BMP-7 blocks mesenchymal conversion of mesothelial cells and prevents peritoneal damage induced by dialysis fluid exposure

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Abstract

Background: During peritoneal dialysis (PD), mesothelial cells undergo an epithelial to mesenchymal transition (EMT), and this process is associated with peritoneal membrane damage. Bone morphogenic protein-7 (BMP-7) antagonizes transforming growth factor (TGF)-β1, modulates EMT and protects against fibrosis. Herein, we analyzed the modulating role of BMP-7 on EMT of mesothelial cells in vitro and its protective effects in a rat PD model.

Methods: Epithelioid or non-epithelioid mesothelial cells were analyzed for the expression of BMP-7, TGF-β1, activated Smads, E-cadherin, Collagen I, α-SMA and VEGF using standard procedures. Rats were daily instilled with PD fluid with or without BMP-7 during five weeks. Histological analyses were carried out in parietal peritoneum. Fibrosis was quantified with Van Gieson or Masson’s Trichrome staining. Vasculature, activated macrophages and invading mesothelial cells were quantified by immuno-fluorescence analysis. Quantification of infiltrating leukocytes and mesothelial cell density in liver imprints was performed by May Grünwald Giemsa staining. Hyaluronic acid levels were determined by ELISA.

Results: Mesothelial cells constitutively expressed BMP-7 and its expression was down-regulated during EMT. Treatment with recombinant BMP-7 resulted in blockade of TGF-β1-induced EMT of mesothelial cells. We provide evidence of a Smad-dependent mechanism for the blockade of EMT. Exposure of rat peritoneum to PD fluid resulted in inflammatory and regenerative responses, invasion of the compact zone by mesothelial cells, fibrosis, and angiogenesis. Administration of BMP-7 decreased the number of invading mesothelial cells and reduced fibrosis and angiogenesis. In contrast, BMP-7 had no effect on inflammatory and regenerative responses, suggesting that these are EMT-independent, and probably upstream, processes.

Conclusions: Data point to a balance between BMP-7 and TGF-β1 in the control of EMT and indicate that blockade of EMT may be a therapeutic approach to ameliorate peritoneal membrane damage during PD.
Introduction

Peritoneal dialysis (PD) is a form of renal replacement that is based on the use of the peritoneum as a semi-permeable membrane across which ultrafiltration and diffusion take place\textsuperscript{1,2}. Chronic exposure to non-physiologic PD solutions and episodes of infection cause inflammation and injury to the peritoneal membrane, which progressively undergoes fibrosis, angiogenesis and hialynizing vasculopathy\textsuperscript{3-8}. These morphological alterations are associated with increased small-solute transport rate and with ultrafiltration dysfunction of the peritoneal membrane\textsuperscript{3,6,7}. Inflammatory cells and myofibroblasts are considered the main entities responsible of the structural and functional alterations of the peritoneum during long-term PD. In response to PD-induced inflammation and injury, myofibroblasts may originate from mesothelial cells by epithelial to mesenchymal transition (EMT)\textsuperscript{7-8}. The EMT of mesothelial cells is a complex process that is characterized by the disruption of intercellular junctions, adoption of a front-back polarity and increased migratory/invasive capacity\textsuperscript{7,10}. In addition, trans-differentiated mesothelial cells acquire the capacity to synthesize pro-inflammatory and pro-angiogenic factors as well as extracellular matrix components (ECM)\textsuperscript{11-13}, which may contribute to peritoneal membrane worsening\textsuperscript{7}. In this context, it has been shown that the presence of trans-differentiated mesothelial cells either in the PD effluent or peritoneal tissue of PD patients correlates with high transport rates\textsuperscript{11,13,14}. It can be hypothesized that the EMT of mesothelial cells is an important process in peritoneal membrane dysfunction, and, thus, it might be considered a potential target for therapeutic intervention.

Transforming growth factor (TGF)-β\textsubscript{1}, a strong pro-fibrotic cytokine\textsuperscript{15}, appears to be a master molecule in peritoneal membrane structural and functional deterioration\textsuperscript{16,17}. In addition, TGF-β\textsubscript{1} is a well-characterized inducer of EMT\textsuperscript{8,12,18}. The relevance of TGF-β\textsubscript{1} in both EMT of mesothelial cells and peritoneal membrane worsening has been further demonstrated in an \textit{in vivo} rat model, which reproduced the structural and functional alterations observed in PD patients\textsuperscript{19,20}. It is worthwhile to point out that EMT is a reversible process and that there are molecules, such as bone morphogenetic protein-7 (BMP-7), that negatively regulate EMT and that promote mesenchymal to epithelial transition\textsuperscript{21}. BMP-7 is an endogenous protein, belonging to the TGF-β superfamily that prevents and reverses fibrosis in several diseases affecting organs such as the kidney, liver and heart\textsuperscript{22-26}. In these diseases, TGF-β\textsubscript{1} and BMP-7 maintain a delicate balance in the control of EMT\textsuperscript{21}. TGF-β\textsubscript{1} and BMP-7 bind to specific type I serine-threonine kinase receptors (ALK receptors) and trigger distinct intracellular signalling pathways mediated by Smad proteins. Smad-2 and
Smad-3 transduce TGF-β1 action, whereas Smad-1, Smad-5 and Smad-8 mediate BMP-7 signalling. Smad-4 is common to both pathways27-29.

