VERSITA

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 10 November 2013
Final form accepted: 25 May 2014
Published online: June 2014

Volume 19 (2014) pp 315-329
DOI: 10.2478/s11658-014-0199-z

Research article

MiR-30b IS INVOLVED IN METHYLGLYOXAL-INDUCED EPITHELIAL–MESENCHYMAL TRANSITION OF PERITONEAL MESOTHELIAL CELLS IN RATS

HONG LIU[1], NING ZHANG[2] and DA TIAN[3,] *
[1]Department of Emergency Medicine, People's Hospital of Lishui, Zhejiang Province, 15 Dazhong Street, Liandu District, Lishui, 323000, Zhejiang Province, P.R. China, [2]Department of Critical Care Medicine, People's Hospital of Lishui, Zhejiang Province, 15 Dazhong Street, Liandu District, Lishui, 323000, Zhejiang Province, P.R. China, [3]Department of Neurosurgery, People's Hospital of Lishui, Zhejiang Province, 15 Dazhong Street, Liandu District, Lishui, 323000, Zhejiang Province, P.R. China

Abstract: Epithelial–mesenchymal transition (EMT) of peritoneal mesothelial cells (PMC) is a major contributor to the pathogenesis of peritoneal fibrosis. EMT is at least in part caused by repeated exposure to glucose degradation products (GDPs), such as methylglyoxal (MGO). MiRNA contributes greatly to the EMT of PMCs. In this study, we tried to profile whether differences exist between the peritoneal membrane (PM) miRNA expression seen in control rats and that seen in rats injected intraperitoneally with MGO. We assessed whether miR-30b has a possible role in MGO-induced EMT of PMCs in rats. Comparative miRNA expression array and real-time PCR analyses were conducted for the control group at the start of the experiment and for the MGO group after 1 and 2 weeks. During the second week, the MGO rats were treated with: a chemically modified antisense RNA oligonucleotide (ASO) complementary to the mature miR-30b (ASO group); an miR-30b mismatch control sequence (MIS group); or a citrate buffer (EMT group). Bioinformatic

* Author for correspondence. Email: datiandeemail@163.com, phone: 86 578-2780150

Abbreviations used: 3'-UTR – 3' untranslated region; α-SMA – α-smooth muscle actin; ASO – antisense RNA oligonucleotide; BMP7 – bone morphogenetic protein 7; EDTA – ethylene diamine tetraacetic acid; EMT – epithelial-mesenchymal transition; ESRD – end-stage renal disease; GDPs – glucose degradation products; HRP – horseradish peroxidase; MGO – methylglyoxal; PD – peritoneal dialysis; PDFs – peritoneal dialysis fluids; PM – peritoneal membrane; PMC – peritoneal mesothelial cells; RPMCs – rat peritoneal mesothelial cells; RRT – renal replacement therapy

analyses indicated that the 3' untranslated region (3'-UTR) of bone morphogenetic protein 7 (*BMP7*) mRNA did contain a putative binding site for miR-30b. We also tried to investigate whether miR-30b targeted *BMP7* in vitro by transfection. Of the upregulated miRNAs, miR-30b expression demonstrated the greatest increase. The administration of miR-30b ASO for two weeks significantly reduced α-SMA excretion and upregulated E-cadherin and BMP-7 expression. Our in vitro study showed that miR-30b directly targeted and inhibited *BMP7* by binding to its 3'-UTR. Our results revealed that miR-30b is involved in MGO-induced EMT of PMCs in rats.

Keywords: Methylglyoxal, miR-30b, Epithelial-mesenchymal transition, Peritoneal dialysis, Rats, Peritoneal fibrosis, MicroRNA, Bone morphogenetic protein 7, E-cadherin, Peritoneal membrane

INTRODUCTION

Peritoneal dialysis (PD) is a blood-purifying treatment for patients with end-stage renal disease (ESRD). Despite recent advances in PD, peritoneal fibrosis, a serious complication for long-term PD patients, still exists and has largely limited the development of PD as a method.

