

Bone morphogenetic protein-7 (BMP7) in chronic kidney disease

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1. ABSTRACT

Bone morphogenetic protein-7 (BMP7) is a member of the BMP-subfamily of perhaps a dozen proteins within the TGF β – superfamily of cysteine-knot fold cytokine – growth factors. BMP7 has pivotal functions during renal and eye development. In adult organisms, BMP7 is heavily expressed in kidney, specifically in podocytes, distal tubules and collecting ducts. The activity of BMP7 is reduced by inhibitors including some members of the dan – cerberus group and CTGF but can be enhanced by endoglin and KCP. Renal BMP7 disappears early in fibrogenic renal diseases which may facilitate progression. Exogenous administration of rhBMP7 or transgenic overexpression reduces renal fibrogenesis and apoptosis as well as transdifferentiation of epithelial cells. BMP7 improves maintenance of nephron function and structural integrity. These antifibrogenic activities result from inhibition of the nuclear translocation of TGF β -activated smad3 by smad6 downstream of BMP7-activated smad5. Although at present the beneficial effects of BMP7 have only been studied in rodent models of chronic renal diseases, there is promise for therapeutic utility of rhBMP7 or small molecule BMP7 agonists in patients.

2. INTRODUCTION

Bone morphogenetic proteins were discovered as a biologically defined activity of demineralized bone extracts to induce endochondral osteogenesis in muscle in-vivo (1). Bone morphogenetic protein-7 (BMP7), formerly called osteogenic protein-1 (OP-1), was first isolated from bone and characterized based on the bioactivity to induce osteogenesis (2).

BMP7 is a member of the BMP-family of proteins within the large TGF β -superfamily of cysteine-knot cytokines. This superfamily of cytokine – growth factors is defined by a single structural component, the cysteine knot fold (Figure 1). Individual subfamilies within the TGF β superfamily are somewhat more loosely defined by structural and sequence similarities (3, 4). There are at least 30 members to this superfamily and the number continues to grow as further peptides are discovered, structurally classified or re-classified. Individual subfamilies include: TGF β s, BMPs (except BMP-1), growth and differentiation factors (GDFs), activins and inhibin, glial-derived neurotropic factor (GDNF), Müllerian inhibitory substance (MIS), Lefty, Nodal and others. Based

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on the presence of a cysteine – knot fold, members of the dan-cerberus group of secreted inhibitors may also be included in this large protein superfamily. Members of this protein superfamily play pivotal roles during development and BMP7 specifically is required in renal and eye development. Postnatally, levels of BMP7 decrease in most organs and tissues with the kidney being an exception. Specific interest in this protein has been stirred by the observation that BMP7 may have antifibrotic as well as phenotype-preserving roles and reduces renal fibrogenesis (5).

3. THE BMP-FAMILY OF CYSTEINE-KNOT FOLD PROTEINS AND BMP7

There are at least 12 proteins that have been included within this subfamily. However, BMP1 does not share the required structural similarities and is not a true BMP. Among this multitude of BMPs with quite divergent functions, BMP2, -4, -5, -6 and -7 are expressed in the kidney; the kidney also expresses several proteins from other subfamilies including TGFβs.

The product of transcription and translation of the BMP7 gene is a large precursor protein of 431 amino acids. It consists of a small leader sequence (29 amino acids) which targets the protein to the excretory pathway of the cell. A pro-domain (262 amino acids) that may contribute to the folding and may help with dimerization of the mature peptide is proteolytically cleaved off giving birth to the active protein of 139 amino acids and a molecular weight of 15.7. kDa. This protein contains 7 cysteine residues (Figure 1). Six of these cysteines form three intra-molecular disulfide bonds forming the characteristic cysteine knot fold (Figure 1 a). The seventh cysteine residue, cys103, forms an intermolecular disulfide bond neighboring the cysteine knot and, thence, gives rise to the mature heterodimer of 31.5. kDa (Figure 1 b). Each monomer contains four potential N-glycosylation sites, and glycosylation is thought to be the reason for a somewhat greater apparent molecular weight in SDS-PAGE analysis of about 34-37 kDa.

4. RENAL EXPRESSION OF BMP7

Several members of the BMP-subfamily are expressed in the kidney. These include BMP2, BMP4, BMP5, BMP6, and BMP7. BMP7 expression in adults is quite selectively limited to kidney and bone. In the kidney, high levels of BMP7 are seen in distal convoluted tubules in the renal cortex. BMP7 is also localized to outer medullary collecting ducts (6-8). This pattern of tubular BMP7 expression is generally accepted by experts in this field (Figure 2). BMP7 is also expressed in glomeruli, specifically and exclusively in glomerular visceral epithelial cells (podocytes) as has been shown by different laboratories (8-13) (Figure 3). We had previously confirmed this immunohistology finding by quantitative PCR on glomeruli that had been isolated by microdissection (10). Moreover, cultured murine podocytes express BMP7 when differentiated but not or only at low levels when cultured under proliferation conditions (unpublished observation). Wetzels and associates

confirmed the tubular expression of BMP7 in normal human kidney but dispute any expression of BMP7 in glomeruli (7). This not only contrasts the findings in rodents as reported by several other laboratories but is also at odds with findings in humans recently reported by Petris and coworkers (8). These latter investigators demonstrated the presence of BMP7 mRNA in glomeruli that were isolated from normal human kidneys or subjects with diabetic nephropathy by laser capture microscopy. BMP7 mRNA levels were reduced in diabetic compared to control glomeruli. Moreover, glomerular BMP7 in these subjects is exclusively localized to podocytes as shown by immunohistochemistry by these same investigators (8). Thus, in totality, data from human and rodents indicate expression of BMP7 in glomerular podocytes in rodents and more likely than not also in humans.

BMP7 is secreted with normal rat urine at an apparent molecular weight of 37 kDa (Figure 4). The origin of urinary BMP7 is virtually beyond doubt mainly or exclusively the distal nephron, perhaps mostly distal tubules. Although plasma BMP7 could undergo glomerular ultrafiltration based on its molecular size, perhaps not quantitatively, but there has been no direct evidence that BMP7 is present in circulating blood in measurable amounts even in mice with transgenic over-expression of BMP7 (10).

5. EXPRESSION OF BMP7 – RECEPTORS AND SIGNALING PATHWAYS IN THE KIDNEY

BMPs interact with heterodimeric serine/threonine kinase receptors that are closely related to TGFβ receptors (Figure 5). The ligand binds to a type II receptor. This event is followed by a conformational change leading to recruitment of a type I receptor into a heterodimeric receptor complex and transphosphorylation of the type I receptor by the type II receptor kinase. There are several type II and type I receptors that can interact with ligands of the TGFβ superfamily. BMP7 interacts with BMP-receptor II (BMPRII) which forms a heterodimer either with activin-like kinase (Alk)-3 or -6; or with activin receptor II (ActRII) in a complex with Alk-2. BMP6 can also activate these same receptor complexes whereas BMP2 and -4 preferably interact with receptor complexes composed of BMPRII and Alk-3 or Alk-6.

The type I receptor, Alk1, plays a specific role in BMP and TGFβ signaling. Alk-1 is mainly expressed in endothelial cells. It can form complexes with TBRII or with ActIIB, a BMP type II receptor. In the endothelium, Alk-5, a TGFβ type I receptor, is also expressed. Endoglin which is expressed exclusively in endothelial cells, appears to act as a support receptor directing TGFβ to the Alk1/TBRII complex as compared to the Alk5/TBRII complex. This is of major importance because activation of the Alk1/TBRII receptor complex induces BMP-like cell signals whereas Alk5/TBRII activation induces TGFβ-typical cellular responses. Moreover, endoglin also binds BMP6 & -7 and increases binding to and activation of the ActRIIb/Alk1 receptor complex with some amplification in cellular effects (see below).