Whereas the role of TGF-β1 in EMT of mesothelial cells and in peritoneal membrane worsening has been well documented7,17, the possible protective function of BMP-7 in these processes has not been explored in depth. Vargha et al demonstrated a reversion from mesenchymal to epithelial phenotype of effluent-derived mesothelial cells with fibroblast-like characteristics by BMP-730. In a recent work, it has been demonstrated that BMP-7 prevented and reversed high glucose-induced EMT of mesothelial cells in vitro and further decreased peritoneal fibrosis during a peritoneal resting period in PD fluid-exposed rats31. However, the ability of BMP-7 to block TGF-β1-mediated EMT of mesothelial cells and to prevent peritoneal membrane deterioration during PD fluid exposure has not been addressed. Herein, we demonstrate that mesothelial cells constitutively express BMP-7 and show activation of BMP-7-specific Smad proteins. During EMT the expression of BMP-7 is down-regulated, but addition of exogenous BMP-7 completely blocks TGF-β1-induced EMT of mesothelial cells in vitro and ameliorates peritoneal membrane worsening in a rat model of PD fluid exposure. Our results provide evidence that blockade of EMT, by using agonists of the BMP-7 signalling pathway, may be a therapeutic approach to preserve peritoneal membrane integrity during PD.
Materials and Methods

Culture of mesothelial cells and treatments
Mesothelial cells were obtained from PD-effluents and from omentum samples using the methods described previously\(^\text{32}\). To standardize effluent mesothelial cell harvesting, the cells were obtained from a long dwell (generally overnight) with a PD fluid containing approximately 2.3% glucose (\textit{Dianeal} from Baxter Healthcare Corporation, Deerfield, IL or \textit{Stay Safe} from Fresenius Medical Care, Germany). Definition of Epitheliod (\(n=9\)) and Non-epitheliod (\(n=7\)) phenotypes of effluent-derived mesothelial cells was based on cellular morphology of confluent cultures and on the expression levels of epithelial (E-cadherin, cytokeratins) and mesenchymal (\(\alpha\)-SMA, collagen I) markers as previously described\(^{8;11;13;32}\). Cells were cultured in Earle’s M199 medium, supplemented with 20% foetal-calf serum, 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin and 2% Biogro-2 (Biological Industries, Israel). The purity of effluent and omentum-derived mesothelial cell cultures was determined by the expression of standard mesothelial markers: intercellular adhesion molecule-1, cytokeratins, and calretinin. These mesothelial cell cultures were negative for von-Willebrand factor excluding endothelial cell contamination\(^\text{32}\). The present study is adjusted to the Declaration of Helsinki and was approved by the Ethics Committee of Hospital Universitario de la Princesa (Madrid, Spain). Written consent was obtained from the PD patients included in this study to use effluent samples. Oral informed consent was obtained from omentum donors submitted to elective surgeries.

To induce EMT in vitro, omentum-derived mesothelial cells were seeded on wells coated with 50 \(\mu\)g/ml of collagen I (Roche Boehringer GmbH, Mannheim, Germany) and treated for 24, 48 or 72 hours with human-recombinant transforming growth factor (TGF)-\(\beta\) (1 ng/mL) (R&D Systems Inc, Minneapolis, MN), which has been proven to be a good model of EMT in vitro\(^{6;11-13;18}\). Where indicated, recombinant human BMP-7 (rhBMP-7) (Prospec, Rehovot, Israel) was used at a final concentration of 0.5 \(\mu\)g/mL. This dose of rhBMP-7 was similar to that employed by others\(^\text{24}\). To analyze the effect of GDPs on BMP-7 and TGF-\(\beta\) expression, omentum-derived mesothelial cells were incubated for 24, 48 or 72 hours with standard PD fluid composed of 4.25% glucose and buffered with lactate (\textit{Stay Safe}, Fresenius Medical Care) or low-GDPs solutions composed of 4.25% glucose and buffered with lactate (\textit{Balance}, Fresenius Medical Care) diluted one half with culture medium.

Western blot, enzyme-linked immunoassays and immunofluorescence analysis
For Western blotting, mesothelial cell cultures were lysed in a buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) and total protein quantified using a
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total-protein assays kit (Pierce, Cambridge, MA). Mesothelial cell proteins (50 μg) were resolved in 8-10% sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred on to nitrocellulose membranes, which were blocked with fat free-milk and then incubated with specific antibodies against E-cadherin, collagen I, α-SMA, Smad-1, 5 and 8, P-Smad-1, 5 and 8, Smad-2, P-Smad-2, Smad-3, P-Smad-3, (Cell Signaling Technology, Beverly, MA, USA), BMP-7 (Santa Cruz biotechnology CA, USA) and β-actin (Becton & Dickinson, Frankling Lakes, NJ). Membranes were incubated with goat anti-mouse IgG antibody conjugated with peroxidase (Pharmigen, San Diego, CA) and developed with enhanced chemiluminscence (ECL) detection kit (Amersham Biosciences, Freiburg, Germany). Blot images were acquired with an LAS-1000 Charge Coupled Device camera (Fujifilm, Cedex, France).

