migrate into the inner of the liver and acquire HSC/MFB features allowing them to produce ECM components (161). In all these processes, TGF-beta acts profibrogenic while BMPs (especially BMP-7) have opposing effects. The balance of both cytokines is further modulated by several sequestering proteins including members of the CCN family (e.g. CTGF, NOV, CYR61) that modulated the activities of TGF-beta and BMPs.

modifiers, it is known that Noggin, Chordin, and Follistatin can physically interact with BMP-7. Therefore, it is reasonable that these antagonists interfere with BMP signaling by sequestering BMP-7. The recent finding that Gremlin was up-regulated in asbestos-exposed mouse lungs and combined with a down-regulation of BMP signaling indicated by reduced levels of Smad1/5/8 and enhanced Smad2 phosphorylation suggests that Gremlin is potentially involved in blockade of BMP signaling (142). However, a direct interaction of Gremlin with BMP-7 was not reported. Therefore, the effects of Gremlin on BMP-7 might be attributed as indirect. Another suppressor of BMP-7 activity is Sclerostin (also known as SOST) that was originally identified as the sclerostenosis-causing gene. It contains six conserved cysteine residues and one conserved glycine residue that are essential to form the cystine knot which binds to BMP-7 with high affinity and with unique ligand specificity (186). A similar protein containing such a sclerostin domain that is commonly known as uterine sensitization-associated gene-1 (USAG-1 or SOSTDC1 for Sclerostin domain-containing protein 1) inhibits BMP-2, BMP-4, BMP-6, and BMP-7 activity in a mouse preosteoblast cell line (187). Interestingly, the ratio of USAG-1 to BMP-7 expression decreased with kidney damage but increased after subsequent kidney regeneration (187). In line with this finding, it was shown that the loss of USAG-1 ameliorates disease in a mouse model of the progressive hereditary kidney disease Alport syndrome again demonstrating that USAG-1 acts fibrogenic by sequestering antifibrogenic BMP-7 activity (188).

Additionally, there are several proteins that increase the activity of BMP-7. We have reported that the accessory type III receptor Endoglin enhances BMP-7 signaling and *vice versa* suppresses the activity of TGF-beta$_1$ (29). In this study we further demonstrated that the