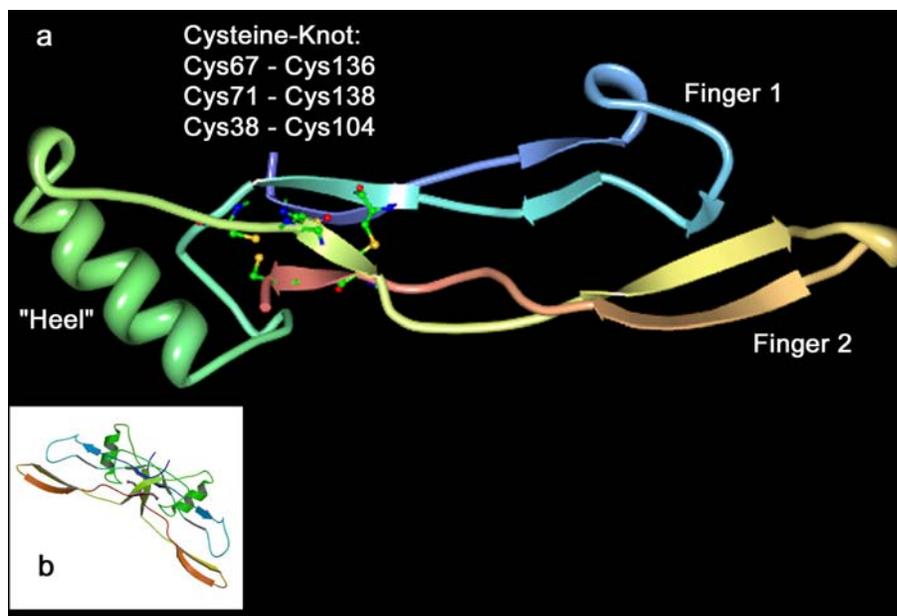


Figure 1. Presumed structure of BMP7. a) The BMP7 monomer has the cysteine-knot that is characteristic for all members of the TGF β superfamily. This knot results from a fold that is induced by 3 intramolecular disulfide bonds. The ‘heel’ region is characterized by a α -helix whereas two ‘fingers’ contain a total of eight β -pleated sheets (indicated by flat bands). A seventh cysteine residue, cys103, which directly neighbors the loop within the knot forms an intermolecular disulfide bond resulting in the bioactive dimeric BMP7 depicted in the insert in b). (54).

Endothelial cells express BMPRII as well as ActRIIB, as well as the ligands BMP2, -4 and -6 (but not BMP7). Alk-1 and -2 are also expressed in these cells (unpublished observation), and Alk-1/ActRIIB or Alk-2/BMPRII receptor heterocomplexes can be activated by autocrine and paracrine modes inducing ‘BMP-type’ cell signals and responses. It appears that BMP6 is the major BMP in endothelial cells and may have functions similar to BMP7. Thus, endothelial cells are unique in that TGF β can cause activation of TGF β -typical pathways and responses as well as BMP-like cellular events.

Both type II receptors, BMPRII and ActRII are widely expressed in the kidney. Among the three BMP7 – sensitive type I receptors, only Alk2 and -3 are expressed in the kidney (Figure 6). Alk2 is expressed in tubules, mesangial cells and podocytes whereas Alk3 is relatively enriched in podocytes.

Receptor binding of BMP7 in rat kidney has been studied elegantly by Bosukonda and associates using in-vivo autoradiography (14). These studies demonstrate BMP7 receptor distribution and binding in tubules in the outer cortex and intensely in glomeruli including mesangial cells but likely also glomerular podocytes. Radiolabeled BMP7 also binds to tubular segments in the outer medulla. This apparent receptor distribution appears to differ somewhat from the cells that express BMP7 in kidney. Thus, autocrine and paracrine modes of actions of BMP7 can be predicted. This is especially true for glomerular actions of BMP7. Clearly, BMP7 binds strongly to receptors in the mesangium and displays activity in mesangial cells but is not or, if any, only minimally

expressed in these cells. Autocrine and/or paracrine actions of podocyte BMP7 is also supported by several in-vivo observations (9, 10, 14). Paracrine modes of action on the glomerulus may also occur through BMP7 that is heavily expressed in distal convoluted tubules which neighbor the glomerulus, but this is hypothetical and unproven by experiments. There is little direct evidence for endocrine actions of BMP7, simply because measurements of BMP7 in serum or plasma have been unsuccessful thus far, at least in this laboratory (10). However, there is circumstantial evidence that BMP7 may also act by endocrine modes. Recent studies have shown distant target actions of endogenous BMP7 (15). Such systemic functions of (renal) BMP7 has also been proposed by Davies and associates based on extra-renal findings in rodents with chronic renal diseases (16). BMP7, similar to other peptides within the TGF β superfamily, binds avidly to extracellular matrix proteins, especially fibronectin and, hence, basement membranes could serve as a local reservoir (17). Taken together, current evidence supports that endogenous BMP7 acts on target cells by autocrine, paracrine and potentially endocrine modes.

5.1. BMP-activated Smad signaling

Ligand binding activates BMP-receptor heterodimers and the phosphorylated receptor complex activates specific cellular substrates, namely smads. Smad2 and -3 are phosphorylated upon activation of Alk5/TBRII complexes by TGF β and smad1, -5 and -8 are BMP-receptor smads (Figures 5, 7) but are also activated upon binding of TGF β to the Alk1/TBRII complex. Smad3 has derived as the major fibrogenesis mediator of TGF β but novel, “non-canonical” pathways have more recently been

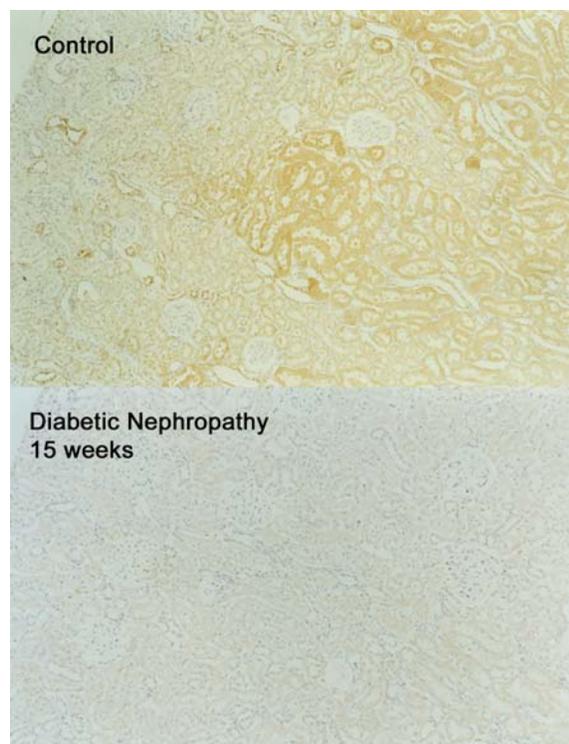


Figure 2. Renal expression of BMP7. In normal rat kidney (top panel) BMP7 is strongly expressed in cortical distal convoluted tubules and in tubules within medullary rays, likely collecting ducts. Expression of BMP7 disappears early in chronic renal diseases, such as in streptozotocin-induced diabetic nephropathy in the rat (bottom panel).

