Bone morphogenetic protein-7 expression and activity in the human adult normal kidney is predominently localized to the distal nephron

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Bone morphogenetic protein-7 (BMP)-7 plays an important role during fetal kidney development. In the adult, BMP-7 is most strongly expressed in the kidney compared to other organs, but the exact expression pattern as well as the function of BMP-7 is unclear. The major aim of the present study was to define which parts of the human kidney do physiologically express BMP-7 and which cells appear to be targets of BMP activity by showing phosphorylated BMP-receptor-associated Smads 1, 5, or 8 and inhibitor of differentiation factor 1 (ID1) expression. BMP-7 expression was localized by immunohistology to the epithelia of the distal tubule as well as the collecting ducts (CDs). Phospho-Smads 1/5/8 and ID1 expression largely colocalized with BMP-7 and was also localized in the epithelia of the distal tubule and the CDs. This was confirmed by polymerase chain reaction-based mRNA expression analysis. *In vitro*, proximal tubular cells (PTCs) expressed BMP receptors and BMP-receptor-associated Smads and were reactive to BMP-7. Our data indicate that BMP-7 expression in the adult human kidney appears to be more restricted than in the fetal situation and predominantly found in the distal nephron. Also, evidence of *in vivo* BMP signalling (i.e. phospho-Smads and ID1 expression) was found there. These findings suggest that BMP-7 plays a physiological role mostly in this part of the kidney. Still, as reported previously, PTCs are responsive to BMP-7, but presumably not in an autocrine or paracrine mode in normal adult kidneys.

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Bone morphogenetic protein (BMP)-7 represents one member of the BMP/transforming growth factor-β-superfamily, which is thought to play important roles during development, but also in adult physiology and disease. BMP-7 itself seems to play specifically an important role during fetal kidney development and is expressed multifocally within the embryonic metanephric mesenchyme and epithelia (for a review see Simic and Vukicevic[1]). BMP-7 knockout mice die owing to renal failure perinatally as a result of severe kidney dysplasia. Thus, overall, BMP-7 is one of the most potent renal morphogenic factors during kidney development.

In the adult, BMP-7 is most strongly expressed in the kidney at least in mice.[2] The exact expression pattern as well as the function of BMP-7 are unclear in the adult. However, BMP-7 was repeatedly reported to be protective in acute and chronic kidney disease (for a review see Simic and Vukicevic[1]): Thus, BMP-7 improved significantly the recovery rate after acute renal injury in terms of histomorphology as well as function.[3] Also, chronic injuries including diabetic nephropathy are counteracted.[4] Although the exact molecular mechanisms are still unknown, BMP-7 appears to reduce the release of proinflammatory cytokines,[5] may be via direct mitogen-activated protein kinase signaling,[6] and is able to reverse epithelial–mesenchymal transitions as well as to counteract the fibrotic activity of transforming growth factor-β.[7,8] Altogether, BMP-7 appears to help to restore the tubular homeostasis in a surprisingly efficient way.[1]

BMP activity in general is on the cellular level mediated via binding of BMP to BMP-specific receptors IA, IB, and II on the cell surface (for a review see Sebald *et al.*[9] and Chen *et al.* [10]). Activated receptor complexes recruit and phosphorylate BMP-receptor-associated Smads 1, 5, and 8, which than bind to common-Smad 4. The Smad complexes then translocate into the nucleus where they act in concert with other factors as transcription factors. Among others, the inhibitory Smads 6/7 and the inhibitor of differentiation family member 1 (ID1) are known as positively regulated gene targets of BMP activity.

The aim of the present study was to define which parts of the human kidney do physiologically express BMP-7 and

which cells appear to be targets of BMP activity by showing phosphorylated BMP-receptor-associated Smads 1, 5, or 8 and ID1 expression *in situ* in adult human kidney tissue.

RESULTS

Gene expression analysis of BMP-7 in normal adult human kidney using conventional and quantitative PCR

By conventional polymerase chain reaction (PCR), transcripts for BMP-7 mRNA could be detected in all RNAs isolated from normal adult kidney (Figure 1a). Quantitative PCR confirmed these results and showed low expression levels of BMP-7 in total kidney RNA extracts (Figure 1b). These were, however, higher than those detectable in other organs (Figure 1b).

