Author’s response to reviews

Title: Bone Morphogenetic Protein (BMP)-7 expression is decreased in human hypertensive nephrosclerosis

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Author’s response to reviews: see over
Dear Dr. Shipley,

thank you for your encouraging e-mail regarding the potential acceptability of our revised manuscript entitled “Bone Morphogenetic Protein (BMP)-7 expression is decreased in human hypertensive nephrosclerosis.” We are thankful to the referees for their detailed comments, which helped us to improve this manuscript substantially. Please find enclosed our responses to the reviewers’ comments point for point. Changes to the manuscript are included with additional words or phrases underlined and deleted words crossed out.

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“Bone Morphogenetic Protein (BMP)-7 expression is decreased in human hypertensive nephrosclerosis.”
**Reviewer Derek Brazil Comments**

Bramlage et al. present data showing that BMP-7, an anti-fibrotic mediator, is decreased in hypertensive sclerosis and is regulated by AT-II in HK-2 cells. The data are of value but the following needs to be addressed before acceptance.

**Major Compulsory Revisions**

1. If the expression of BMP-7 in vivo is mainly in the CD and DCT, why use the proximal tubule cell line HK-2 for experiments?

   **Answer:** Indeed, it would be interesting to examine the expression of BMP-7 in cells of the collecting duct or of the distal collecting tubule. Therefore, we started our experiments in human distal tubule cells, isolated by Patrick Baer of the University of Frankfurt (Baer PC et al. Kidney Int. 1997). However, the number of available distal tubule cells was limited and we were not able to cultivate these cells long enough to perform stimulation experiments. Due to the unavailability of a human distal tubule or distal collecting tubule cell line, we used the proximal tubule cell line HK-2 comparable to several other working groups examining the BMP-7 expression in the human kidney (Xu Y, J Nephrol 2009; Veerasamy M, Am J Physiol Renal Physiol 2009). Although expression level of BMP-7 is lower in proximal tubule cells compared to CD and DCT, expression is present as demonstrated by immunostaining and real-time PCR.

   To clarify this point, the following sentence was added into the discussion. “In preliminary experiments, we were able to detect BMP-7 mRNA in primary isolated human distal tubule cells [22], but the number of cells were limited; moreover, we were not able to cultivate these cells long enough to perform stimulation experiments.”
2. Fig. 1 refers to RNA expression – this is incorrect and should be changed throughout the text.

Answer: The description was changed according to your comment throughout the text.

3. Fig. 3 - not clear what samples were used here-why not compared control to sclerosis in these stainings?

Answer: Double immunofluorescence with BMP-7 was conducted only in control kidneys (Figure 2 & 3). The aim of the staining was the localization of BMP-7 in human kidneys rather than the comparison between nephrosclerotic and normal human kidneys which was done in the immunohistochemistry (Figure 4 of the old manuscript).

However, following your comment, we performed double immunofluorescence with BMP-7 and aquaporin-1 or -2 in nephrosclerotic kidneys. Comparable to the results of the immunohistochemistry, staining intensity of BMP-7 was lower compared to the control kidney samples as demonstrated by the lower signal intensity of BMP-7 despite a longer exposure time and therewith a stronger intensity of AQ-1 and AQ-2. The experiment was added into the manuscript and the pictures of the staining were added as a new figure (Figure 4).

Material: “Double immunofluorescence (DIF) with anti BMP-7 (goat polyclonal IgG, Santa Cruz, USA) was performed to identify the expression localization of BMP-7 inside the normal and nephrosclerotic kidney.”

Results: “As illustrated by double immunofluorescence (Figure 2 and 3) and immunohistochemistry (Figure 4), localization of BMP-7 expression was unchanged in patients with nephrosclerosis, but intensity was lower compared to controls.”

Legend Figure 4 - Immunofluorescence of BMP-7 and Aquaporin-1 and Aquaporin-2 in nephrosclerotic kidneys showing decreased expression of BMP-7: Double immunofluorescence of BMP-7 with aquaporin-1 (AQ-1) and aquaporin-2 (AQ-2): BMP-7 staining is red (rhodamine red, A, B), double-labeling of BMP-7 and AQ-1 (C) or AQ-2 (D) is
stained organge. Expression of BMP-7 was lower than in the control kidneys demonstrated by the lower staining intensity of BMP-7 despite a longer exposure time and therewith a stronger intensity of AQ-1 and AQ-2. Original magnification: x 200."