For the detection of TGF-β or VEGF in culture supernatants, the media of mesothelial cells cultured under the different conditions were replaced and eighteen hours later supernatants were collected and stored at –80 ºC until their analysis. The concentrations of TGF-β or VEGF in supernatants were assessed by a standard enzyme-linked immunoassay (ELISA) kit (R&D Systems Inc). The expression of BMP-7 under different conditions was measured in cell lysates by an ELISA-based assay according to Merrihew et al(33) using two anti-BMP-7 antibodies (Abnova Corporation, Taipei, Taiwan; Santa Cruz Biotechnology CA, USA).

For immunofluorescence analysis, staining with antibodies to P-Smad-1, 5 and 8 (Cell Signaling Technology, Beverly, MA, USA) and BMP-7 (Santa Cruz biotechnology CA, USA) was performed using alexa-labeled secondary antibodies (BD Biosciences, USA). Cells were fixed for 15 minutes in 4% formaldehyde in PBS, and blocked with 10% horse serum for 60 minutes in PBS with 0.3% Triton X-100. First antibody was incubated in PBS with 0.3% Triton X-100 for 60 minutes and then secondary alexa-labeled antibody was incubated under same conditions. Finally, the preparations were mounted with a 4, 6-diamidino-2-phenylindole (DAPI) nuclear stain (Vectorashield; Vector Laboratories). Negative controls for immunofluorescent staining were conducted using 10% rabbit serum instead primary antibody. Images were analysed by computerized digital image analysis (AnalySIS, Soft Imaging System).

Animals and experimental design of PD fluid exposure
Male Wistar rats (Harlan CPB, Horst, The Netherlands) weighing 250-275 gr. at the beginning of the experiment were used throughout the study (n=44). They were allowed one week of acclimatization before the start of the experiment. Animals were housed under conventional laboratory conditions and were given food and water ad libitum. Fluids were instilled via a peritoneal catheter connected to an
implanted subcutaneous mini access port as previously described. Rats that were not surgically treated and received no fluid instillation, served as control group (Control; n=8). The animals implanted with a peritoneal access port (n=36) received 2 ml of saline with 1 IU/ml heparin to allow wound healing during the first week after operation. Thereafter, during a five week period, 14 rats were daily instilled (one per day) with 10 ml of standard PD fluid (Dianeal® PD4, 3.86% glucose, pH 5.2, Baxter R&D, Utrecht, The Netherlands) (PDF; n=14). The remainder of the animals received daily treatment (one per day) with rhBMP-7 (0.25 mg/kg body weight) either in 10 ml of PD fluid (PDF + BMP-7; n=14) or in 1 ml saline (Control + BMP-7; n=8) which were both given i.p. via the peritoneal access port. The dose of rhBMP-7 was similar to that employed by others. Drops out were 0, 6, 2, and 4 for the Control, PDF, PDF + BMP-7 and Control + BMP-7 groups; respectively, being the cause catheter obstruction with omental wrap. There were no significant differences in weight gain among the different groups. Since the number of rats in the Control + BMP-7 group at the end of the study was only four, insufficient for appropriated statistical analysis, they were employed to study possible side effects (e.g. immune response) derived from rhBMP-7 treatment (Supplementary Figure S1 and Table S1). The experimental design was approved by the Animal Care Committee of the Vrije Universiteit of Amsterdam.

Morphological analysis of peritoneal samples
For histological analyses specimens of the parietal peritoneum were obtained from the contralateral side to the tip of the implanted catheter. Cryostat sections (7 μm) were cut and stained with Van Gieson or Masson’s Trichrome (Merck, Darmstadt, Germany) to quantify fibrosis. The thickness of submesothelial tissue was determined by blinded microscope analysis using a metric ocular, and was expressed as the mean of 10 independent measurements for each animal. Frozen sections were stained for immuno-fluorescence analysis with antibodies to visualize vasculature (CD31) and activated macrophages (ED2) (Serotec, Oxford, UK). Background control staining was performed by incubating secondary antibodies alone (Invitrogen, Carlsbad, USA) (omitting the first antibodies) and proved to be negative. Images were analysed by computerized digital image analysis (AnalySIS, Soft Imaging System). The positive area for CD31 and ED2 was calculated as a percentage of the total area of the tissue. To detect the presence of invading mesothelial cells that have undergone an EMT, the frozen section were stained with antibodies specific for cytokeratins and FSP-1 (Dako; Glostrup, Denmark).
Peritoneal leukocyte infiltration, Hyaluronic acid content and liver imprint