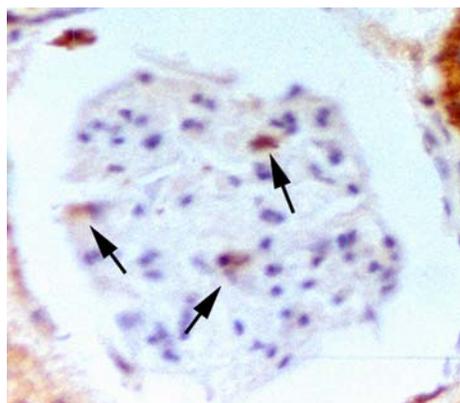


Figure 3. Expression of BMP7 in glomeruli. Within glomeruli, BMP7 is only expressed in glomerular visceral epithelial cells (red-brown) such as in the depicted glomerulus from a normal rat. There is generally agreement as to this distribution of glomerular BMP7 expression in rodents. Although there is moderate controversy, the available data favor similar expression of BMP7 in human glomeruli.

described such as activation of Pak-2 downstream of abelson by TGF β specifically (and exclusively) in fibroblasts (18). Moreover, the strict separation of cellular

smad pathways as TGF β -activated (smad2, 3) or BMP-activated (smad1, 5, 8) has also lost some of its validity: Recent studies, for example, provide strong evidence for a TGF β pro-fibrogenic pathway involving Erk1/2 and smad1 activation for the transcription-driven increase in CTGF levels (19). There is some overlap between TGF β smads and BMP smads; it also continues to be an enigma how ligand-specific cellular events are induced given a very limited number of receptor smads but a multitude of ligands within the BMP-family and especially the TGF β superfamily with diverse and sometimes opposing functions in different cell types. Better understanding of these specificities will undoubtedly derive from network approaches in the study of cell signaling. One of the proposed but perhaps not widely accepted possibilities is formation of heteromeric receptor complexes involving TBR11, Alk5 and Alk1 with consequent activation of smad1 in fibroblasts causing fibrogenic events (19). One of the questions arising is that thus far Alk1 is thought to be specifically expressed in endothelial cells.

Activated receptor smads heterodimerize with common smad4, a non-phosphorylated partner that functions most likely as a transcriptional co-activator. The R-smad/sm4 complex is translocated to the nucleus through the importin- β mechanism and assembles into larger transcriptional complexes at specific gene promoter targets (Figures 5, 7). It was conventionally assumed that smad4 may be required for translocation to the nucleus, but its role appears to be more specifically that of a transcriptional co-regulator. Still, smad4 may also facilitate transport through the nucleopores.

The activation of smad signals by BMP7 has been examined in renal cells, specifically in mesangial cells and glomerular podocytes in this laboratory (20, 21). In both cell types BMP7 specifically phosphorylates smad5 but not smad1 (Figure 8). Smad1, traditionally thought to be a BMP-specific cell signal that is also induced by BMP7 may be more typical for BMP2 and -4 and also a TGF β signal downstream of the Alk1 receptor. There is also evidence that a TGF β -induced profibrogenic regulator, namely connective tissue growth factor (CTGF or CCN2) is transcriptionally activated through TGF β - (and perhaps BMP2/4-) induced smad1 (19). Abe and associates reported observations in renal biopsies from patients with diabetic nephropathy demonstrating increased expression of Alk1 and smad1 in the glomerular mesangium (22). Interestingly, smad1 is induced in mesangial cells by incubation with glycated BSA presumably through TGF β . In these experiments smad1 was shown to contribute to mesangial fibrosis by inducing collagen type IV (22). Thus, there is at least circumstantial evidence that smad1 is a dual TGF β and BMP (2, 4) cell signal that appears to positively regulate fibrogenesis. In contrast, BMP7 does not activate smad1 in renal cells, rather it utilizes smad5 (Figure 8).

6. RENAL BMP7 DISAPPEARS DURING RENAL FIBROGENESIS

One of the observations that support a beneficial effect of endogenous renal BMP7 is that the expression of

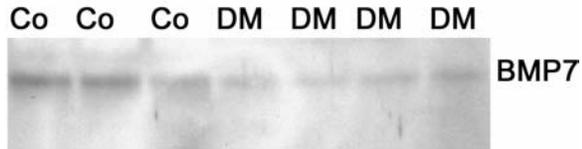


Figure 4. BMP7 is excreted with urine. The depicted Western Blot assay shows intact, dimeric BMP7 at an apparent molecular weight of 36-37 kDa. Urinary excretion of BMP7 is diminished in diabetic rats (DM) compared to normal control animals (Co).

this peptide decreases early at the onset of diabetic nephropathy in rodents. This has been shown by at least two laboratories (6, 23). At 15 weeks after induction of diabetes with streptozotocin in rats, renal BMP7 expression is decreased by two-thirds (Figure 2) (6). One group of investigators found a transient increase in renal BMP7 early in the course of diabetes in rats but in agreement with findings from other laboratories, renal BMP7 levels subsequently decreased at 16 weeks of diabetes below control values (24). The decrease in renal BMP7 is paralleled by the appearance and subsequent increase in TGF β giving rise to the possibility that this latter cytokine may transcriptionally down-regulate BMP7.

The decreased expression of BMP7 pre-dates the onset and progression of proteinuria in these animals. Given the widely accepted evidence that glomerular proteinuria is a result of podocyte injury, the loss of BMP7 in glomeruli and the loss of its autocrine action on podocytes gives rise to the possibility that among the functions of BMP7 in the adult kidney is the preservation of the highly differentiated phenotype of these cells. Such a view would be consistent with the role of BMP7 during renal development, namely inducing and maintenance of a specialized epithelial cell type (25). This is further supported by a series of experimental findings that are discussed later in this narrative.

BMP7 is also excreted with urine, and urinary BMP7 is most likely “spill over” from the distal nephron. The loss of BMP7 expression in the kidney is also reflected by a decrease in urinary BMP7 in experimental diabetic nephropathy in rats (Figure 4). Perhaps, the measurement of urinary BMP7 such as a ratio over the urinary creatinine concentration, might be a clinical marker for the onset and/or progression of diabetic nephropathy. However, this is a speculative thought and any series of measurements in patients of this kind has not been published. Such a study would require prospective and repeated measurements in patients with recent onset of diabetes mellitus and would require many years of follow-up for completion.

The loss of BMP7 expression in the kidney is not limited to diabetic nephropathy but has also been described in the obstructed kidney in unilateral obstructive nephropathy in rats (26). Although not every disease model in rodents or humans has been examined, it is reasonable to presume that a decrease and finally loss of renal BMP7 expression occurs in most fibrotic renal diseases and may be TGF β -driven. Consistent with this

view is the finding that high glucose actually tends to upregulate BMP7 promoter activity in podocytes and distal tubule-like epithelial cells whereas TGF β reduces promoter activity (unpublished observation). This data may suggest that hyperglycemia in-vivo per-se is not responsible for the down-regulation of BMP7 expression during early diabetic nephropathy; rather, glucose-induced TGF β reduces BMP7 transcription. This view is also consistent with the finding that BMP7 is reduced in non-diabetic renal diseases, such as obstructive nephropathy.