4. Fig. 4-staining needs to be quantified for the decrease in BMP-7

Answer: Thank you for this interesting advice. According to your comment, the staining intensity was scored and therewith quantified. As expected, the quantification showed a significantly lower expression of BMP-7 in nephrosclerotic kidneys compared to normal samples. The results were added into the manuscript:

Material: “Intensity of BMP-7 expression in tubules were evaluated by an established semiquantitative score as follows: 0 = no staining; 1 = weak staining; 2 = moderate staining and 3 = strong staining (Koziolek 2001, Kidney Int.). For evaluation, five visual field areas per kidney slice were analysed, and the results are presented as the mean ± standard error.”

Results: “As illustrated by double immunofluorescence (Figure 2, 3, 4) and immunohistochemistry (Figure 4), localisation of the BMP-7 expression was unchanged in patients with nephrosclerosis, but the mean intensity was significantly lower (0.86 ± 0.17) compared to controls (2.5 ± 0.07) (95% confidence interval (CI) 0.23 – 3.10, p<0.05).”

5. Fig. 9 Western blot needed to show decrease in TGF-b1 receptor

Answer: The decreased expression of the TGF-b1 receptor was demonstrated at mRNA level by real-time PCR and at protein level by the determination of the staining intensity in immunofluorescence. We agree with your comment, that the quantification with western blotting would be an additional interesting and more precise quantification of the expression. However, we failed to establish western blotting of the TGF-b1 receptor despite three different attempts.
6. For the effect of BMP-7 on TGF-b type I receptor, the authors need to show a functional consequence of this effect. HK2 cells should be treated with BMP-7 for 24 hr to reduce the type I receptor level, then TGF-b1 should be added for 60 min and pSmad2 / 3 measured. Only then can they link this effect to the mechanism of inhibition of TGFb1 by BMP-7.

**Answer:** According to your comment, we performed the experiment. However, following the suggested incubation times of 24 hours for BMP-7 and 60 min. for TGF-β, we were not able to detect significant differences of the pSmad-2 levels in the western blot.

In contrast, pSmad-2 expression was significantly decreased compared to TGF-β alone by expanding the incubation time of TGF-β and BMP-7 to 36 hours. The results were added into the manuscript:

**Methods:** “Western blot analysis of phosphorylated Smad 1/5/8 (pSmad 1/5/8, diluted 1:1000; rabbit polyclonal IgG, Cell Signalling, Beverly, USA) and phosphorylated Smad 2 (pSmad 2, diluted 1:1000; rabbit polyclonal IgG, Cell Signaling, Beverly, USA) was performed as previously described [16].... Phosphorylated Smad 2 was determined in HK-2 cells (1,000,000/ml, 25 µg/blot) after stimulation with 10 ng/ml TGF-β alone and in combination with BMP-7 (100 ng/ml).”

**Results:** “Decreased pSmad-2 expression in HK-2 cells after stimulation with BMP-7:

To further test the hypothesis that the decreased expression of the TGF-β receptor type I by BMP-7 may have functional consequences, phosphorylated Smad 2 was determined by western blotting after stimulation with TGF-β in presence or absence of BMP-7. Compared to unstimulated HK-2 cells, phosphorylated Smad 2 was increased after stimulation with TGF-β alone (323.9 ± 96.4%) and significantly decreased after co-stimulation with TGF-β and BMP-7 (95% CI: 34.8 – 52.8, p<0.05; Figure 9B).”

**Discussion:** “The functional aspect may be demonstrated by the decreased expression of phosphorylated Smad 2 after stimulation with TGF-β and BMP-7 compared to TGF-β alone.”
7. I don’t understand the data on p12 first paragraph. The Western blot data on pSmad 1/5/8 in HK-2 cells in response to AT-II needs to be shown - addition of AT-II decreases pSmad1/5/8-and yet profibrotic BMPs such as BMP-2, -4 also signal through pSmad1/5/8 so this data needs to be 1. shown and 2. better explained.