In situ localization of BMP-7 in the adult kidney – mRNA expression analysis of microdissected glomeruli and tubuli

Immunostaining localized BMP-7 expression to the epithelia of the distal tubule (Figure 2a and c). This specific distribution pattern was confirmed by double-labelling experiments with calbindinD28k for the identification of the distal convolute and collecting ducts (CDs) (Figure 2b, and d). This showed a large number of double-positive tubuli, distal-convoluted tubule (DCT), and connective tubule (CNT) as well as few of only calbindinD28k (CD) or only BMP-7-stained tubuli. No expression was detected in the proximal tubuli and the glomeruli. Of interest, the most prominent immunostaining was found in the cytoplasm at the luminal side of tubular epithelial cells.

Quantitative PCR was performed on microdissected human glomeruli and tubules: significant BMP-7 expression was mostly detectable in dissected tubular cells and hardly detected in glomeruli (<0.01 molecules/molecules glyceraldehyde-3-phosphate dehydrogenase).

Proximal tubular epithelia lack endogenous BMP-7, but do respond to rhBMP-7 *in vitro*

According to our immunostainings, BMP-7 is not expressed in proximal tubular cells (PTCs) and glomeruli (Figure 2a and c). Also, an established cell line of PTCs, HK-2,[11] did not express BMP-7 even if investigated with highly sensitive PCR. However, this cell line expressed significant levels of BMP receptors IA, IB, and II as well as BMP activity mediating R-Smads 1, 5, and 8 (Figure 3a). Stimulation of HK-2 cells

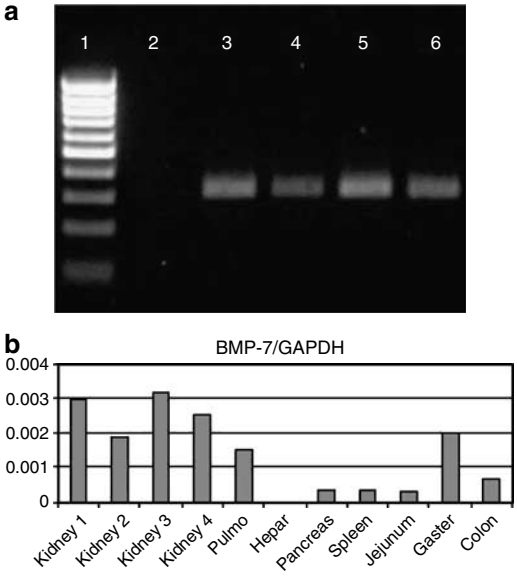

Figure 1 | mRNA expression of BMP-7 in normal adult kidney. (**a**) Detection of a BMP-7 amplification product (500 bp) in four different kidney samples by conventional PCR (lane 1: DNA marker, lane 2: H_20 control; lanes 3–6: four different kidney specimens). (**b**) Real-time PCR analysis of the BMP-7 expression level demonstrates a BMP-7 activity in four separate samples of human adult kidney and to a much lower extent in other organ samples.