After five weeks of treatment rats were injected with 30 ml of standard PD fluid into the peritoneal cavity via a direct i.p. catheter (Venflon Pro, BD Medical, New Jersey, USA), under a mixture of Hypnorm (0.05 ml/100 gr. BW) / Dormicum (0.08 ml/100 gr. BW) anaesthesia. After 90 minutes the PD fluid was drained and cells were isolated by centrifugation. Cell number and viability was determined by Trypan Blue exclusion. Cytocentrifuge preparations were stained by May Grünwald Giemsa, and cell subsets were identified and counted. After PD fluid draining, animals were sacrificed and tissues were taken to analyse morphological and cellular parameters. The amount of hyaluronic acid (HA) in the supernatant of the peritoneal effluent was determined in an ELISA-based assay according to Fosang et al39. Liver imprints of the mesothelial monolayer of the liver were taken with 3% gelatin coated slides according to the method described before40 and stained by May Grünwald Giemsa. Mesothelial cell density was counted in 15 visual fields and expressed as cells/mm².

Statistical analysis

All data are presented as median and interquartiles, except Figures 1C, 1D and 3 that are given as means ± SD. Comparisons between data groups were performed using the non-parametric Mann-Whitney rank sum U test. Linear correlation was determined by Spearman regression (figures 5C and 6D). P<0.05 was considered statistically significant. We used SPSS 14.5 (Chicago, IL) and GraphPad Prism 4.0 (La Jolla, CA).
Results

BMP-7 is constitutively expressed in mesothelial cells and down-regulated during EMT

BMP-7 is involved in the modulation of mesenchymal conversion in a wide spectrum of epithelial cells, thus we analyzed the expression of BMP-7 in mesothelial cells and its association with EMT, a key process in peritoneal membrane dysfunction. Omentum-derived mesothelial cells showed cytoplasmic immuno-staining of BMP-7 (Figure 1A, panel a). Treatment of these cells with 1 ng/ml of TGF-β1 during 48 hours to induce EMT, resulted in a clear down-regulation of BMP-7 expression (Figure 1A, panel b). Similarly, effluent-derived mesothelial cells with epithelioid phenotype displayed BMP-7 expression (Figure 1A, panel c), whereas mesothelial cells with non-epithelioid phenotype showed weak, if any, expression of BMP-7 (Figure 1A, panel d). The decrease of BMP-7 during in vitro and in vivo EMT was significant in both cases (Figure 1A, right graphs). The decrease of BMP-7 expression in omentum-derived mesothelial cells treated with TGF-β1 could also be demonstrated by western blot analysis (Figure 1B). These data were further confirmed in a more quantitative manner by measuring the expression levels of BMP-7 by ELISA in cellular extracts from epithelioid and non-epithelioid mesothelial cells obtained from different donors and in lysates from omentum mesothelial cells treated or not with TGF-β1 (Figure 1C). Exposure of omentum mesothelial cells for 24 to 72 hours to standard PD fluid with high content of GDPs, which has been shown to trigger EMT-like changes in mesothelial cells, resulted in significant down-regulation of BMP-7 expression at each time point (High-GDPs vs Control, p=0.0099 at 24h; p=0.0074 at 48h, p=0.0081 at 72h) and in significant induction of TGF-β1 expression at 72h (High-GDPs vs Control, p=0.0077) (Figures 1D and 1E). In contrast, when mesothelial cells were incubated with more biocompatible PD fluids containing low GDPs concentration, with little impact on EMT of mesothelial cells, cells did not show repression of BMP-7 or induction of TGF-β1 (Figures1D and 1E).