Answer: According to your suggestions, we added a figure illustrating the results of the western blot (Figure 9A).

It is difficult to give a distinct answer to the second part of your comment due to the complex and in part unknown signal transduction pathway of the BMP family. It remains unclear, why different BMPs are binding to the same receptors (BMPR-IA, -IB and -II), stimulating the same intracellular pathway, but do have in part contrary biological effects. The most convincing explanation is the complex modulation of the signal transduction e.g. by a) the BMP antagonists like Gremlin and the BMP signalling enhancer KCP (Kielin/chordin-like protein), b) the different binding affinity to the receptors BMPR-IA, -IB and -II, c) the phosphorylation of the Smads, d) the interaction with inhibitory Smads and Co-Smads and at least e) the intranuclear modulation of the transcription and expression of various target proteins. Thereby, only the last two signal transduction modulation steps may be determined, wether pSmad 1/5/8 may act profibrotic or not. Detailed information about the BMP antagonists (Gremlin) is reported by Roxburgh SA et al. (Diabetes 2009) and those about the signal transduction is reviewed by L. Oxburgh in 2009 (Current Genomics).

Indeed, evaluating this single experiment, it remains unclear if AT-II acts profibrotic or not. The following sentence was added into the section discussion: “However, the Smad complex 1/5/8 is also activated by pro-fibrotic BMPs (e.g., BMP-2, -4) and the exact regulation is due to several confounding factor (Roxburgh SA, 2009, Diabetes; L. Oxburgh, 2009, Current genomics).”
However, the Smad complex 1/5/8 is also activated by pro-fibrotic BMPs (e.g., BMP-2, -4) and the exact regulation by several confounding factors are as yet unknown (Roxburgh SA, 2009, Diabetes; L. Oxburgh, 2009, Current genomics).”

8. The figures should be rearranged to that Fig. 9 and Fig.8 are swapped around. I would also include the FACS data in Fig. 9

Answer: The figures 9 and 8 (new manuscript: Figure 9 and 10) were swapped around and the FACS data were included.

9. The methods for how apoptosis was measured in Fig. 8 are not described – is it apoptosis or cell death being measured?

Answer: Thank you for this important comment. Indeed, the staining with annexin-V indicating the cell death including apoptosis and necrosis. However, FACS analysis was evaluated by counting annexin-V positive and propidium iodide negative cells, therewith separating apoptotic cell form necrotic cells.

To clarify this point we changed / added the text into the section Material / Immunofluorescence: “To analyse the influence of BMP-7 on cell death, HK-2 cells (10,000 / ml) were stained for annexin-V (Annexin-V-FLUOS Staining Kit, Roche, Mannheim, Germany)....”. In addition, in the section “FACS analysis of apoptosis” the following sentence was added: Apoptotic cells were classified as annexin-V positive and propidium iodide negative using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).”

Results: “Apoptotic cells were detected by FACScan (FACS) counting annexin-V positive and propidium iodide negative cells (Figure 11A) ... The influence of BMP-7 on TNF-α induced cell death of HK-2 cells was determined by immunofluorescence after staining for annexin-V (Figure 11B).”
10. The manuscript is full of typos, grammatical mistakes and unclear abbreviations. The English needs to be carefully checked and corrected before publication.

*Answer:* The manuscript was sent to a native speaker, which helped us to improve the manuscript substantially.

**Minor Essential Revisions**

1. Methods relating to PB-MNC isolation (p5) described but no data presented

*Answer:* We apologize for our mistake and deleted the section. The experiment was removed due to a comment of another reviewer.

2. Page 10 line 12 please define IMH

*Answer:* The abbreviation IMH was replaced by immunohistochemistry.

3. Page 10 line 17: the statement about BMP-7 levels being low due to the requirement for 25-30 cycles to see a signal is arbitrary-factors such as primer affinity and PCR efficacy could be play here. BMP-7 levels are low-compared to what?

*Answer:* Indeed, the requirement of 25 – 30 cycles is not necessarily indicating the expression level of BMP-7. However, the low expression level is demonstrated by the comparison to the housekeeping gene PPIA and the amount of used cDNA (18 µg/ml).