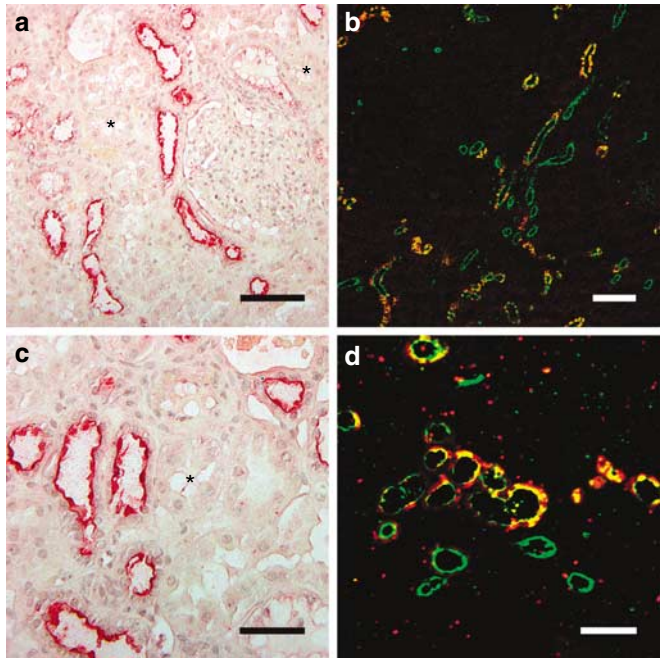

Figure 2 | Immunolocalization of BMP-7 in normal adult kidney. (**a**, **c**) Immunostaining for BMP-7. (**a**) original magnification $\times$ 20 (bar = 100 μm); (**c**) original magnification $\times$ 40 (bar = 50 μm): no signal was detected in glomeruli and proximal tubuli (*). (**b**, **d**) Double immunofluorescence showing the relation between BMP-7 (green) and calbindinD28k (red) (**b**) original magnification $\times$ 10 (bar = 200 μm); (**d**) original magnification $\times$ 20 (bar = 100 μm): there is only a small population of double-stained distal tubuli.

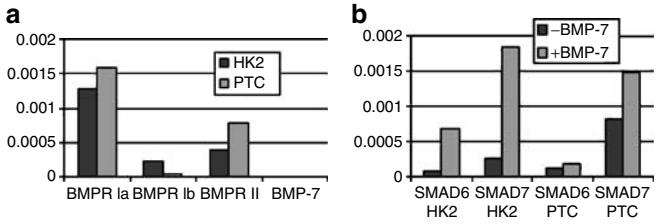

Figure 3 | Quantification of BMP receptor and Smad 6/7 mRNA expression in HK2 cells. (**a**) Quantitative mRNA expression analysis of BMP receptors as well as BMP-7 in HK-2 in primary PTCs. (**b**) Detection of statistically significant Smads 6 and 7 upregulation after stimulation with BMP-7 (100 ng/ml for 16 h) by real-time PCR.

with 100 ng/ml BMP-7 lead to an increased expression of Smads 6 and 7 (Figure 3), which are known to be upregulated by BMPs in a negative feedback manner. Similar results were obtained with primary PTCs (Figure 3b).

Immunolocalization of P-Smads 1, 5, and 8 and ID1 in normal adult kidney – co-immunolocalization with BMP-7

We were then interested in looking for BMP-receptor-associated Smad activation in the normal kidney. Phospho-smads (p-Smads) 1/5/8 and ID1 were detected in the epithelia of the distal tubuli (DCT, CNT) and the CD, based upon the morphological characteristics of the stained tubuli. Co-labelling with Tamm–Horsfall protein as a marker for the thick ascending limb showed a majority of positive p-Smads tubuli and just some tubuli either positive for Tamm–Horsfall protein alone or simultaneously for both. This indicates that the p-Smad-labelled tubuli must be located at the transition of thick ascending limb/DCT to the CNT and CD (Figure 4b and e).

To confirm our p-Smad data, we investigated the ID1 distribution as effector downstream of the p-Smads 1/5/8 signaling. Supporting our former findings, the ID1 stainings showed a pattern similar to that of the p-Smads 1/5/8 (Figure 5).

BMP-7 showed an apical distribution and was localized to the distal tubuli, mostly in close colocalization with p-Smads (see Figure 4c and f, green staining). Other tubuli were either only BMP-7 (i.e. DCT, CNT) or p-Smads 1/5/8 and ID1 positive. BMP-7 was also colocalized with ID1 in DCT and CNT (Figure 5c and f, green staining). Neither p-Smads, ID1, nor BMP-7 was seen in glomeruli.