In agreement with BMP-7 expression pattern, omentum-derived mesothelial cells showed basal activation (phosphorylation) of the BMP-7-specific Smad 1, 5, and 8 (Figure 2A). Treatment of cells with 1 ng/ml of TGF-β1 during 24 or 48 hours resulted in decreased phosphorylation of Smad 1, 5, and 8 (Figure 2A) and in enhanced phosphorylation of TGF-β1-specific Smad 2 and 3 (Figure 2B). Furthermore, untreated mesothelial cells showed nuclear immunostaining of activated forms of Smad 1, 5, and 8 (Figures 2B, left), which decreased upon TGF-β1 treatment (Figure 2B, middle). Interestingly, co-treatment of mesothelial cells with TGF-β1 and rhBMP-7 (0.5 μg/ml) restored the activation and nuclear translocation of BMP-7-
Figure 1: Down-regulation of BMP-7 expression during EMT of mesothelial cells. A: Immunofluorescence microscopy shows cytoplasmic staining of BMP-7 in omentum-derived mesothelial cells (panel a), which is down-regulated upon treatment with 1 ng/ml of TGF-β1 during 48 hours (panel b). Insets in panels a and b show the expression of BMP-7 determined by western blot. Effluent-derived mesothelial cells with epitheloid phenotype display BMP-7 staining (panel c) and mesothelial cells with non-epitheliod phenotype show decreased BMP-7 expression (panel d). Magnification x400. The decrease of BMP-7 during in vitro and in vivo EMT is significant in both cases, as determined by computerized digital image analysis of the percentage of positive area for BMP-7 (right graphs). B: Analysis of BMP-7 expression by western blot in omentum mesothelial cells treated or not with TGF-β1 during 48 hours. C: Analysis of BMP-7 expression by ELISA in untreated omentum mesothelial cells (n=5), omentum mesothelial cells treated with TGF-β1 (n=5), epitheloid effluent mesothelial cells (n=9) and non-epitheliod effluent mesothelial cells (n=7) shows significant down-regulation of this protein during in vitro and in vivo EMT. D-E: Omentum-derived mesothelial cells were incubated for 24, 48 or 72 hours with control medium, standard PD fluid containing high GDPs (High-GDPs) or a solution containing low GDPs (Low-GDPs) diluted one half with culture medium. Cells were also treated with recombinant TGF-β1 (1 ng/ml). The synthesis of TGF-β1 was measured in culture media supernatant by ELISA and results are depicted as nanograms per milligrams of total cellular proteins (D). The expression of BMP-7 was measured in cell lysates by ELISA and results are depicted as picograms per milligrams of total cellular proteins (E). The experiment was repeated five times and results are presented as mean ± SD. Box Plots represent 25% and 75% percentiles, median, minimum and maximum values. Symbols show statistical differences between groups.
specific Smads (Figure 2B, right). These results indicated that TGF-β1-mediated blockade of Smad1, 5 and 8 activation could be mediated by BMP-7 down-regulation or by modulation of BMP-7 signalling, and that exogenous addition of rhBMP-7 prevented the effect of TGF-β1.

**BMP-7 counteracts TGF-β1-induced EMT of mesothelial cells**
Since exogenous BMP-7 prevented the inactivation of Smad 1, 5 and 8 by TGF-β1, we investigated the role of BMP-7 in the maintaining of the epithelial phenotype of mesothelial cells. To this end, we analyzed the effect of rhBMP-7 on EMT of

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**Figure 2**: Inactivation of BMP-7-specific Smad proteins during EMT of mesothelial cells.
A: Omentum-derived mesothelial cells were either left untreated or treated with 1 ng/ml of TGF-β1 for 24 or 48 hours. Western blot analysis shows basal activation (phosphorylation) of the BMP-7-specific Smad 1, 5, and 8, which decreases upon TGF-β1 treatment at both time points. In contrast, exposure to TGF-β1 results in enhanced phosphorylation of Smads 2 and 3. B: Immunofluorescence microscopy shows nuclear staining of activated forms of Smad 1, 5, and 8 in untreated mesothelial cells (left), which decreases upon treatment with TGF-β1 during 48 hours (middle). Co-treatment of mesothelial cells with TGF-β1 and BMP-7 (0.5 μg/ml) restores the activation and nuclear translocation of BMP-7-specific Smads (right). The experiment was repeated at least three times and a representative experiment is shown. Magnification x630.
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Figure 3: BMP-7 blocks TGF-β1-induced EMT of mesothelial cells. Omentum-derived mesothelial cells were treated for 48 hours with 1 ng/ml TGF-β1, in the presence or absence of 0.5 μg/ml of BMP-7. A: Phase-contrast microscopy shows that BMP-7 treatment prevents non-epitheliod phenotype acquisition of mesothelial cells. Magnification x200. B-D: Western blot analysis shows that BMP-7 treatment prevents TGF-β1-induced E-cadherin down-regulation (B) as well as collagen I (C) and α-SMA up-regulation (D). The experiment was repeated three times and results are depicted as means plus SD. E: A representative experiment is shown. F: Analysis of VEGF expression by ELISA demonstrates that BMP-7 prevents TGF-β1-mediated induction of this growth factor. Bars represent values obtained by ELISA and are depicted as picograms per milligrams of total cellular proteins (F).
mesothelial cells *in vitro*. Treatment of omentum-derived mesothelial cells with 1 ng/ml of TGF-β1 during 48 hours resulted in morphological change to a fibroblast-like shape and in E-cadherin down-regulation, which were prevented by co-treatment with 0.5 μg/ml of rhBMP-7 (Figures 3A and 3B). In addition, rhBMP-7 completely blocked TGF-β1-mediated up-regulation of the mesenchymal markers collagen I, α-SMA and VEGF (Figures 3C to 3F). These results demonstrated that BMP-7 was involved in the epithelial maintaining of mesothelial cells and prevented the mesenchymal conversion of these cells.