“To mitigate the statement, the sentences in the section “Results – Regulation of BMP-7 expression by the renin-angiotensin system” were changed into: “...BMP-7 mRNA level in HK-2 cells was quantified by real-time PCR (18 µg/ml cDNA; 25 - 30 cycles) after stimulation with AT-II, telmisartan or both in combination for 12 hours.” The following sentence was deleted: “BMP-7 mRNA levels were low in HK-2 cells as demonstrated by the
necessity of up to 25 - 30 PCR cycles to detect BMP-7 signals despite the use of 18 μg/ml cDNA for PCR analysis.”

4. Page 10 last line, 30 μg should read 30 μM

*Answer: The unit was changed.*


*Answer: DIF is the abbreviation for double immunofluorescence. The abbreviation was replaced. Regarding the description of the methods see point 9 of the” major revisons”.

6. Page 12 for the FACS data please refer specially to Annexin V staining rather than “a different experiment”

*Answer: The section was changed and the the phrase “a different experiment” removed.

7. Page 14 line 10-what does progredient mean? Remove and replace with a clearer term

*Answer: The word progredient was replaced by „concomitant”; “Thus, progression of kidney fibrosis is associated with a concomitant loss of BMP-7 expression in later stages of kidney disease [21].”

8. In vitro and in vivo are written without a hyphen

*Answer: The term was changed.

9. Fig. 7 should be a statement

*Answer: We apologize, but we can not interpret the comment. Figure 7 is described in the section results, the discussion and the figure legend. Moreover, the heading of the figure
legend state the conclusion of the Figure legend: “BMP-7 is able to reverse EMT in human proximal tubule cells (HK-2).

10. Fig. 8 apoptosis spelt wrong

*Answer: The word was replaced*

11. Reference to “t-test” in the paper - Student t-test is a more standard description

*Answer: The term was replaced.*
Reviewer Leif Oxburgh Comments

The authors have fundamentally revised the manuscript, focusing on BMP-7 in nephrosclerosis. This is a great improvement and they have a number of interesting findings that are very worthy of publication. However, the incomplete nature of the manuscript again makes it difficult to assess as a whole, and I recommend another revision addressing the following questions.

Major Compulsory Revisions

Samples

Are the tubulointerstitial and glomerular samples from the same individuals?

*Answer:* Indeed, the tubulointerstitial and glomerular samples are from the same individuals.

To clarify this point, the following words were added into the section Material / Real time PCR: “Thereby, tubulointerstitial and glomerular samples were from the same individuals.”

Table 1: male/female distribution indicate a sample size of 42 rather than 32.

*Answer:* We apologize this mistake. Indeed, 25 male instead of 35 male patients were examined. We changed the sample size in table 1.

Also, could some normal reference values be added to this table for readers who do not have clinical experience?

*Answer:* We added the reference values in the legend of table 1: “Reference values: Creatinine Clearance: 80-140 ml/min., Proteinuria: <150 mg/day”

QPCR

The assay detecting BMP-7 does span an intron, but the authors do not list a DNase treatment step in the sDNA protocol. The authors need to provide some proof that they are not detecting
To exclude genomic contamination, we performed a DNase treatment step. The step was added into the section material. “Total RNA from both tissue and cells was extracted using the Qiagen RNaseasy Mini Kit, including a treatment with RNase-Free DNase (both Qiagen, Hilden, Germany) and reverse transcribed using random priming.”

In addition, to exclude the possibility to detect genomic contamination despite the use of a DNase treatment step, we conducted a “no-RT control” in every single real-time PCR, using not reversed transcribed mRNA instead of cDNA. Only real-time PCRs with a negative “no-RT control” were evaluated. To clarify this point, the following sentence was added into the section Material / Real-time PCR: “To exclude the possibility of genomic contamination, we conducted a “no-RT control” in every single PCR using non-reverse transcribed mRNA instead of cDNA. Only real-time PCRs with a negative “no-RT control” were evaluated.”

**Fluorescent colocalization of BMP-7 with molecular markers**

Both the anti-BMP-7 antibody and the Aquaporin antibodies used in costaining are rabbit polyclonals. Fluorophore conjugated anti-rabbit secondary antibodies were used to label these. Using the approach described, it is not possible to colocalize two different antigens in tissue.