7. RENAL EXPRESSION OF BMP-INHIBITORS

The activity of members of the TGF β superfamily including BMPs is not only regulated by the expression and levels of these cytokines but also by several series of extracellular regulators. These include the small, secreted proteins of the dan/cerberus group (gremlin, cerberus, dan, DCR5, DCR6, DCR7, cCar), noggin, chordin and follistatin. These proteins function as ligand binding and inhibiting proteins and reduce their interaction with signaling receptors. Two other proteins, namely inhibin and BMP3, are BMP-receptor blockers. Connective tissue growth factor (CTGF), Kielin/chordin-like protein (KCP) and endoglin are TGF β - and BMP7-activity modifiers.

7.1. Secreted, extracellular BMP-inhibitors

Renal BMP7 does not only decrease in diabetic nephropathy but there is also emergence and increased expression of BMP antagonists including those of the dan/cerberus group of proteins as well as noggin, chordin and follistatin. Proteins within this family are secreted into the extracellular space where they bind to BMPs and possibly other members of the TGF β protein superfamily. There is very limited information about the affinity and specificity of these inhibitors and it remains an open question as to whether these are specific BMP inhibitors. Noggin binds and inhibits several BMPs including BMP7; chordin probably binds and blocks BMPs with only modest affinity whereas follistatin clearly binds and blocks BMP7. Chordin and follistatin may also bind TGF β . Gremlin blocks BMP2 and -4 but not -6 and most likely also not BMP7. The only specific inhibitor within this family appears to be cerberus which blocks selectively nodal with the consequence of crypto signaling inhibition and important implications during development (27).

Noggin, follistatin, chordin and gremlin are all expressed in adult rodent kidney. In whole kidney extracts from normal and diabetic mice noggin mRNA levels are very low and do not change in diabetes compared to control. Follistatin mRNA levels are considerably higher and increase by about 60% in diabetes (unpublished observation). In cultured murine podocytes noggin and follistatin are expressed. Noggin expression increases about 3-fold when cells are exposed to high glucose levels or to TGF β (unpublished observation).

Gremlin is perhaps the best studied member of the dan/cerberus family of antagonists in the

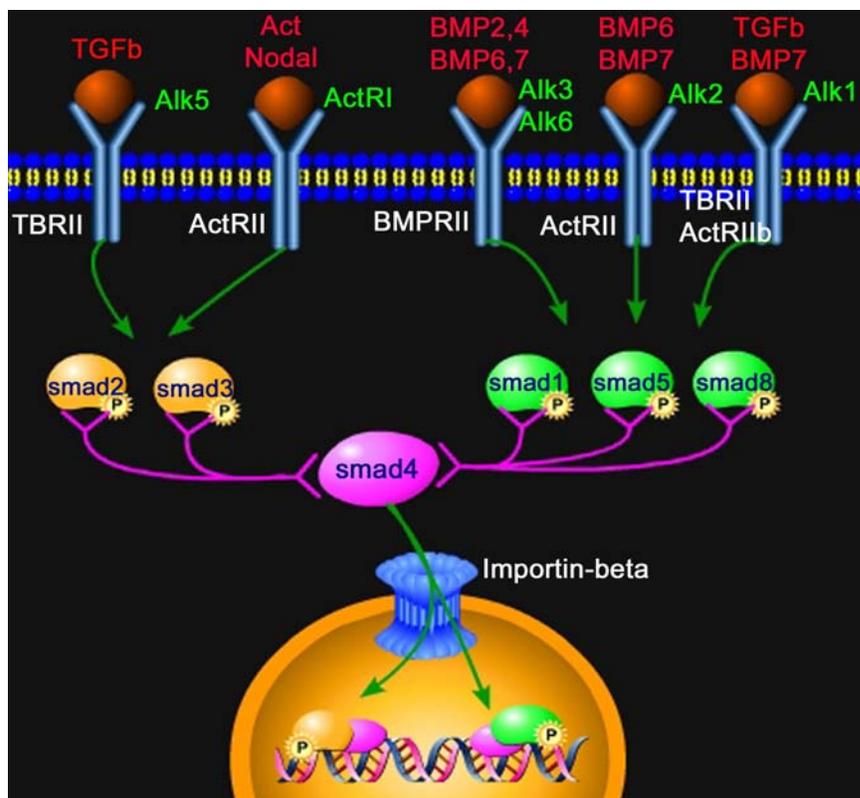


Figure 5. Affinities of TGFβ and BMP ligands for different receptor heterodimers. Different ligands (depicted in red) bind initially to the type II receptor (in white lettering) which leads to recruitment of a type I receptor (activin receptor I or one of several activin receptor – like kinases, ALKs). Different BMPs interact preferably or exclusively with different receptor heterodimers, as indicated. Ligand-induced activation of the dimeric receptor induces phosphorylation of different receptor smad substrates (R-smad) which form a complex with the common smad, smad4 (which serves as a transcriptional co-regulator). The R-smad/smاد4 complex is translocated into the nucleus through the importin-β mechanism. The cartoon is simplified and does not depict non-smad pathways.

kidney. Its renal expression increases substantially in diabetic rats compared to control animals (6). Although the levels of gremlin mRNA in whole kidney extracts is low, relatively high levels are found in mesangial cells where gremlin is further induced by TGFβ. Mesangial gremlin is upregulated in diabetes in-vivo and by high glucose or TGFβ in mesangial cells in-vitro (28). Gremlin expression has also been described in fibrotic tubulo-interstitial areas in diabetic nephropathy where it is absent in samples from normal controls. In areas of fibrosis gremlin appears to be expressed in transformed tubular cells (29). Gremlin binds and neutralizes, similar to dan, BMP2 and -4, but not BMP5 or-6; gremlin may also neutralize TGFβ; whether it antagonizes BMP7 remains unclear. Gremlin has also activity towards other proteins outside the TGFβ- and BMP-families. It binds slit proteins and augments the activity of Slit1/2 as repulsive guidance molecules. The gremlin-slit complex appears to have aggravated activity on the roundabout (ROBO) receptor in monocytes substantially reducing migration upon chemotactic activators such as by SDF-1 (30). Thus, gremlin has functions that are independent of binding and neutralizing BMPs, such as anti-inflammatory actions at sites of fibrosis.

7.2. BMP-receptor blockers, inhibin and BMP3

Inhibin is secreted in relatively large amounts by granulosa (female) and Sertoli (male) cells under the regulatory control of FSH and functional co-regulator. As such it is present in plasma. However, it also has separate, probably autocrine/paracrine, functions, namely blockade of activin and BMP-receptors. Thereby, inhibin opposes BMP (7) and activin actions. Inhibin is a heterodimer of an α- and either of two β-chains, β_A and β_B, each of which is a product of a distinct gene. Thus, several different molecular forms of inhibin exist, and differences in their functions are yet to be defined. Activins have completely different functions but are structurally very similar to inhibin. Activins are homodimers of inhibin β-β-subunits, either as activin A (β_Aβ_A) or activin B (β_Bβ_B). Activins are expressed in kidney, mainly in interstitial fibroblasts, are up-regulated by TGFβ, inhibited by follistatin and have fibrogenic activity by inducing fibroblast proliferation and other pro-fibrogenic fibroblast activities (31).

The expression of inhibin in cultured murine podocytes has recently been examined in this laboratory. All three inhibin genes are expressed in podocytes in-vitro



Figure 6. Expression of BMP7-sensitive type I receptors in kidney. Alk6 mRNA is virtually not present in whole kidney (Ki) or liver (Li) RNA extracts and is not found in RNA extracts from podocytes by PCR. Alk3 is present in whole kidney extracts albeit at low levels but is highly expressed in podocytes. Alk2 is expressed in kidney and in podocytes. The exact spacial distribution of different BMP7-sensitive type I and type II receptors within the nephron is not known.