Continuous exposure to PD fluids (PDFs) is believed to be a non-physiological, bioincompatible state, and such exposure may cause inflammation and injury to the peritoneal membrane (PM), which would then progressively undergo peritoneal fibrosis [1, 2]. Glucose is widely used as an osmotic agent in PDFs. Glucose degradation products (GDPs), such as methylglyoxal (MGO) and glyoxal, are generated from the degradation of glucose during heat sterilization and storage [3]. MGO is an extremely toxic GDP that may cause peritoneal injury during the epithelial-mesenchymal transition (EMT) of peritoneal mesothelial cells (PMC) [4, 5].

EMT is a dynamic process in which epithelial cells undergo a phenotypic conversion that gives rise to a fibroblastoid appearance. The transformed cells, which express α-smooth muscle actin (α-SMA), possess a mesenchymal phenotype and the increased ability to proliferate, migrate and synthesize extracellular matrix while they lose epithelial markers [6]. Recent in vivo and in vitro evidence suggested that the EMT of PMCs is a key mechanism in peritoneal fibrosis [7, 8]. New experimental approaches confirming the effects of MGO-induced EMT of PMC are needed.

MicroRNAs (miRNAs) are a group of endogenous, noncoding small RNAs that have recently been implicated in the regulation of gene expression by translation repression or transcript degradation of specific target genes in multiple biological processes [9]. To date, more than 1000 human miRNAs have been experimentally identified, and it is estimated that they regulate more than one-third of cellular mRNA by binding to the 3' untranslated regions (3'-UTRs) of their target genes or by inducing mRNA degradation [10]. Recent evidence suggested that miRNAs contribute greatly to EMT, especially to the EMT of

PMCs. This provided critical new information that advanced the knowledge of peritoneal fibrosis [11, 12]. MiRNAs are distributed in a tissue- and time-specific manner, but their roles in the MGO-induced EMT of PMCs in vivo have not been explored in depth.

Previous research showed that MGO may induce peritoneal fibrous thickening with the proliferation of mesenchymal-like mesothelial cells, which may be transdifferentiated from PMCs by EMT [13]. In this study, we hypothesize that miRNA expression patterns might serve as a signature of the EMT of PMCs in rats after intraperitoneal injections of MGO. To prove this hypothesis, we profiled the differences between the PM miRNA expressions of control rats and rats that had received MGO intraperitoneally. We found significantly changed miRNA expression in the latter, with the greatest increase in the expression of miR-30b. We also investigated whether miR-30b is involved in MGO-induced EMT of PMCs in rats and expected to find novel insights into the mechanisms underlying peritoneal fibrosis in terms of miRNA.

MATERIALS AND METHODS

Animal studies

49 male Wistar rats (6 weeks old, weighing 180–220 g) purchased from Center of Experimental Animal of Institute of Radiation Medicine of Peking Union Medical College were used in the experiment. The animals were housed in an air-conditioned room at a constant temperature of 23 ± 2ºC and a relative humidity of 50 ± 10% with a 12-h light/12-h dark cycle. They had free access to a standard rat diet and tap water.

PDFs containing 20 mmol/l MGO were prepared by adding MGO to a standard PDF of 2.5% glucose (pH 5.2) from Baxter Healthcare. They were checked to be free of endotoxins. Over a 2-week period, 36 rats were injected intraperitoneally with 10 ml PDF per day containing 20 mmol/l MGO. The remainder of the animals received an intraperitoneal injection of normal saline (CTL group, n = 13). PM were harvested from 3 of the CTL group rats for miRNA microarray analysis at the start of the experiment (W0, n = 3). At an indicated time point in weeks 1 and 2, PM were also harvested for miRNA microarray analysis from one control rat and two rats receiving MGO (W1, n = 3; W2, n = 3). The rats' body weights were recorded weekly throughout the experimental period. After week 2, the intraperitoneally injected rats were randomly divided into three groups. One group was injected intraperitoneally with a chemically modified antisense RNA oligonucleotide (ASO) complementary to the mature miR-30b sequence (ASO group, n = 10). One group was given the miR-30b mismatch control sequence (MIS group, n = 10). The final group was given an equal volume of citrate buffer (EMT group, n = 10). On week 4, the PM of individual rats was sampled for histological analysis and gene expression analysis.