**Answer:** We apologize for the mistake. Of course, the anti-BMP-7 antibody was a goat polyclonals. The text (Material / Immunofluorescence) was changed into: Double immunofluorescence (DIF) with anti BMP-7 (goat polyclonal IgG, Santa Cruz, USA) was performed to identify the expression localisation of BMP-7 inside the normal and nephrosclerotic kidney.”
Immunohistochemistry

Although the finding that BMP-7 is reduced in nephrosclerosis tissue is based on analysis of multiple sections, only one is shown and there is no control (ie other protein confirming the quality of the section). This analysis would be greatly improved if the authors could quantify the difference in BMP-7 staining between control kidney tissues versus nephrosclerotic tissue, while showing that overall protein levels are not depressed in nephrosclerotic tissue by comparing expression of a housekeeping protein.

**Answer:** Thank you for this interesting but complex comment. First, to illustrate the analysis of multiple sections and to quantify the BMP-7 expression, staining intensity of BMP-7 was evaluated after immunohistochemistry in normal and nephrosclerotic kidneys samples. This may reliable since all stainings were performed according to the same protocol and nephrosclerotic and normal kidney samples were conducted in parallel. As expected, the quantification showed a significant lower expression of BMP-7 in nephrosclerotic kidneys compared to normal samples. The results were added into the manuscript:

**Material:** “Intensity of BMP-7 expression in tubules were evaluated by an established semiquantitative score as follows: 0 = no staining; 1 = weak staining; 2 = moderate staining and 3 = strong staining (Koziolek 2001, Kidney Int.). For evaluation, five visual field areas per kidney slice were analysed, and the results are presented as the mean ± standard error.”

**Results:** “As illustrated by double immunofluorescence (Figure 2, 3, 4) and immunohistochemistry (Figure 4), localisation of the BMP-7 expression was unchanged in patients with nephrosclerosis, but the mean intensity was significantly lower (0.86 ± 0.17) compared to controls (2.5 ± 0.07) (95% confidence interval (CI) 0.23 – 3.10, p<0.05).”

In order to show other stainings of nephrosclerotic kidney samples and to compare the expression of BMP-7 with a control or housekeeping protein, we additional performed double immunofluorescence in nephrosclerotic kidney samples with BMP-7 and aquaporin-1 or -2. Pictures of the staining were added as a new figure (Figure 4). The aim of the new stainings
in nephrosclerotic kidney samples in parallel with normal kidneys was, to use aquaporin-1 and -2 as “housekeeping protein”. However, preliminary quantitative analysis in nephrosclerotic kidneys was not reproducible due to the very low BMP-7 expression in nephrosclerotic kidney samples and the necessity of different and in part longer exposure times. Despite the failed attempt to quantify the BMP-7 expression intensity, we added the staining in a new figure (Figure 4) to demonstrate the lower expression. This may be demonstrated by the lower expression of BMP-7 despite the longer exposure time with a stronger AQ-1 and -2 staining intensity.

Material: “Double immunofluorescence (DIF) with anti BMP-7 (goat polyclonal IgG, Santa Cruz, USA) was performed to identify the expression localisation of BMP-7 inside the normal and nephrosclerotic kidney.”

Results: “As illustrated by double immunofluorescence (Figure 2 and 3) and immunohistochemistry (Figure 4), localisation of BMP-7 expression was unchanged in patients with nephrosclerosis, but intensity was lower compared to controls.”

Legend Figure 4 – “Immunofluorescence of BMP-7 and Aquaporin 1 and Aquaporin 2 in nephrosclerotic kidneys showing decreased expression of BMP-7: Double immunofluorescence of BMP-7 with aquaporin-1 (AQ-1) and aquaporin-2 (AQ-2): BMP-7 staining is red (rhodamine red, A, B), double-labeling of BMP-7 and AQ-1 (C) or AQ-2 (D) is stained organge. Expression of BMP-7 was lower than in the control kidneys demonstrated by the lower staining intensity of BMP-7 despite a longer exposure time and therewith a stronger intensity of AQ-1 and AQ-2. Original magnification: x 200.”
Cell-based experiments

Throughout these experiments, the horizontal lines above the graphs on which the p values are written are confusing – the authors need to specify which actual comparison have been made.