(unpublished observation). Inhibin expression (mRNA by rtPCR) has also been found in extracts from whole kidney of normal and diabetic mice (unpublished observation). Levels of inhibin expression appear to be rather low (perhaps two orders of magnitude lower than activin B mRNA) but are upregulated about two-fold in diabetic mouse kidney. The distribution of inhibin expression within different nephron segments is not known.

BMP3, also termed osteogenin, is extensively expressed in bone matrix but also in the terminal nephron (collecting ducts) (32). BMP3 is an antagonist to BMP7 (and probably other BMPs) and functions by blocking BMP-receptors (33). There is virtually nothing known about the importance of these receptor blockers in the kidney or in renal diseases.

7.3.) *BMP7- and TGFβ-activity modifiers, endoglin, KCP and CTGF*

In addition to the secreted inhibitors of the dan/cerberus group of proteins there are other secreted proteins that modulate TGFβ and BMP7 activity with impact on renal fibrogenesis. Endoglin which has already been discussed earlier and which is mainly expressed by endothelial cells binds TGFβ, inhibits its interaction with Alk5 and increases the activation of Alk1 by TGFβ inducing smad1 and, hence, BMP-like cell responses (34). Another extracellular enhancer of BMP7 has recently been cloned, namely the cysteine-rich, secreted Kielin/Chordin-like protein, KCP (35). KCP is a cysteine-rich protein (it contains 18 cysteine-rich domains) with sequence similarities to murine chordin and the *Xenopus* protein Kielin. This relatively high molecular weight, secreted protein (~150 kDa) binds BMP7 and enhances its interaction with BMP-type I receptors and BMP-R-smad activation. KCP also binds TGFβ and activin A, two pro-fibrogenic proteins, but inhibits their activity (36). Thus, KCP is a BMP7 activity enhancer and TGFβ/activin A inhibitor. Although KCP is expressed in the kidney during development, its expression in the normal adult kidney or changes that may occur in fibrogenic renal diseases have not been published.

CTGF (CCN2) has long emerged as a TGFβ-induced fibrogenesis accelerating mediator but its

mechanisms of action have been elusive due to the lack of an identified signaling receptor. Several years ago we demonstrated that CTGF can act through IGF1-receptors because it binds IGF-I and enhances IGF-I actions (37). This concept that CTGF may actually not act through a specific signaling receptor rather than by binding other proteins and enhancing or inhibiting their interaction with specific receptors has now found further experimental support. CTGF also binds TGFβ as well as BMP7. While the CTGF/TGFβ complex increases binding of TGFβ to Alk5 and smad3-driven cell responses, CTGF inhibits BMP7 signaling by reducing the interaction of BMP7 with its receptors (38). Thus, CTGF is functionally the reverse of endoglin: the former is a BMP7 inhibitor whereas the latter is a BMP7 activity enhancer. CTGF is functionally also the opposite to KCP.

Overall, the regulation of BMP7-activity is complex and cannot easily and exclusively be assumed by assessing BMP7 levels in the normal or diseased kidney. Although many of the secreted inhibitors of the dan/cerberus group are thought to be brought BMP inhibitors, there is little detailed experimental data especially with regard to the inhibition of BMP7 specifically. It may turn out that some of these proteins may actually inhibit TGFβ activity.

8. ANTIFIBROGENIC ACTIVITY OF BMP7 IN KIDNEY DISEASES

8.1. Antifibrogenic actions of exogenously administered rhBMP7 in rodents

TGFβ has long emerged as the master regulator of renal (and other parenchymal organ) fibrogenesis. At least one other member of this large family of proteins, activin, has also been shown to promote renal fibrogenesis (31). TGFβ acts on multiple cell types including mesangial cells and podocytes in glomeruli as well as (proximal) tubular cells and renal interstitial fibroblasts. It activates many pro-fibrogenic genes, usually by transcriptional activation. This leads to extracellular matrix assembly and reduced proliferation and increased apoptosis of differentiated, residential epithelial cells. In renal fibroblasts TGFβ induces proliferation and the transition to the active phenotype, myofibroblasts, and their activity to induce interstitial extracellular matrix accumulation. TGFβ also induces transformation of damaged epithelial cells to a fibrogenic cell phenotype. Very few studies have addressed the functions of other proteins within the TGFβ superfamily in the kidney. Superficially, one might expect that BMPs may have similar, pro-fibrogenic activities in the kidney. Surprisingly, this is not at all the case for BMP7.

The hypothesis that BMP7 has antifibrogenic activity was not obvious in the past but was initially deduced by Keith Hruska and his associates (5) based on observations that BMP7 induces and contributes to the maintenance of mesenchymal cell condensation during early nephron development (25) and that this growth factor reduces tubular cell apoptosis and prevents loss of renal function in a rodent model of acute renal failure (39).

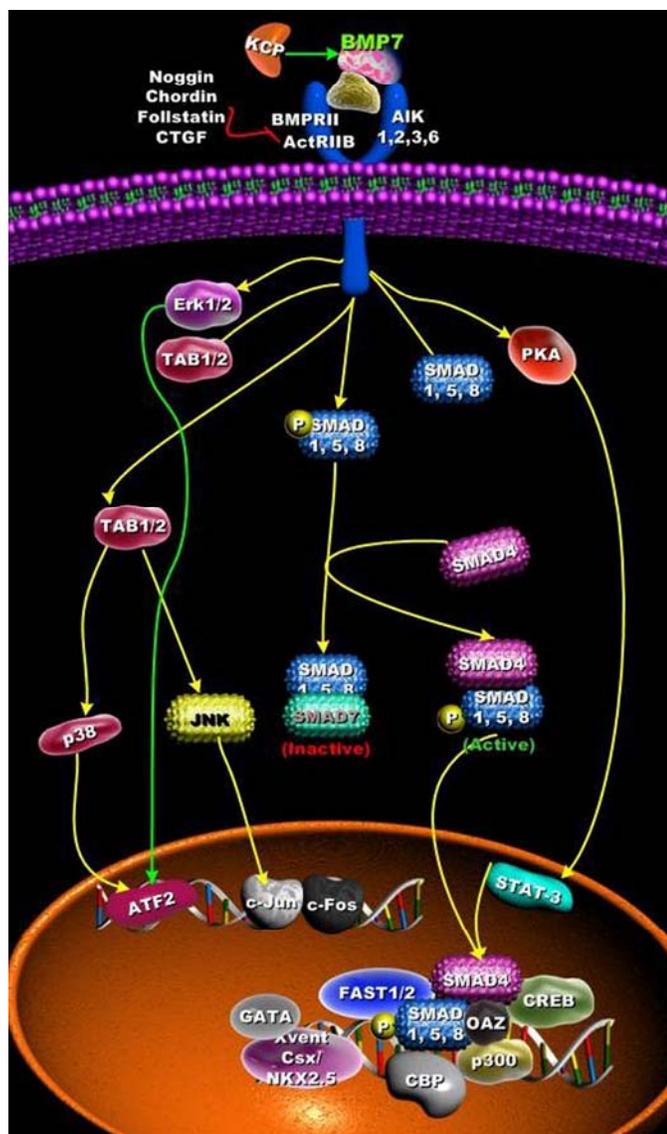


Figure 7. BMP7 signaling pathways: This simplified cartoon provides an overview of the cellular regulation of BMP activity with focus on BMP7.