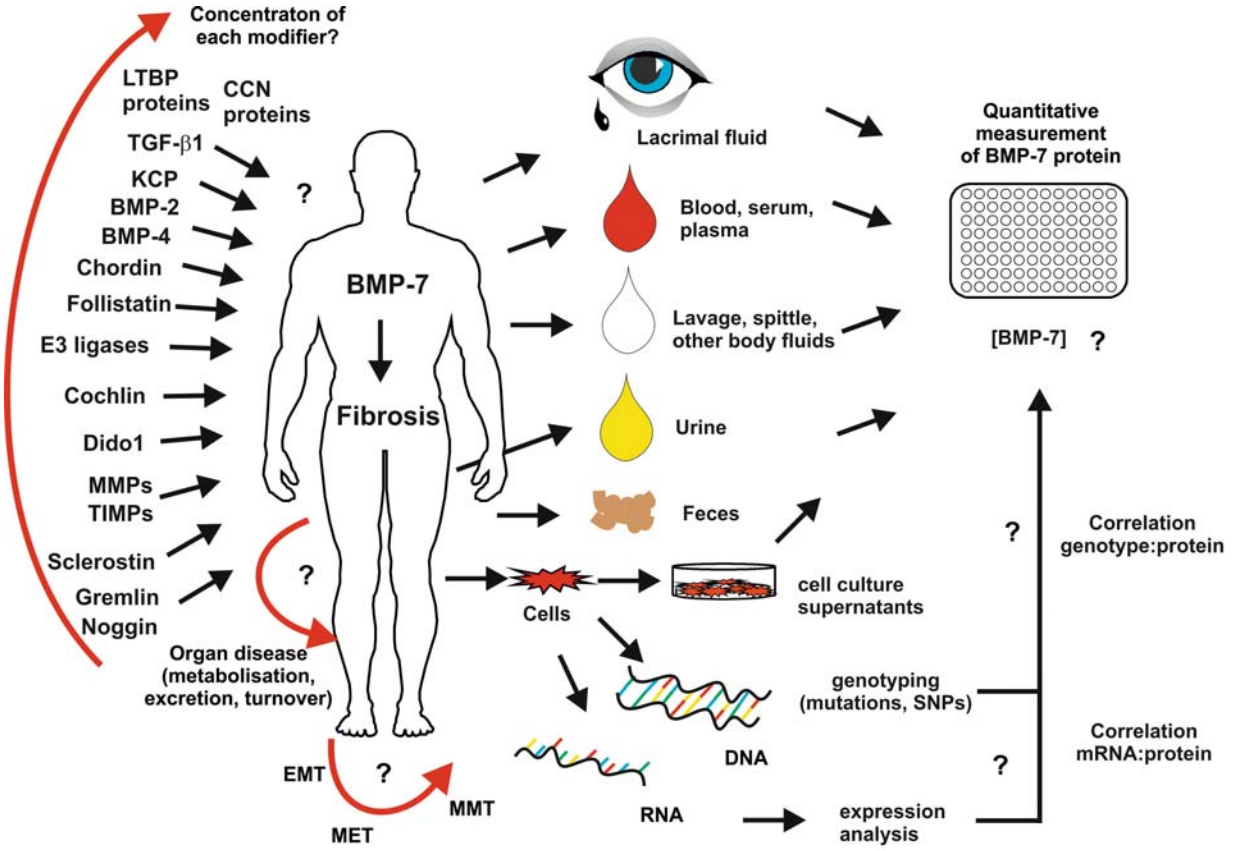

Figure 10. Pitfalls in diagnostics of BMP-7 protein, mRNA and genotyping measurements. In principle it is possible to determine BMP-7 quantities in any kind of body samples including blood, serum, plasma, lavage, spittle, lacrimal fluid, urine, and feces as well as in cellular supernatant of any kind of cells. In addition, mRNA expression of endogenous BMP-7 can be quantified in any organ or cell and genomic mutations or single nucleotide polymorphisms can be identified by genotyping. However, the absolute concentration of BMP-7 in these specimens, the sequence variants as well as the expression data for BMP-7 must be definitely interpreted with caution. Moreover, there are several proteins which modulate the biological activity of BMP-7 in a synergistic or antagonistic/opposing. These signaling modulators themselves are regulated by secondary circuits resulting in a complex interactive network that further influences the overall activity of BMP-7 in the body. In addition, organ disease that may affect metabolisation, excretion or turnover of all these proteins or BMP-7 itself impact the activity of BMP-7. Last but not least endogenous processes including EMT, MET and MMT (cf. Figure 9) will sequester BMP-7 or TGF-beta quantities resulting in an elevated or decreased activity of respective cytokines.

transient overexpression of Endoglin, previously shown to inhibit TGF-beta$_1$-induced ALK-5/Smad3 signaling, enhanced the BMP-7/Smad1/Smad5 pathway suggesting that Endoglin is another attractive target molecule when a lowered BMP-7 activity should be counteracted. The Kielin/Chordin-like protein (KCP) is another protein that was identified as an enhancer of BMP-7 signaling (189). KCP is a high molecular weight protein consisting of a signal peptide, followed by 18 cysteine-rich chordin repeats and a C-terminal von Willebrand factor type D domain. It binds to BMP-7 and enhances binding to the type I receptor. Accordingly, animals lacking KCP are more susceptible to the development of renal interstitial fibrosis and are molecularly characterized by reduced levels of phosphorylated Smad1 again demonstrating that BMP-7 in conjunction with its modifiers is essential for proper organ development and function (189).

3.8. BMP-7 in clinical use and diagnosis

Newly recognized pathogenetic mechanisms that may impact the process of fibrogenesis such as EMT, MET and MMT which are strongly regulated by the concentration of active TGF-beta and its endogenous opposing factor BMP-7 offer several therapeutic options for therapy of fibrogenesis and non-invasive diagnostic strategies. In principle the level of BMP-7 can be measured quantitatively from any kind of body fluid, faeces, cell supernatants and other specimen by ELISA or other techniques (Figure 10). Beside direct measurements of absolute concentrations or biological activities of individual BMPs, recent advance has further forwarded methods that allow the simultaneously measuring of multiple BMPs in complex solutions (190).