Primary culture of rat peritoneal mesothelial cells (PMCs)

Rat PMCs were isolated from the peritonea of rats by enzymatic digestion as previously reported [14]. Briefly, male Wistar rats weighing 180–220 g were humanely euthanized and 30–40 ml of 0.25% trypsinase and 0.02% EDTA-Na_2 were infused into the rats' abdominal cavities. After infusion for 2 h, all of the fluid was collected from the peritoneal cavity of the rats under sterile conditions and centrifuged at 1500 rpm for 5 min at 4ºC. The PMCs for this study were harvested and grown in M199 medium (Sigma) supplemented with 10% FBS, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. PMC identification was based on their morphological polygonal cobblestone appearance and the staining of the mesothelial-specific marker Hector Battifora-mesothelin-1. Cells between passages 3 and 5 were grown as a monolayer to 80% confluence and used for the experiments.

MiRNA microarray analysis

Total RNA was extracted from PM tissue with a mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. The concentrations and purities of the total RNA were assessed with a spectrophotometer and the RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies). Briefly, 5 μg of good quality RNA was used for miRNA expression analysis performed using a TaqMan MicroRNA Array v2.0 (Applied Biosystems).

Real-time PCR

Real-time PCR using miRNA-specific stem-loop primers for reverse transcription and TaqMan probes for mature murine miRNA was performed according to the manufacturers' protocols with a 7500 FAST Real-Time PCR System (Applied Biosystems). First-strand cDNAs were generated using SuperScript III (Invitrogen). Mature miRNA-specific primers and probes were obtained from Applied Biosystems. Primers specific for miR-136, miR-703, miR-30b, miR-107, miR-653 and miR-598 were used, and the values were normalized to the housekeeping gene U6. Data analysis was performed using the Applied Biosystems SDS Software package, version 2.2.

Reagents

A chemically modified antisense RNA oligonucleotide complementary to the mature miR-30b and miR-30b mismatch control oligonucleotides for the in vivo applications were purified using Sigma-Genosys reagents. The miR-30b ASO sequence was UGUAAACAUCCUACACUCAGCU; and the miR-30b mismatch control sequence was CGAGCUCAUCCUAUACUCAGUC. All oligonucleotides were 2'-OMe modified and free of endotoxin for in vivo delivery. MiR-30b ASO or miR-30b mismatch control oligonucleotides were injected at 80 mg/kg body weight intraperitoneally two weeks after the MGO injection.

MiR-30b mimics, anti-miR-30b, and normal control molecules were synthesized by Shanghai Genepharma Co. Ltd. The primers of full-length *BMP7* with target

3'-UTR were forward, 5'-GTCGAGATGCTGCCTTACTGGCTTGTAGTC-3'; and reverse, 5'-CGTCTGCAGTACCTGGAATGCTACGTTGTC-3'.

Liposome-mediated plasmid transfection

The 3'-UTR portion of *BMP7* mRNA containing the predictive binding site (nt 550-640) for miR-30b was amplified and subcloned into pmirGLO luciferase plasmid using PmeI and XbaI restrictions. The putative miR-30b binding site UGUUUAC (nt 582-588) was mutated into CGUGCAG via oligonucleotide-directed PCR (Fig. 5B). The cells were seeded in 24-well plates for 24 h and co-transfected with 800 ng of pGLO-BMP7-3'-UTR or pGLO-BMP7-3'-UTR-mut and miR-30b mimics, the miR-30b mimics negative control, anti-miR-30b molecules, or the anti-miR-30b negative control at a final concentration of 20 nmol/l by using Lipofectamine 2000 (Invitrogen) for 24 h. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Specific miR-30b mimics were transfected into rat PMCs at a final concentration of 20 nmol/l using Lipofectamine 2000. The transfection efficiency was detected using real-time PCR or western blotting.