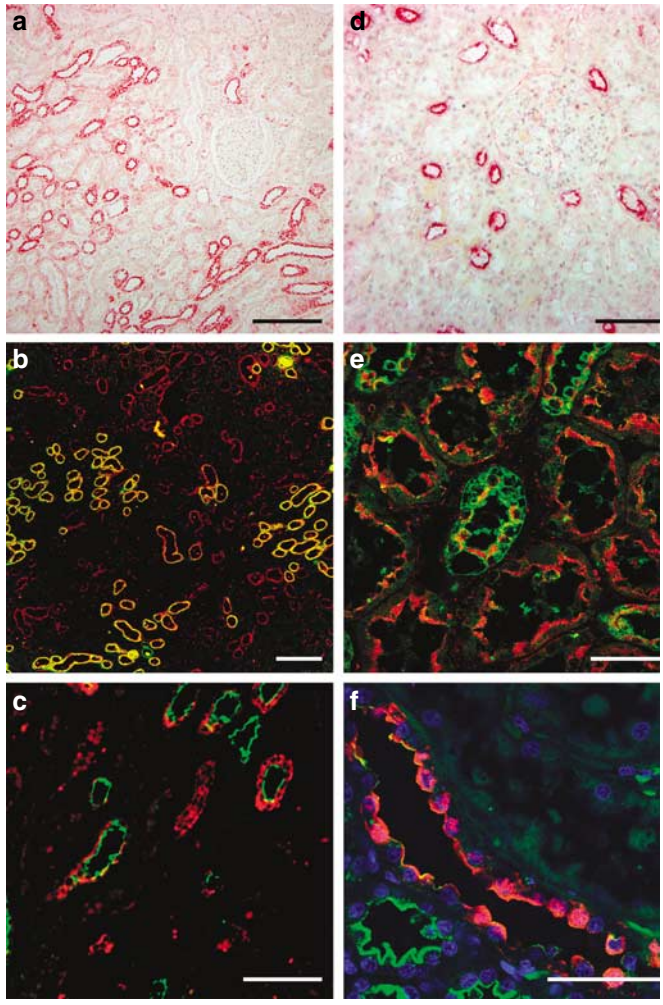

Figure 4 | Immunolocalization of phosphor-Smads 1/5/8 in normal adult kidney. (**a**, **d**) Immunolocalization of phospho-Smads 1/5/8 (p-Smad) **a**: original magnification × 10 (bar = 200 μm), **d**: original magnification × 20 (bar = 100 μm) in normal adult kidney: positive staining for p-Smad was observed in distal tubular structures, whereas glomeruli and the proximal tubuli remained unstained. (**b**) Double immunofluorescence for p-Smads 1/5/8 (red) and Tamm–Horsfall–protein (THP; green): original magnification × 8 (bar = 200 μm); **e**: original magnification × 40 (bar = 50 μm): only a small number of double-positive tubuli was observed. (**c, f**) Double immunofluorescence for p-Smads 1/5/8 (red) and BMP-7 (green) **c**: original magnification × 10 (bar = 100 μm); **f**: original magnification × 40 (bar = 50 μm, nuclear staining: 4′,6-diamino-2-phenylindole-2HCl): a higher number of double-positive tubuli besides tubuli positive only for p-Smads 1/5/8.

DISCUSSION

BMP-7, a well-known important morphogenetic factor during nephrogenesis, is also expressed and active in the adult human kidney. Interestingly, our data indicate that BMP-7 expression in the adult kidney is much more restricted than in the fetal situation and predominantly found in the distal nephron. This is in line with previous studies in mice and rat documenting the kidney as a major site of BMP-7 expression,[2] with the most prominent staining seen in the distal tubulus and the CDs.[5,12–14] Other reported localizations include glomerular cells and renal arteries[13] or weak staining in some PTCs.[14] These findings, however, appear to be different in between the species. According to our data in human kidneys, BMP-7 is hardly found in these cells and was not detectable by our antibody staining.

BMP-7 activity is mediated by BMP receptors and phosphorylation of R-Smads 1, 5, and 8. Interestingly, phosphorylation of the latter was also specifically found in our study in the distal nephron and the CDs basically overlapping with the BMP-7 expression. This, together with the restricted expression of BMP-7 suggests that BMP-7 plays a physiological role mostly in these kidney areas. Also, the mostly luminal distribution of the immunostaining of BMP-7 suggests urinary excretion of BMP-7, further supporting that physiological activity of BMP-7 is largely restricted to distal nephron areas. Interestingly, BMP-7 knockout mice show adequate glomerulogenesis, but poor nephron development: whereas the proximal convoluted tubule appears to be well formed, the DCT is largely deficient.[15]