**BMP-7 ameliorates peritoneal membrane structural alteration in a rat model of PD fluid exposure**

Since EMT of mesothelial cells is an important process in peritoneal membrane dysfunction and that BMP-7 is able to block the EMT process, we analyzed the effects of this protein in a model of PD fluid exposure in rats. With this purpose rats were daily instilled via catheters with glucose-based PD fluid and treated or not with rhBMP-7 by intraperitoneal route (PDF and PDF+BMP-7 groups). Rats that were not surgically treated and received no fluid instillation, served as control group (Control). Peritoneal inflammation and tissue repair are early responses to PD fluid exposure, which in turn may promote the induction of EMT of mesothelial cells, the activation of fibroblasts, the accumulation of ECM and angiogenesis. Thus, we first analyzed if rhBMP-7 might have any effect in the recruitment of inflammatory cells into the peritoneal cavity, in the local production of hyaluronic acid and in the regenerative response. As shown in Table 1, rhBMP-7-treatment did not affect significantly any of these processes induced by PD fluid exposure, except for a tendency to reduce hyaluronic acid production (p=0.054), which suggested a decrease of peritoneal fibrosis.

**Table 1: Composition of peritoneal leukocytes, liver imprints and hyaluronic acid**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PDF</th>
<th>PDF+BMP-7</th>
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<tr>
<td>Total peritoneal cells (x10⁶)</td>
<td>23.5 +/- 3.6</td>
<td>214.5 +/- 51.7^a</td>
<td>205.4 +/- 182.4^a</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>72.8 +/- 6.1</td>
<td>65.6 +/- 35.7</td>
<td>74.75 +/- 21</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.5 +/- 0.7</td>
<td>0.8 +/- 0.5</td>
<td>0.25 +/- 0.25</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>2.4 +/- 1.7</td>
<td>27.3 +/- 31.4^b</td>
<td>21.25 +/- 20.5^a</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>11.5 +/- 3.3</td>
<td>4.6 +/- 8.1</td>
<td>1.5 +/- 2^a</td>
</tr>
<tr>
<td>Mast cells (%)</td>
<td>12.25 +/- 5.5</td>
<td>0.1 +/- 0.3^a</td>
<td>0.25 +/- 0.25^a</td>
</tr>
<tr>
<td>Liver imprints (cells/mm²)</td>
<td>1168 +/- 273</td>
<td>2045 +/- 548^a</td>
<td>2193 +/- 544^a</td>
</tr>
<tr>
<td>Hyaluronic acid (ng/ml)</td>
<td>21.4 +/- 24.6</td>
<td>2424.9 +/- 2896.8^a</td>
<td>1090 +/- 1131.6^a,^c</td>
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All data is presented as Median +/- Inter Quartiles

^a versus Control: p<0.01; ^b versus Control: p<0.05; ^c versus PDF: p = 0.054.
The histological analysis of parietal peritoneum biopsies showed that PD fluid exposure resulted in increased thickness compared with control rats (Figure 4) and in the accumulation of trans-differentiated mesothelial cells in the submesothelial space (cytokeratin positive cells), some of which also showed expression of the activated fibroblast marker fibroblast specific protein-1 (FSP-1)\(^{21}\) (Figure 5). The administration of rhBMP-7 significantly reduced the thickness and the presence of submesothelial cytokeratin positive cells (Figures 4 and 5).

**Figure 4:** Effect of BMP-7 on thickness of parietal peritoneum of PD fluid exposed rats. 
A: Masson’s trichrome staining of parietal peritoneum shows a marked increase of extracellular matrix deposition in rats exposed to standard PD-fluid (PDF) and BMP-7 treatment significantly reduces this effect (PDF+BMP-7). B: The peritoneal thickness (\(\mu m\)) is increased in group PDF (n=8) compared with control rats (n=8), and the group PDF + BMP-7 (n=12) shows a significant reduction of thickness when compared with group PDF. Box Plots represent 25% and 75% percentiles, median, minimum and maximum values. Symbols show statistical differences between groups. Magnification x200.
Interestingly, a correlation between thickness and numbers of cytokeratin positive cells in the compact zone was observed, reinforcing the notion of the relevance of EMT of mesothelial cells in peritoneal fibrosis (Figure 5C). PD fluid exposure also resulted in recruitment of activated macrophages (ED2) and in new vessel formation (CD31) in the parietal peritoneum compared with control rats (Figure 6A). Treatment with rhBMP-7 resulted in a significant reduction of angiogenesis but did not affect the influx of activated macrophages (Figure 6A). As above, a correlation between vessel formation and numbers of cytokeratin positive cells was observed (Figure 6B), suggesting that EMT of mesothelial cells is a key process not only in peritoneal fibrosis but also in angiogenesis.