Immunohistochemical analysis

PMs were removed from rats and fixed in 10% phosphate buffered formalin. Paraffin-embedded tissues, cut into 2- to 3-μm thick slices, were deparaffinized with xylene and microwave-treated at 98ºC for 10 min. Then the slides were preincubated for 10 min with a blocking solution containing bovine serum albumin (Zhongshan Goldenbridge). Staining was performed using the avidin biotin-peroxidase complex technique in accordance with the manufacturer's protocol. The sections were incubated with the primary antibody, rabbit anti-rat BMP-7 polyclonal antibody (1:200, Bosider), overnight at room temperature, followed by a 40-min incubation with the secondary antibody, PV-6001 goat anti-rabbit IgG-HRP multimer (Zhongshan Goldenbridge). The negative control sections were incubated with PBS instead of the primary antibody.

Western blot

For the western blot analysis, the PMs were homogenized in RIPA buffer and subjected to centrifugation at 10,000 and 14,000 rpm. Proteins in the supernatant were determined using a Bio-Rad assay kit, and 50 μg was mixed with 5x SDS-PAGE sample buffer (500 mmol/l DTT, 0.2% bromophenol blue, and 50% glycerol) and boiled for 3 min. The proteins were separated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane, which was blocked with fat-free milk and incubated with rabbit anti-BMP-7 polyclonal antibody (1:200, Bosider), goat anti-E-cadherin monoclonal antibody (1:200, Santa Cruz Biotechnology, Inc.), and rabbit anti-α-SMA monoclonal antibody (1:500, Abcam Inc.). The membranes were washed with Tris-buffered saline containing Tween 20 and incubated with horseradish peroxidase-conjugated antiserum. Protein was then detected using enhanced chemiluminescence.

Statistical analysis

All of the statistical analyses in this study were performed using SPSS 15.0 software with $p < 0.05$ considered statistically significant. The data are presented as the means ± SEM and compared using Student's t test or ANOVA as appropriate.

RESULTS

Changes in specific miRNA expressions in the PMs of rats that received intraperitoneal injections of MGO

To determine whether miRNAs play a role in the initiation and progression of MGO-induced EMT of PMC in rats, we performed miRNA expression profiling of rats that had received intraperitoneal injections of MGO and compared them to profiles for control rats. We found 44 miRNAs that were altered 1 and 2 weeks after MGO injection. Significantly upregulated miRNAs were miR-136, miR-703, miR-30b and miR-107, while miR-653 and miR-598 were significantly downregulated. Due to the involvement of miRNAs in the pathogenesis of EMT, it is suggested that specific miRNAs might participate in the MGO-induced EMT of PMCs in rats (Fig. 1).

In order to confirm the findings with the miRNA array analysis, we tested those miRNAs that showed the greatest alterations in the PMs of MGO-injected rats (miR-136, miR-703, miR-30b, miR-107, miR-653 and miR-598; Fig. 2). Consistent with the miRNA array data, the expressions of miR-136, miR-703, miR-30b and miR-107 were significantly increased, whereas the expressions of miR-653 and miR-598 were significantly decreased relative to the control group. In both the miRNA array and the real-time PCR analysis, miR-30b expression showed the greatest increase in the PMs of rats injected with MGO.

MiR-30b ASO counteracts MGO-induced EMT of PMCs in rats

The expression of E-cadherin significantly decreased after week 4 in the EMT and MIS groups compared to the CTL group ($p < 0.05$). Treatment with miR-30b ASO significantly increased E-cadherin expression ($p < 0.05$). The expression of α-SMA significantly increased after week 4 in the EMT and MIS groups relative to the CTL group ($p < 0.05$), and treatment with miR-30b ASO significantly decreased α-SMA expression ($p < 0.05$; Fig. 3A–D). Real-time PCR analysis indicated that miR-30b expression was significantly upregulated in the EMT and MIS groups compared with the CTL group ($p < 0.05$) after week 4 and was downregulated significantly after the administration of miR-30b ASO ($p < 0.05$; Fig. 3E).