Despite the absence of detectable BMP-7 expression in glomeruli and PTCs as well as the absence of sufficiently high levels of BMP-7 in the circulating blood to stimulate cells, all epithelia in the kidney have BMP receptors at least in adult mice[16] and rats[5] and appear to be responsive to BMP activity.[5,11,12] In line with these previous studies, also our

study shows that immortalized well-differentiated PTCs (HK-2) express the BMP receptors, the BMP-signalling Smads, and are responsive to BMP-7. Thus, Smad 6 and 7, which are known to be responsive to BMP stimulation in many cells,[17–22] and the ID-1 gene (own unpublished data) were upregulated. A very intriguing speculation is that under adverse (e.g. hypoxic) conditions BMP-7 can act as a physiological protector of the distal tubule,[5] whereas the proximal tubule is prone to ischemic injury. This hypothesis is supported in many facets by our data as discussed above, whereas a protection of PTCs by BMP-7 synthesized in the glomeruli[12] is at least in physiological circumstances unlikely according to our data.

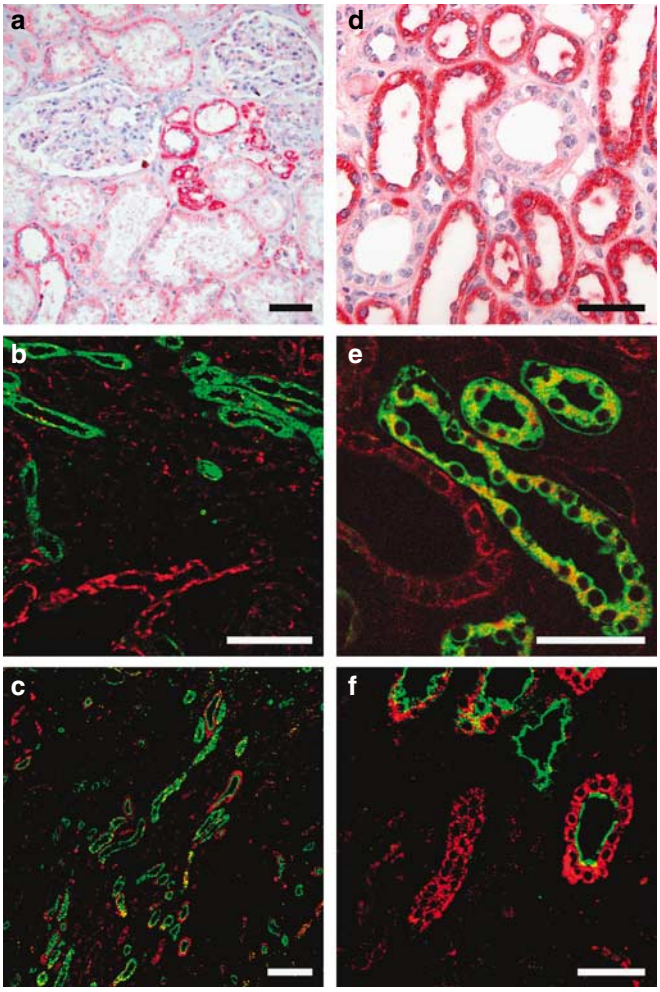

Figure 5 | Immunolocalization of IDI in normal adult kieney. (**a**, **d**) Immunolocalization of ID1 **a**: original magnification × 20 (bar = 100 μm); **d**: original magnification × 40 (bar = 50 μm) in normal adult kidney: positive staining for ID1 was observed in distal tubular structures, whereas glomeruli and the proximal tubuli remained unstained. (**b**, **e**) Double immunofluorescence for ID1 (red) and Tamm-Horsfall-protein (THP; green) **b**: original magnification × 20 (bar = 50 μm); **e**: original magnification × 40 (bar = 200 μm): again only a small number of double-positive tubuli is seen. (**c, f**) Double immunofluorescence for ID1 (red) and BMP-7 (green) **c**: original magnification × 8 (bar = 200 μm); **f**: original magnification × 40 (bar = 50 μm): again a higher number of double-positive tubuli besides tubuli positive only for ID1.