*Answer:* The horizontal lines were removed and replaced by * indicating significant changes. The p-values and the actual comparison were added into the figure legends.

Smad expression

Although an immunoblot experiment measuring the expression of smads is reported in the results, no data is presented.

*Answer:* We completed the section results and added a figure (Figure 9) about the western blot results.

TNF-α induced apoptosis data

The authors have quantified the effect of BMP-7 treatment on TNF-α induced apoptosis in HK-2s by double immunofluorescence, but there is no description of what they have stained in the results section or in the figure legend.

*Answer:* According to your suggestion, we added the method of staining in the result section and the figure legend.

Results: “The influence of BMP-7 on TNF-α induced cell death of HK-2 cells was determined by immunofluorescence after staining for annexin-V (Figure 11B).”

Legend: “Induction of apoptosis (A) and cell death (B) in proximal tubular cells (HK-2) after stimulation with 20 ng/ml TNF-α alone and/or in combination with BMP-7 (1, 10, 100 ng/ml). Apoptotic cells were detected by FACS analysis counting annexin-V positive and propidium iodide negative cells (n=3). Cell death was detected after staining with annexin-V (n=3)”
TGFbR1 immunostaining

The anti-TGFbR1 immunofluorescence seems to show a change in localization of the receptor to the nucleus after BMP-7 treatment. If that is really the case, it seems worthy of discussion.

Answer: Thank you for this comment, which will make the discussion more interesting. Indeed, beside the decreased expression of the TGF-β receptor type 1, the localization of the receptor may play an additional function in the signal transduction of TGF-β. This hypothesis may underlined by a review of Chen YE (Cell Research 2009). He summerized that TGF-β R1 is internalized in clathrin-coated vesicles and that the process of being expressed on the cell surface plays an important regulatory role in TGF-β signalling. Based on our findings, we suggest that the decreased TGF-β signal transduction by BMP-7 is regulated by a) the decreased receptor expression and b) the decreased expression on the cell surface.

According to your comment, we added the following sentence into the section “Results”: “Moreover, the expression pattern of TGF-βRII changed after stimulation with BMP-7 to a localisation that was more intracellular than on the cell surface.” Discussion: “Thereby, the attenuated TGF-β signalling may be caused by the decreased expression level as well as by the decreased receptor expression on the cell surface. This hypothesis may be in line with previous findings suggesting that TGF-β R1 is internalised in clathrin-coated vesicles and that the process of being expressed on the cell surface plays an important regulatory role in TGF-β signalling (Chen YE, Cell Research 2009)”.

Minor essential revisions

Figure legends for the new figures need to be more informative – for example, it is very difficult to understand what antibodies have been used, how the quantification has been performed.
Answer: The figure legend of the new figures (TGFβRI immunofluorescence and Smads) was extended.

The conclusion of the abstract is cryptic and should be revised.

Answer: The conclusion of the abstract was changed into: “The findings suggest a protective role for BMP-7 by counteracting the TGF-β and TNF-α-induced negative effects. The reduced expression of BMP-7 in patients with hypertensive nephrosclerosis may imply loss of protection and regenerative potential necessary to counter the disease.”

In the statistical analysis section, the sentence on significant changes being regarded as descriptive needs to be explained.

Answer: The statistics were conducted by the department of medical statistics of our university. Some of the cell culture experiments were conducted with the sample size of 3 (at least). Out of the view of a statistician, a higher sample size is preferable, although the analysis were leading to significant results. To indicate, that the statement would be more tightened if more sample size were used, the phrase “regarded as descriptive” were added.

Discretionary Revision

It would help the flow of the manuscript if the authors included a couple of sentences on the rationale for doing experiments at the beginning of each results section.

Answer: According to your comment, we added a short rationale at the beginning of each result section.
In summary, we have addressed all the comments from the reviewers and revised the manuscript to reflect those changes. We believe our revised manuscript has improved, and are convinced that you will find the current version acceptable for publication in BMC Nephrology.

Sincerely,

Carsten Bramlage