Expression changes in BMP-7 in the PMs of rats injected with MGO

To assess the effect of miR-30b ASO on BMP-7 expression, we performed immunohistochemistry and western blot assays. In the EMT and MIS groups, the BMP-7 levels were significantly lower than in the CTL group ($p < 0.05$),

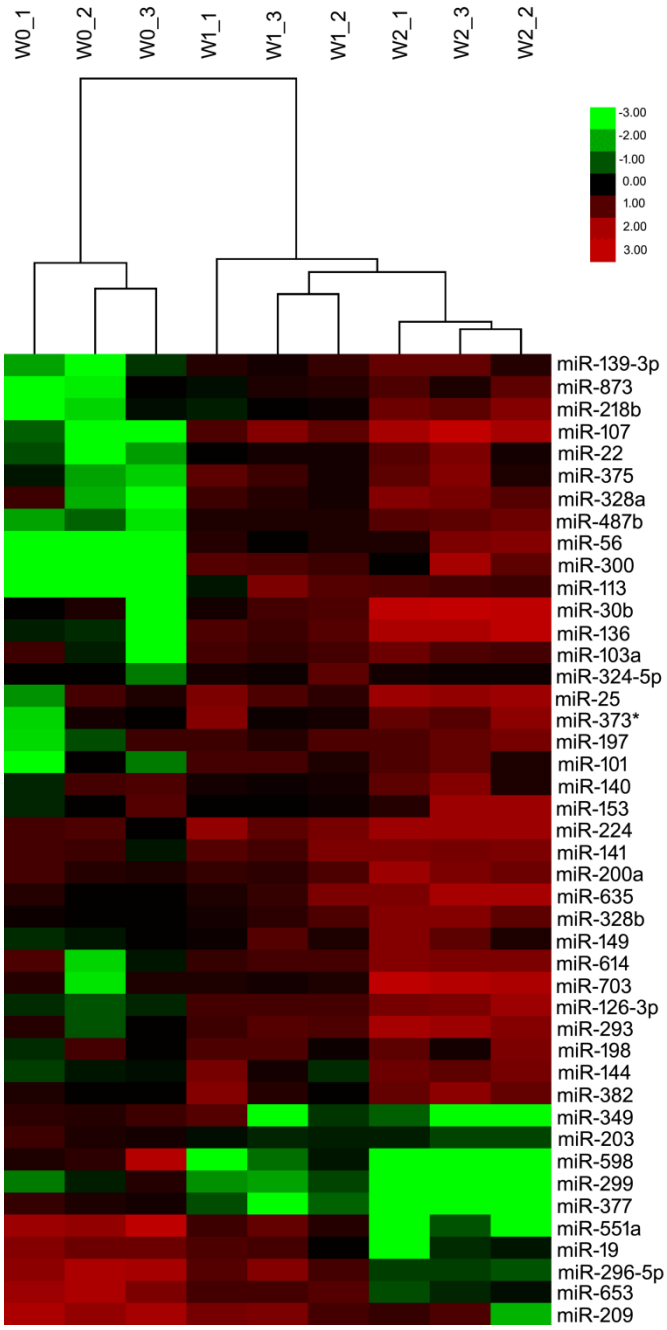

Fig. 1. Analysis of miRNA microarray data. MiRNA expression is altered in the peritoneal membranes of rats that received intraperitoneal injections of MGO. The analysis categorized 3 clusters corresponding to the control group at the start of the experiment (W0), rats after 1 week of MGO injections (W1), and rats after 2 weeks of MGO injections (W2). The method of complete linkage was used as a clustering method (n = 3).