Hruska and his associates tested their hypothesis in rats with unilateral obstructive nephropathy, a rodent model of TGF β -driven, accelerated renal fibrosis. These simple but illustrative studies demonstrated convincingly that BMP7 reduces tubular cell dropout by apoptosis and diminishes the expression and accumulation of interstitial extracellular matrix proteins (5).

These investigators also tested the action and possible utility of exogenously administered rhBMP7 in a rodent model of streptozotocin-induced diabetic nephropathy. In this model the administration of BMP7 reduced and postponed some of the early changes associated with diabetic renal disease. Even more exciting is the observation that BMP7 can induce some regression of diabetic nephropathy-associated pathology when the treatment is started at 16 weeks after induction of diabetes

(23). These beneficial, antifibrogenic effects of exogenous BMP7 in diabetic nephropathy were also shown by Sugimoto and associates (40). These latter investigators used a novel rodent model of diabetic renal disease, namely CD1-mice receiving streptozotocin. These mice develop more severe diabetic renal pathology than in more conventional rodent models consistent with the notion that the genetic background determines onset and severity of nephropathy in diabetes. Despite of the more rapid and severe progression of renal pathology, exogenous rhBMP7 ameliorated the renal disease, especially the fibrosis.

The beneficial effects of BMP7 are not limited to these two disease models, obstructive nephropathy and diabetic nephropathy but have also been shown in other forms of chronic renal disease in rodents. Examples include the lupus-prone mouse model of renal disease with

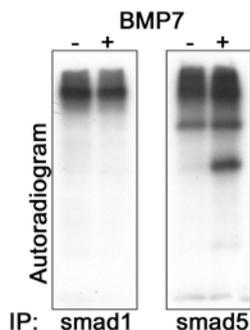


Figure 8. Activation of smad5 but not smad1 by BMP7: The depicted autoradiogram demonstrates that immunoprecipitation of smad1 from lysates from metabolically radio-labeled cells which were incubated with or without rhBMP7 does not show a phosphorylated band. In contrast, BMP7 effectively phosphorylates smad5. This is similar in mesangial cells and in podocytes (21, 44).

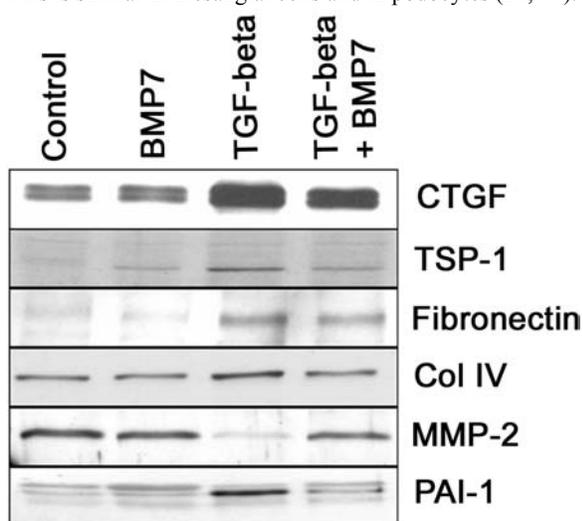


Figure 9. BMP7 reverses the pro-fibrogenic program that is induced by TGFβ. The displayed series of Western Blots indicates that incubation of mesangial cells with TGFβ increases the levels of CTGF (connective tissue growth factor), TSP-1 (thrombospondin-1), fibronectin, col IV (collagen type IV), and PAI-1 (plasminogen activator inhibitor-1) and reduces those of MMP-2 (matrix metalloproteinase-2, collagenase A). BMP7 prevents these changes.

chronically progressing nephropathy resembling a mixed form of lupus nephritis; and col4A3 knock-out mice which develop renal glomerular and interstitial fibrosis with death from renal failure at about 4 months of age (41). Several novel and interesting observations were made in these two genetic models when mice received vehicle or rhBMP7 at three different dosages for up to 16 weeks. Findings not only indicated dose-dependent reductions in the accumulation of interstitial depositions of extracellular matrix proteins and improved functional outcome as assessed by serum creatinine measurements. Interestingly, BMP7 also ameliorated the proliferative, crescentic

glomerular lesion in mice with the genetically determined lupus-like renal disease (41). To the knowledge of the author of the present narrative, this experimental series by Zeisberg and his co-workers utilized the longest period of BMP7 administration (16 weeks). This provides an opportunity to assess possible major side effects of the exogenous administration of rhBMP7. The importance of this latter assessment becomes apparent if one considers another known activity of BMP7, namely activation of osteoblast activity and bone formation upon topical administration at bone fracture sites. In fact, rhBMP7 is clinically used topically for this specific indication. Thus, long-term systemic administration was feared to potentially induce extrasosseous bone formation such as at the injection site, or vascular calcifications. This was explicitly assessed by Zeisberg *et al* and did not occur. Moreover, these potential complications were also not reported by any other group using rhBMP7 in rodent models of renal diseases. More recently, Hruska and associates demonstrated that BMP7 reduces vascular calcifications (42). In studies from our laboratory in mice with regulated, transgenic expression of BMP7 which were examined for up to one year extrasosseous calcifications or bone formation were also not observed (10).

Not all investigators examining anti-fibrogenic efficacy of BMP7 in experimental renal disease models reported beneficial results. Ikeda and associates examined the effects of exogenous BMP7 in rats with BSA-overload nephropathy (43). These investigators did not observe a decrease in the endogenous expression of BMP7 in renal tubules. Moreover, exogenous BMP7 failed to reduce extracellular matrix protein accumulation in the interstitium to any significant degree. The reasons for a different result from these latter studies compared to many other observations in several different laboratories and different reno-fibrogenic disease models is not clear to this narrator.

8.2. Transgenic expression of BMP7: focus of diabetic nephropathy

The in-vivo studies that have been reviewed thus far convincingly demonstrate that BMP7 ameliorates progression of renal diseases and renal (glomerular and interstitial) fibrogenesis. All these studies had been performed with exogenous, pharmacological administration to rodents with renal diseases. This is a suitable experimental approach given also that administration of rhBMP7 to humans, if such a therapy would be attempted, would also be done parenterally.

However, it is evenly appropriate to ask a slightly different question, namely, “does the maintenance of endogenous BMP7 expression in the kidney, such as by transgenic means, during the early evolution of renal diseases which reduces renal BMP7, prevent onset and progression of renal fibrogenesis?” Studies from this laboratory attempted to address this specific question. Given that BMP7 is down-regulated in many renal disease processes, transgenic expression would have to be regulated by a non-physiological promoter. Moreover, an attempt to design a transgenic approach that leads to forced