The protective effect might be hampered, if BMP-7 expression is significantly reduced, that is, after acute or prolonged ischemia[13,23] or in diabetic nephropathy,[14] as suggested by diverse animal studies. In line with this, in many conditions of acute ischemia and chronic kidney disease, the delivery of external BMP-7 appears to be protective and regenerative: thus, BMP-7 represses proinflammatory genes[5] and has a transforming growth factor-β competing anti-fibrotic activity,[24,25] stabilizes the epithelial phenotype of tubular cells *in vivo* and *in vitro*,[7,13] and significantly improves renal function.[26]

Altogether, the data suggest that BMP-7 plays a physiological role mostly in the distal tubulus. Still, as reported previously, PTCs might be responsive to BMP-7, but not in an autocrine or paracrine mode.

MATERIALS AND METHODS

Clinical cases

Samples of six normal human kidneys were obtained immediately during surgical nephrectomy for neoplastic kidney disease. By visual control, only tumor-free healthy areas of the kidney were used.

Conventional immunohistochemistry

Tissues samples were fixed with 10% formalin and embedded with paraffin. Thick tissue sections of 4 μm were used for immunostaining (for pretreatment and antibody dilutions, see Table 1). All primary antibodies were applied at 4 °C in a moist chamber over night. Primary antibodies were linked with a streptavidine-conjugated secondary antibody and labelled with biotin-conjugated alkaline phosphatase. Staining was performed with a fast red containing solution.

Negative controls were performed by replacing the primary antibodies with Tris-buffered saline and with the immunizing protein.

Immunofluorescence analysis

Tissue samples were fixed with 10% formalin and embedded in paraffin. Tissue sections (4 μm thick) were used for immunolocalization studies (for pretreatments and antibody dilutions, see

Table 1 | Primary antibodies and enzymatic pretreatments used for immunohistochemical analyses

	Species	Dilution	Digestion	Source
BMP-7 (N-19)	g	1:100	—	Santa Cruz Biotechnologies, Santa Cruz, CA, USA
p-Smads 1/5(8)	r	1:100	pt	US Biological, Swampscott, MA, USA
Calbindin D28k	r	1:2000	—	Chemicon, Temecula, CA, USA
THP	g	1:2000	—	ICN Biomedicals, Aurora, OH, USA
ID1 C-20	r	1:500	—	Santa Cruz Biotechnologies, Santa Cruz, CA, USA

BMP, bone morphogenetic protein; ID1, inhibitor of differentiation factor 1; pt, protease XXIV (Sigma, FRG; 0.02 mg/ml in PBS, pH 7.3, 60 min at RT); r/g, rabbit/goat polyclonal; THP, Tamm–Horsfall protein.

Table 1). All primary antibodies were applied at 4°C in a moist chamber over night. Primary antibodies were detected using secondary antibodies labeled either with Alexa-488 or Cy-5 (Dianova, Hamburg, FRG). Nuclear counterstain was performed with 4′,6-diamino-2-phenylindole-2HCl. Seventy percent glycerin was used as mounting medium.

Double-labelling fluorescence microscopy

For double-labelling immunofluorescence using goat and rabbit polyclonal antibodies (Table 1), histological sections were pre-treated with protease (2 mg/ml in Tris-buffered saline (pH 7.3) for 60 min at 37°C; Sigma, Taufkirchen, FRG) and incubated with a mixture of both antibodies with goat primary antibodies used at a 10 times higher concentration than in conventional immunohistochemistry. The goat antibodies were detected using Alexa-488-labelled donkey-anti-goat (Dianova, Hamburg, FRG). For the detection of the rabbit antibodies sections were first incubated with biotin-labelled donkey-anti-rabbit antibodies (Dianova) and then with peroxidase-labelled streptavidin. Subsequently, the tyramide amplification system (Perkin-Elmer, Boston, USA) was used. Finally, the signals were detected using Cy5-labelled streptavidin (Dianova). Nuclear staining was again performed using 4′6-diamino-2-phenylindole-2HCl.

The sections were evaluated by a (fluorescence) microscope (Olympus AX70) and digitally photographed.

Laser scanning confocal microscopy

Confocal scanning microscopy was performed using a Leica TCS SP-II microscope (Leica, Wetzlar, Germany). For detection, Cy-5 was used as fluorochrome (Dianova, Hamburg, Germany) as renal tissue did not show any autofluorescence at the Cy-5 excitation wavelength.