Figure 5: Effect of BMP-7 on the number of trans-differentiated mesothelial cells in the compact zone of parietal peritoneum.
A: Immunofluorescence microscopy analysis of parietal peritoneal sections stained for cytokeratin (green) and FSP-1 (red) with DAPI counterstaining shows accumulation of trans-differentiated mesothelial cells in the submesothelial space (cytokeratin positive cells), some of which also show expression FSP-1 (arrows) in the PDF group. The administration of BMP-7 reduces the number of submesothelialcytokeratin positive cells per field (PDF+BMP-7). Magnification x200. B: The reduction of the number of invading mesothelial cells by BM-7 is significant. Box Plots represent 25% and 75% percentiles, median, minimum and maximum values. Symbols show statistical differences between groups. C: Correlation between peritoneal membrane thickness (μm) and number of cytokeratin positive cells per field at the compact zone in the whole group of rats treated with PD fluid (Spearman regression, p=0.0047; n=20).
Figure 6: Effect of BMP-7 on macrophages recruitment and angiogenesis at the parietal peritoneum.
A: Immunofluorescence microscopy analysis of parietal peritoneal sections stained for ED2 (green) and CD31 (red) shows accumulation of activated macrophages and an increase in vasculature in rats exposed to PD-fluid (PDF). Magnification x400. B-C: The administration of BMP-7 (PDF+BMP-7) does not affect the recruitment of macrophages (B) but reduces significantly the number of vessels (C). The positive area for ED2 and CD31 is calculated as a percentage of the total area of the tissue. Box Plots represent 25% and 75% percentiles, median, minimum and maximum values. Symbols show statistical differences between groups. D: Correlation between CD31 staining (% area) and number of cytokeratin positive cells per field at the peritoneal compact zone of rats treated with PD fluid (Spearman regression, p=0.0058; n=20).
Discussion

The presence of mesothelial cells that have undergone an EMT in the effluent and in the peritoneum of PD patients was first demonstrated in a landmark paper published in 2003. During the last few years emerging evidences have suggested that the mesenchymal conversion of mesothelial cells is an important mechanism of peritoneal structural and functional deterioration. From clinical nephrologists points of view, perhaps the most important aspect of the identification of the EMT of mesothelial cells as a key event in peritoneal membrane failure is that this process can be modulated with a number of endogenous factors and pharmaceutical agents. In this context, the endogenous factor BMP-7 has been demonstrated to block and reverse the mesenchymal conversion of different types of epithelial cells and of endothelial cells in vitro by activating Smad-5, which interferes with TGF-β-activated Smad-2/3. It has been also shown in various animal models that BMP-7 treatment prevents and reverses diverse acute or chronic fibrotic diseases. However, only few works about the role of BMP-7 in mesenchymal conversion of mesothelial cells or in peritoneal membrane alteration have been reported. It has been demonstrated that BMP-7 is able to promote a reversion from mesenchymal to epithelial phenotype of effluent-derived mesothelial cells and to block high glucose-induced EMT of omentum-derived mesothelial cells in vitro. The therapeutic strategies to preserve the peritoneal membrane during PD may be designed either to prevent or to reverse the EMT. It has been shown that BMP-7 treatment further reverses peritoneal fibrosis during a peritoneal resting period in PD fluid-exposed rats. However, the ability of BMP-7 to counteract TGF-β1-mediated EMT of mesothelial cells and to prevent peritoneal membrane deterioration during PD fluid exposure has not been addressed.

In the present study, we show that mesothelial cells constitutively express BMP-7 and display basal activation of Smads 1, 5, and 8. Induction of EMT with different stimuli results in down-regulation of BMP-7 and inactivation of BMP-7-specific Smad proteins. Mechanistically, the TGF-β1-mediated inhibition of BMP-7 signalling might be explained by BMP-7 down-regulation itself or, alternatively, by the up-regulation of modulators of BMP-7 and TGF-β1 pathways. In this context, it has been shown that connective-tissue growth factor (CTGF), a cytokine that is induced in mesothelial cells upon TGF-β1 treatment, inhibits BMP-7 and activates TGF-β1 signals by direct binding in the extracellular space. In addition, mesothelial BMP-7 signalling might also be influenced by other members of the TGF-β superfamily and by others BMP-7 modulators such as gremlin, noggin, kielin/chordin-like protein, or uterine
sensitization-associated gene. Thus, the relative contribution of these different factors in the inhibition of BMP-7 pathway by TGF-β1 remains to be established and deserves further studies. We could demonstrate that exposure of mesothelial cells to PD fluid with high content of glucose degradation products (GDPs), results in down-regulated expression of BMP-7 and in up-regulation of TGF-β1. Interestingly, when mesothelial cells are exposed to fluids with identical concentration of glucose but with low GDPs content, which have been proved to have little impact on EMT of mesothelial cells, there is no repression of BMP-7 and no induction of TGF-β1 synthesis. In contrast, a recent study has shown that culture media with high glucose induced EMT and BMP-7 down-regulation. The authors concluded that these effects were due to D-glucose per se and not to high osmolality or to GDPs, because L-glucose did not induce EMT. An explanation to these apparent discrepancies could be that different types of GDPs, with different impacts on EMT, were generated from D-glucose and L-glucose during sterilization process or during their storage. We believe that both high glucose and GDPs may have a role on EMT in vitro, depending on experimental conditions, and certainly have additive effects on PD-fluid-induced peritoneal damage.

The data presented in this work demonstrate that addition of exogenous rhBMP-7 prevents the inactivation of Smad 1, 5 and 8 and by TGF-β1 and completely blocks TGF-β1-induced EMT of mesothelial cells in vitro. These results suggest that BMP-7 has a role in preserving the epitheliod phenotype of mesothelial cells. Administration of rhBMP-7 to rats exposed to PD fluid results in preservation of the mesothelial monolayer and in reduction of invading mesothelial cells, demonstrating that rhBMP-7 is also effective in vivo in the prevention of EMT. Furthermore, treatment with rhBMP-7 reduces extracellular matrix deposition and new vessel formation in the parietal peritoneum, indicating an association between EMT of mesothelial cells.