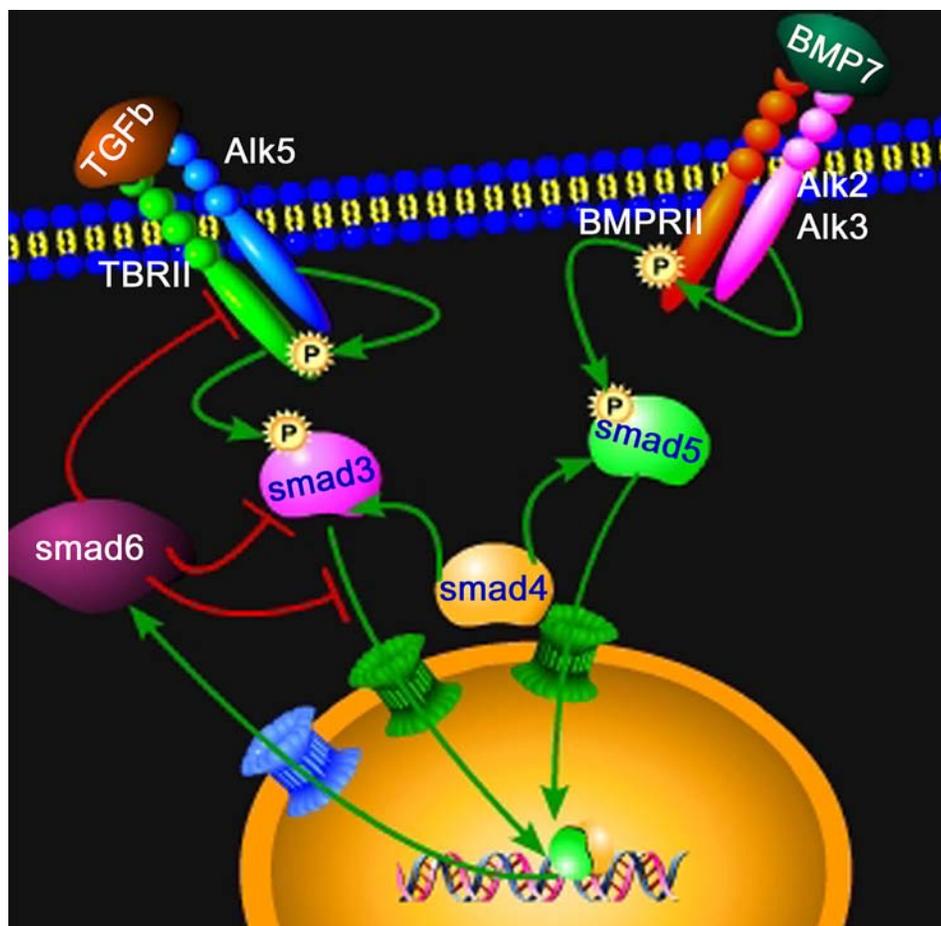


Figure 10. Simplified model of the mechanism of inhibition of TGF β signals by BMP7: Many of the pro-fibrogenic effects of TGF β are transduced by smad3 which forms a heterodimer with smad4 which undergoes nuclear translocation and acts at specific promoter response elements of many genes. BMP7 activates smad5 which transcriptionally induces smad6. This inhibitory smad interacts with smad3, possibly by forming a smad3/smاد6 heterodimer, which prevents nuclear translocation of the smad3/4 complex and, hence, actions on gene targets.

overexpression of BMP7 only at nephron sites that normally express the gene (distal tubules, podocytes) is also impractical. We settled on a strategy using the phosphoenol-pyruvate carboxykinase promoter to regulate BMP7 expression in mice in studies in diabetic nephropathy (10). However, this promoter also induces transgene expression in proximal tubules (perhaps substantially less effectively) and in non-renal tissues including liver, muscle, brown fat and low levels in the jejunum. In a series of studies in mice bearing this transgene we tested its effect on diabetic nephropathy after inducing diabetes with streptozotocin. Diabetic and control transgenic and non-transgenic littermates were followed for up to 12 months. As expected, levels of TGF β were increased in the kidney as well as in serum in diabetic mice, and TGF β expression is unaltered by the BMP7 transgene. Bioefficacy of transgenic BMP7 is demonstrated by phosphorylation of smad1/5 in renal tissue and by increased Id-1 levels (known to be driven through a BMP7-sensitive promoter element). Endogenous, renal transgenic BMP7 reduces renal collagen accumulation and virtually

quantitatively blocks glomerular fibrosis and interstitial fibrosis as examined by quantitative histology assays (10). Are these anti-fibrogenic effects of endogenous BMP7 of functional significance? Transgenic BMP7 substantially reduces albuminuria and appears to reduce renal insufficiency at one year as tested by measuring serum urea nitrogen and creatinine serum levels. These latter measurements should be interpreted with some caution and in the context that the diabetic animals, even at one year, develop only modest renal insufficiency. Nevertheless, the substantially reduced albuminuria in transgenic diabetic mice is a strong indicator of improved functional glomerular and renal integrity.

9. MECHANISMS OF BMP7: INHIBITION OF TGF β SIGNALS

Most of the in-vivo findings that have been reviewed thus far convincingly show that BMP7 reduces renal fibrogenesis in various animal models where glomerular and renal interstitial fibrosis is heavily (perhaps

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not exclusively) TGF β -dependent. This raises ground for the hypothesis that BMP7's mechanism of action involves opposition to TGF β -induced cell signals. Indeed, in-vitro experimental findings strongly support this notion.

9.1. BMP7 reduces the TGF β -induced pro-fibrogenic program in mesangial cells

In mesangial cells TGF β induces increased expression of several extracellular matrix genes and other genes with known pro-fibrogenic functions. Mesangial cells do not express BMP7 in-vitro nor in-vivo, but express BMP7-sensitive receptors and signaling systems. Thus, these cells comprise an excellent model system for the study of BMP7-mechanisms.

Co-incubation of cultured mesangial cells with TGF β in the presence of BMP7 reduces the TGF β -induced changes in collagen IV, fibronectin, CTGF, TSP1, MMP2 and PAI-1 (Figure 9) (20). Most of these proteins increase upon TGF β , and this rise is blocked by BMP7. The exception is matrix metalloprotease-2, which decreases during incubation with TGF β ; and it is the decrease in MMP2 which is prevented by co-incubation with BMP7. This observation provides some evidence that BMP7 inhibits TGF β signals upstream of transcriptional activators or repressors. To further examine this latter point we performed experiments in mink lung epithelial cells that are stably transfected with a minimal PAI-1 promoter coupled with a luciferase reporter. The minimal promoter contains the TGF β response element. In this model, TGF β dose-dependently raises luciferase activity which is inhibited by BMP7, giving further rise to the hypothesis that BMP7 inhibits the levels or activity of a downstream TGF β -induced signal (20).

As already discussed in this narrative BMP7 activates smad5, but not smad1 in mesangial cells and podocytes (Figure 8); although we did not test other renal cell types, there appears to be a scheme that BMP7 may differ somewhat from other BMPs by utilizing this BMP – R-smad selectively. Perhaps, the activation of either smad1, -5 or -8 or co-activation of more than one of these R-smads is a mechanism that determines the differences between cellular responses to different BMPs.

An important aspect of the cellular effects of TGF β and BMP7 is the transcriptional induction of inhibitory smads. In mesangial cells TGF β induces expression of smad7 but reduces smad6. In contrast, BMP7 has no effect on smad7 but strongly induces smad6 (44). This latter activity requires activation of smad5; in fact, smad5 is fully sufficient for inducing smad6. Thus, the upregulation of the inhibitory smad6 by BMP7 through smad5 could be the mechanism by which BMP7 reduces TGF β signals (44).

9.2. BMP7 inhibits smad2/3 signal propagation through smad6

Incubation of mesangial cells with TGF β induces strong nuclear translocation of the TGF β – R-smad, smad3, and nuclear translocation of smad3 is partially blocked by co-incubation with BMP7 (44). Thus, BMP7 reduces

smad3 signals by reducing its nuclear availability at transcription regulating sites. Further experiments show that smad6 which is induced by BMP7 downstream of smad5 inhibits the translocation of smad3 into the nucleus; and smad3 has long emerged as the primary profibrogenic TGF β signal (45). As further proof of this hypothesis we performed experiments on the transcriptional activation of PAI-1 by TGF β . In cells that overexpress smad5 or smad6, TGF β fails to induce PAI-1; whereas BMP7 inhibits the induction of PAI-1 by TGF β , this action of BMP7 is reduced during smad5 knock-down. In concert, these experimental observations give rise to the model shown in Figure 10. Although not proven at present, it appears likely that smad6 forms a heterodimeric complex with activated smad3 that is excluded from nuclear translocation through the importin- β mechanism.