Elevated serum levels of both BMP-7 (repressor of EMT) and TGF-beta (inducer of EMT and MMT) are found for example in serum and plasma of patients suffering from hepatic fibrosis. This is most likely due to an altered transcriptional control in cells relevant for fibrogenesis, release from necrotic hepatocytes and an overall reduced hepatic clearance by the insured (fibrotic) organ that may affect metabolisation, excretion and turnover of BMP-7 (191-193). In addition, it should be kept in mind that the body contains several (possibly hundreds) of other proteins and cytokines within the body fluid that either have antagonistic or agonistic functions or even physically interact with BMP-7 thereby preventing its accurate measurement. Moreover, it is potentially possibly that a general altered expression of BMP-7 might also result from genomic sequence variants (mutations, single nucleotide polymorphisms) that might be identified by genotyping or analysing the relative expression of BMP-7 at the RNA level. Presently there is no clear data available how these variants or altered mRNA quantities correlate to the measured cytokine concentration. Therefore, it is comprehensible that the measurement of either TGF-beta or BMP-7 or the analysis of BMP-7 mutations or gene variants alone is not sufficient to predict the outcome or predisposition of hepatic fibrogenesis and respective measurements are yet only partially incorporated into daily routine in clinics.

4. SUMMARY AND PERSPECTIVES

Many independent studies in experimental animal models have congruently shown that recombinant BMP-7 is therapeutically applicable to attenuate ongoing fibrogenesis in liver, lung heart, and skin. More basically studies had shown that BMP-7 acts as an opposing factor TGF-beta$_1$, thereby reducing inflammation and ECM synthesis that are hallmarks of organ fibrosis. BMP-7 is further effective in preventing EMT representing a crucial process in which epithelial cells transit into mesenchymal, myofibroblastic cells that lose cell-cell adhesion and express large quantities of alpha-SMA and profibrotic molecules such as collagen types I and III and fibronectin. Although the functionality of TGF-beta and the opposing activity of BMP-7 is well documented in the setting of several *in vitro* experiments, the *in vivo* occurrence of EMT and the relevance for fibrogenesis is presently matter of controversy and needs further investigations. Likewise, the recent observation that TGF-beta and its signaling cascade triggers MMT in lung and liver during which mesothelial cells acquire a mesenchymal phenotype needs critical approval. In particular, it will be interesting to see if BMP-7 can block MMT and prevent the formation of surplus quantities of profibrogenic cells that are formed in the course of organ insult.

At the end it should be mentioned that the rapidly growing numbers of reports that describe novel findings about BMP-7 function and regulation including its receptors, modifiers, intracellular pathways, and its signaling crosstalk with other cytokines or signaling molecules shows that BMP-7 is an interesting multifunctional molecule. Based on its properties this cytokine and its downstream signaling pathways offer definitely a large number of valuable drug targets that might be relevant for the treatment of fibrotic lesions. It will be interesting to see how this novel knowledge will be translated into clinic and help to develop effective novel antifibrotic therapies.

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Abbreviations: ALK: activin-like receptor-kinase: BMP: Bone morphogenetic protein; BRSmads: BMP specific RSmads; Col: collagen; CTGF: connective tissue growth factor; CYR61: Cysteine-rich protein 61; ECM: extracellular matrix; EMT: epithelial-to-mesenchymal transition; FSP1: fibroblast specific protein 1; GDF: growth and differentiation factor; Id: inhibitor of differentiation; ILK: Integrin-linked kinase; KCP: Kielin/Chordin-like protein; MET: mesenchymal-to-epithelial transition; MMT: mesothelial-mesenchymal transition; NOV: Nephroblastoma-overexpressed protein; PAI-1: plasminogen activator inhibitor-1; rhBMP-7: recombinant human BMP-7; RSmad(s): receptor-regulated Smad(s); Runx: runt domain transcription factors; SBE: Smad binding elements; TGF-beta: transforming growth factor-beta; USAG-1: uterine sensitization-associated gene-1; XIAB: X-linked inhibitor of apoptosis protein.

Key Words: Bone morphogenetic protein, transforming growth factor-beta, CCN proteins, intracellular signaling, signaling modulators, Smad proteins, epithelial-to-mesenchymal transition, mesothelial-mesenchymal transition, fibrosis, therapy, Review

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