Isolation of primary human tubular cells

Primary human adult tubular cells were isolated according to a protocol published by Trifillis *et al.*[27] Briefly, 2 × 2 cm measuring tumor-free pieces were taken under sterile conditions from the cortex of a tumor nephrectomy sample. After removal of the capsule and the medulla, the cortex was cut into 5 mm pieces, which were washed in Hank's balanced salt solution and digested by sterile collagenase type 1a. The digest was filtered through two cell sieves with a 100 mash and a 300 mash screen. The cell suspension was washed twice in Hank's balanced salt solution and plated at 1.5 million cells on a Petri culture dish and kept in Dulbecco's modified Eagle's medium/F12 containing 5% fetal calf serum, 1% insulin/transferrin (Sigma), 0.1% hydrocortisone, 1% penicillin/streptomycin, 1% glutamate, and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid. After 4 days, the cells were splitted 1:5.

Cell culture

Immortalized cell lines of PTCs *(HK-2)* were cultured in keratinocyte serum-free medium containing 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract. Primary tubular cells were cultured in the medium as described above. Before starting the experiments confluent cells were kept for 24 h under starvation (without epidermal growth factor/bovine pituitary extract or fetal calf serum).

RNA isolation from cultured distal tubular cells and intact renal tissue

RNA was isolated from cultured tubular cells using the RNeasy minikit (Qiagen, Germany) (with an on-column DNAse digestion step, according to the manufacturer's instructions). Briefly, cells were passed through a Qiashredder (Qiagen, Germany) and the eluted lysate was mixed 1:1 with 70% ethanol. The lysate was applied to a minicolumn, and after washing and DNAse digestion, the RNA was eluted in 30–50 μl of RNAse-free water.

RNA was isolated from normal *renal tissue* according to a protocol previously published using the RNeasy minikit (Qiagen, Germany) (with an on-column DNAse digestion step, according to the manufacturer's instructions).[28]

The quantity and quality of RNA was assessed by ethidium bromide staining of RNA separated on 1.2% agarose gels.

cDNA synthesis – conventional PCR

First-strand cDNA was synthesized using 2 μg of total RNA, 400 U Moloney murine leukemia virus Reverse Transcriptase, RNase H Minus (Promega, Mannheim, FRG), 2 mM deoxynucleoside triphosphates (Roth, FRG) and 200 ng random primers (Promega) in a total volume of 40 μl. For conventional PCR, cDNA equivalent to 50 ng total RNA was first denatured (94°C, 4 min) and then amplified in a 35 cycle protocol (94°C/30 s–60°C/30 s–72°C/60 s)

Table 2 | Sequences of primers and probes for quantitative online-PCR experiments

Gene	Acc. No.	Primers	Probe	[$MgCl_2$]
GAPDH	NM_002046	fw: GAAGGTGAAGGTCGGAGTC rv: GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC	5.5
Smad 1	NM_005900	fw: CACAAACATGATGGCGCCT rv: CATAGTAGACAATAGAGCACCAGTGTTT	CCCTGCCCTCAGAAATCAACAGAGGAGA	6.5
Smad 5	NM_005903	fw: ACCAGCCCAACAACACTCCT rv: AGCTGAAATGGACTTCCTGGTC	TTCCCTTATCTCCAAACAGCCCTTATCCCC	4.5
Smad 8	NM_005905	fw: TCCTGGCCAAGTTCCGC rv: AGTGCAGAGCACGGAGGC	CCTCCCTGCACAGTGAGCCACTCA	4.5
BMP-RIA	NM_004329	fw: CCTTTATTGGTTCAGCGAACTATTG rv: TTGCCCATCCATACTTCTCCATA	AAACAGATTCAGATGGTCCGGCAAGTTG	6
BMP-RIB	D89675	fw: CAAAGGTCTTGCGTTGTAAATGC rv: CAATATCCGTCTGTGCTGCAAA	ACCACCATTGTCCAGAAGACTCAGTCAACA	4.5
BMP-RII	NM_001204	fw: CTACCATGGACCATCCTGCTG rv: CGCACATAGCCGTTCTTGATT	AGCACTGCGGCTGCTTCGCA	5.5
BMP-7	M60316	fw: ACGCTTCGACAATGAGACGTT rv: TTCCCTGCCCAAGTGCTC	CGGATCAGCGTTTATCAGGTGCTCCA	7