**Supplementary Figure S1:** Effect of BMP-7 on thickness of parietal peritoneum of PD fluid exposed rats. The peritoneal thickness (μm) is increased in group PDF (n=8) compared with control rats (n=8), and the group PDF+BMP-7 (n=12) shows a significant reduction of thickness when compared with group PDF. Rats treated with BMP-7 alone (BMP-7, n=4) also show a significant increase of peritoneal thickness compared with control group. Box Plots represent 25% and 75% percentiles, median, minimum and maximum values. Symbols show statistical differences between groups. Magnification x200.
and both fibrosis and angiogenesis. Interestingly, rhBMP-7 treatment has no effect on inflammatory and regenerative responses. It can be hypothesized that these two processes are EMT-independent and probably up-stream to EMT.

In a recent study using a cohort of 50 new PD patients, it has been shown that the levels of BMP-7 in the effluents, measured at four weeks after PD starting, correlated with peritoneal transport characteristics, and that a high BMP-7 level was associated with a gradual increase in peritoneal transport parameters with time\(^49\). At a first glance, these observations may seem contradictory with the expected beneficial effect of BMP-7. However, the elevated levels of BMP-7 during the early stages of PD might simply represent an intense reparative response of injured peritoneal tissue, which resulted in increased mass of mesothelial cells and in enhanced production of BMP-7, as we and others\(^31\) have demonstrated that mesothelial cells are a site of BMP-7 synthesis.

The main limitation of this study is the use of a recombinant human protein (rhBMP-7) in a rodent model of PD fluid exposure, which might trigger an immune/inflammatory response against this protein. In fact, we observed that rhBMP-7 treatment induced per se a significant increase of hyaluronic acid production and a slight but significant increase of fibrosis at the parietal peritoneum (Supplementary Table S1 and Figure S1). In addition, although BMP-7 treatment alone did not result in an increase of total leukocytes recruitment into the peritoneal cavity, it promoted a switch in the percentages of some leukocyte subpopulations; increased neutrophils and decreased eosinophils and mast cells proportions, in a similar manner to that

| Suppementary table 1: Composition of peritoneal leukocytes, liver imprints and hyaluronic acid |
|---------------------------------|---------|---------|---------|---------|
| Total peritoneal cells (x10\(^6\)) | Control | PDF | PDF+BMP-7 | BMP-7 |
| 23.5±3.6 | 214.5±51.7\(^a\) | 205.4±182.4\(^a\) | 33.5±14.51 |
| Macrophages (%) | 72.8±6.1 | 65.6±35.7 | 74.75±21 | 75.61±7.9 |
| Lymphocytes (%) | 0.5±0.7 | 0.8±0.5 | 0.25±0.25 | 0.6±0.21 |
| Neutrophils (%) | 2.4±1.7 | 27.3±31.4\(^b\) | 21.25±20.5\(^a\) | 23.91±22.28\(^b\) |
| Eosinophils (%) | 11.5±3.3 | 4.6±8.1\(^a\) | 1.5±2\(^a\) | 6.1±2.3\(^b\) |
| Mast cells (%) | 12.25±5.5 | 0.1±0.3\(^a\) | 0.25±0.25\(^a\) | 1.7±1.04\(^a\) |
| Liver imprints (cells/mm\(^2\)) | 1168±273 | 2045±548\(^a\) | 2193±544\(^a\) | 1431±113 |
| Hyaluronic acid (ng/ml) | 21.4±24.6 | 2424.9±2896.8\(^a\) | 1090±1131.6\(^a, c\) | 886.4±434.1\(^a\) |

All data is presented as Median +/- Inter Quartiles.
\(^a\) p<0.01 versus Control; \(^b\) p<0.05 versus control; \(^c\) p=0.054 vs PDF.
observed in PDF-treated animals (Supplementary Table S1). It can be speculated that this immune response could mask and/or neutralize part of the protective effects of rhBMP-7.

In summary, our results indicate that TGF-β1 and BMP-7 pathways maintain a balance in the control of EMT of mesothelial cells and that addition of exogenous rhBMP-7 completely blocks the mesenchymal conversion of mesothelial cells. To our knowledge, this work is the first to show the preventive effect of BMP-7 on peritoneal membrane damage in an animal model of PD fluid exposure. We believe that our results about the beneficial effects of rhBMP-7 provide evidences about the feasibility of considering the EMT of mesothelial cells as a therapeutic target to ameliorate peritoneal membrane deterioration in PD patients. Further studies of the BMP-7 signalling pathway will provide more specific strategies of interventions (e.g. by using agonists of the BMP-7 receptors) with minimum side effects.

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BMP-7 and peritoneal membrane protection

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