These mechanisms of BMP7-opposition to TGF β are not limited to mesangial cells but are apparently a generalized scheme. Zeisberg and associates performed a series of experiments in tubular epithelial cells to examine how BMP7 blocks epithelial-to-mesenchymal transition (EMT). The loss of the epithelial phenotype of tubular cells and the gain of a fibroblastic gene expression program has emerged as an important contributory mechanism in renal interstitial fibrogenesis (46). This phenotype transition can be induced by TGF β (and TGF β is most likely the main driver of tubular cell EMT) but not by BMPs, and EMT requires the reprogramming of a complex gene expression network by TGF β (47). BMP7 can prevent and reverse tubular cell EMT in-vivo and in-vitro and this BMP7 effect is also mediated through smad5 and smad6 (48).

The regulation of BMP7 activity beyond its levels appears to have importance for renal fibrogenesis. Whereas there are no studies available to the knowledge of the current narrators that would demonstrate that defects or absence of the 'classic members' of the dan-cerberus group of BMP-inhibitors would reduce fibrogenesis, there is clearly experimental evidence that the BMP7 inhibitor and TGF β enhancer, CTGF, aggravates renal fibrosis (49, 50). In contrast KCP which increases BMP7 activity independent of its levels, reduces renal fibrosis in unilateral obstructive nephropathy in mice (35).

10. ANTI-APOPTOSIS EFFECTS OF BMP7: IMPORTANCE FOR THE MAINTENANCE OF PODOCYTES

Podocyte injury and loss of podocytes are highly important causes of glomerular proteinuria in many diseases. In experimental or human diabetic nephropathy, injury and drop-out of glomerular podocytes is among the earliest structural abnormalities (after initial nephron hypertrophy) that can be observed, and is associated with loss of support for glomerular capillary epithelium. In diabetic mice with early nephropathy the number of podocytes is substantially reduced. This could be caused, in part, by reduced podocyte proliferation, and there seems to be a subtle reduction in PCNA-positive podocytes in comparison to non-diabetic controls (10). However, in-vitro studies from this laboratory are more consistent with

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podocyte apoptosis as the main mechanism of podocyte loss (21). Both, incubation of murine podocytes with high glucose (25 mM) or with TGF β increases the number of apoptotic cells and raises caspase-3 activity three- to five-fold. Caspase-3 activation is inhibited by BMP7 through mechanisms that are also smad5 dependent (21). In-vitro, TGF β (or high glucose) induces injury to podocytes which is largely inhibited by BMP7 (8, 21). In experiments from this laboratory, TGF β or high glucose do not induce major loss of podocyte-specific or general epithelial phenotype markers as indicated by the maintenance of levels of podocin, CD2AP, Neph1 and synaptopodin as well as E-cadherin (21). In contrast, Petris and associates found reduced synaptopodin and podocin mRNA levels in podocytes that were incubated with high glucose for 1-2 weeks and which was completely prevented by incubation with BMP7 (8). Thus, there is some discrepancy in the reported experimental findings from similar in-vitro studies. Whether and to what extent the levels of podocyte differentiation marker expression decreases in-vivo in human diabetic nephropathy is unclear and controversial. The limited published data that is available to answer this question suggests that nephrin mRNA and protein levels decrease in human diabetic nephropathy which is consistent with findings from in-vitro studies in podocytes that were exposed to high glucose (unpublished observation). Koop and associates found reduced levels of nephrin, podocin and podocalyxin, but increased mRNA levels encoding these proteins in microdissected glomeruli from diabetic patients. In contrast, Benigni and his coworkers found only and selective reductions in nephrin but not in other podocyte markers in specimen from patients with diabetic nephropathy (51, 52). Thus, whether a major loss of most slit diaphragm – associated proteins occurs in-vivo in diabetic nephropathy is questionable, except for nephrin. Moreover, it appears that podocytes, at least early in diabetic nephropathy or when incubated with high glucose in-vitro, do not lose their specific phenotype, although some structural changes do occur.

High glucose or TGF β induces a subtle change in the appearance of podocytes in-vitro which is heralded by a departure from the normal ‘stress fiber’ distribution of actin filaments. These changes in the cytoskeleton are also prevented by BMP7 (21). Overall, the data from two laboratories support that BMP7 is a podocyte differentiation maintenance and survival factor, and the loss of endogenous BMP7 in diabetes and perhaps other glomerular diseases likely facilitates podocyte pathology.

11. SUMMARY & OUTLOOK

BMP7 reduces progression of experimental chronic renal diseases mainly by inhibiting profibrogenic signals of TGF β as well as by promoting phenotype maintenance in epithelial cells. Studies in rodents suggest broad activity towards reducing fibrosis independent of the individual disease process, perhaps with the caveat that its activity is limited to TGF β -driven fibrogenesis; however TGF β is causative in renal fibrosis across most or all chronic renal diseases.

Perhaps, there is now enough experimental data to consider studies in humans. An early fear that rhBMP7 may cause extraosseous calcifications or ossification at injection sites or in the systemic vasculature appears to be somewhat resolved. The narrators of the present review are not aware of any studies in humans using either rhBMP7 or small molecule agonists have been performed or are ongoing. Nevertheless, the totality of experimental in-vitro and in-vivo data is highly encouraging that BMP7 could have tremendous benefit in patients with chronic renal diseases.

There is another question that arises from the data that has been discussed in this review, namely, “are BMP7, some or all of its receptors, signaling proteins or activity regulators risk genes for progressive renal fibrogenesis and, hence, the onset and rate of progression of chronic renal failure?” The search for risk genes for the onset but also severity and progression of diseases is an important, ongoing endeavor. For example, the ongoing ‘Family Investigation of Nephropathy of Diabetes (FIND)’ study is an attempt to find genetic pathways that define the development of nephropathy in diabetic patients (53). Generally, risk genes may be defined as genetic mutations that determine, in part or in full, that given injuries induce pathology and/or clinical disease and others that determine disease severity and progression. In this context, it is unlikely that BMP7 is a risk gene for the development, for example, of nephropathy in diabetics. This not because BMP7 may be of little importance; in fact, findings reviewed above may convincingly support an important role of BMP7. However, loss of function of BMP7 has been shown to be embryologically lethal due to severe developmental defects. Although not experimentally tested, it is likely that loss of function mutations of BMP-receptors or smad signaling proteins will cause even severe phenotypic abnormalities and are not likely risk genes.

Better risk gene candidates appear to be those proteins that regulate or direct the activity of TGF β and BMP7, but their absence (loss of function mutation) does not cause any phenotype under normal conditions. One such candidate for a ‘renal fibrogenesis risk gene’ might be KCP, the novel, secreted BMP7 activity enhancer kielin/chordin-like protein, that had been discussed earlier in this narrative. KCP-null mice have a normal phenotype and are healthy, and do not develop any renal disease during normal life conditions. However, when challenged with a fibrogenic renal insult, these KCP-defective mice develop more severe disease than their normal littermates.

12. ACKNOWLEDGEMENTS

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