BMP, bone morphogenetic protein; fw, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; rv, reverse.

using SilverStar-DNA-Polymerase (Eurogentec, Belgium). Buffers and nucleotides were used in standard concentrations and purchased from Roth (FRG). $MgCl_2$ was added at 1.5 mM. The following primers were used for amplifying a 500 bp fragment of BMP-7 cDNA, fw 5′-TTC CCC TCC CTA TCC CCA ACT TT-3′, and rv 5′-TTT TCC TTT CGC ACA GAC ACC-3′.

TAQMAN PCR. TAQMAN PCR was used to detect human Smads 1, 5, 8, BMP-RIA, -RIB, and RII and BMP-7. The primers (MWG Biotech, Germany) and TAQMAN probes (Eurogentec, Belgium) were designed using PRIMER EXPRESS TM software (Perkin-Elmer). In order to be able to obtain quantifiable results for all genes, specific standard curves using sequence-specific control probes were performed in parallel to the analyses. Thus, for each gene a gene-specific cDNA fragment was amplified by the gene-specific primers (Table 2) and cloned into pGEM T Easy (Promega) or pCRII TOPO (Invitrogen, Karlsruhe, FRG). The cloned amplification product was sequenced for confirmation of correct cloning. Cloned standard probes were amplified using the Qiagen amplification kit (Qiagen, FRG), linearized, and used after careful estimation of the concentration (gel electrophoresis, UV-photometry, and a fluorimetric assay for DNA (Picogreen; Invitrogen, Karlsruhe, FRG)). For the standard curves, concentrations of 10, 100, 1000, 10 000, 100 000, as well as 1 000 000 molecules per assay were used (all in triplicates).

For the analyses of the different genes, a separate master-mix was made up for each of the primer pairs and contained a final concentration of 200 μM nucleotide triphosphates, 600 nM Roxbuffer, and 100 nM TAQMAN probe. For all genes the final reaction mix contained besides cDNA and 1 U polymerase (Eurogentec, Belgium), forward and reverse primers, the corresponding probes, and $MgCl_2$ at concentrations given in Table 2. All experiments were performed in triplicates.

The assay for glyceraldehyde-3-phosphate dehydrogenase was described previously.[29]

Laser-assisted microdissection and RNA isolation

Frozen tissue specimens from human control kidneys were cut as a series of 5-μm-thick sections with a clean blade and mounted on slides coated with a special membrane. To minimize degradation, slides were fixed with 100% ethanol for 1 min, then 70% ethanol for 1 min, washed in diethylpyrocarbonate-treated deionized water, and stained with the hematoxylin, then washed with diethyl-pyrocarbonate-treated deionized water, and dipped for 1 min in 70, 96, and 100% ethanol. The excess ethanol was removed by gently tapping the slide on an absorbent surface, air-dried for about 10 min at room temperature, and stored at -80°C before use. Glomerular and tubular structures (distal and proximal tubuli) were dissected by focal melting of the membrane with a UV laser beam. Microdissected fragments were selected and laser caption microdissection was performed by transferring the samples into adhesive caps (Molecular Machines & Industries AG, Switzerland) on RNAse-free conditions as described elsewhere in detail.[30]

For RNA isolation from the microdissected tissue, the RNeasy microkit was used (Qiagen, Hilden, Germany) with lysis buffer being supplemented with ExpressArt RNA Care N-Carrier (1 μl/100 μl buffer). Hundred microliter of lysis buffer was added and reaction tubes with laser caption microdissection samples were incubated for about 15 min. Then, total RNA from laser caption microdissection of glomeruli and tubuli was isolated with spin columns following the manufacturer's protocol.

For RNA quality control and for estimates of RNA quantity, aliquots of the isolated RNA samples were analyzed using the RNA 6000 Pico Assay kit (Agilent Technologies, Böblingen, FRG) in the Agilent 2100 bioanalyser.

First-strand cDNA was synthesized and quantitative PCR was performed as described above.

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