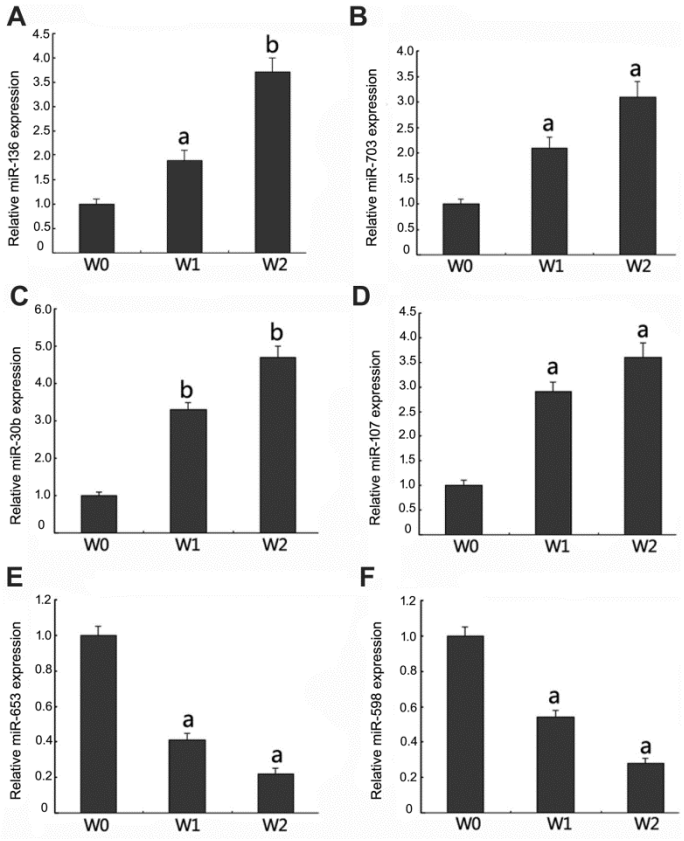

Fig. 2. The expression of specific miRNAs is altered in the peritoneal membranes of rats that received intraperitoneal injections of MGO. The expression was determined using real-time PCR. A, B, C, D – upregulated miRNAs; E, F – downregulated miRNAs. W0: control group at the start of the experiment; W1: rats after 1 week of MGO injections; W2 rats after 2 weeks of MGO injections. [a]$p < 0.05$ vs. the control group at the start of the experiment, [b]$p < 0.01$ vs. the control group at the start of the experiment ($n = 3$).

whereas rats treated with miR-30b ASO had significantly greater expressions of BMP-7 ($p < 0.05$). Consistent with the above findings, immunohistochemical analysis also showed decreased BMP-7 staining in the EMT and MIS groups, but the signal was markedly stronger in the miR-30b ASO group (Fig. 4).

MiR-30b targets *BMP7*

Bioinformatic assessments using Targetscan showed that miR-30b was the predicted miRNA target site in *BMP7* 3'-UTRs. To test whether miR-30b is involved in the regulation of *BMP7*, we first transfected miRNA mimics into rat PMCs. As detected by western blot analysis, miR-30b transfection downregulated the protein levels of BMP-7 significantly ($p < 0.05$; Fig. 5A).

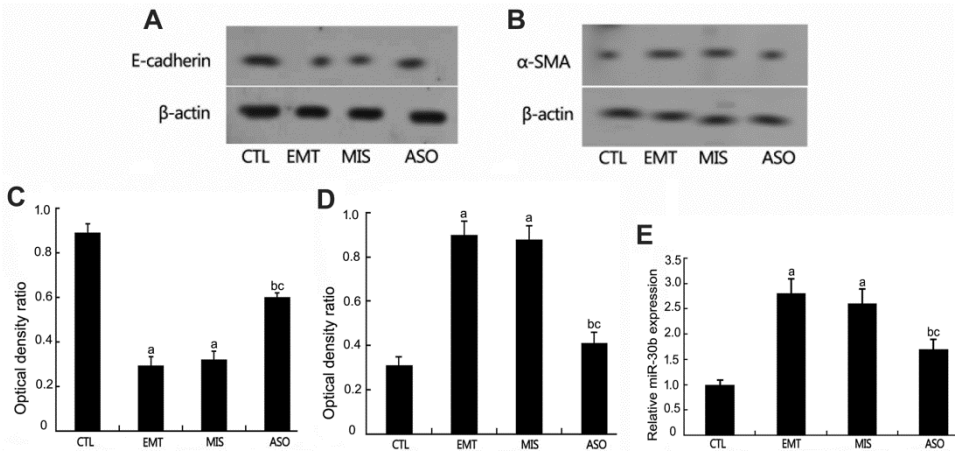

Fig. 3. The effect of miR-30b ASO on the expression of E-cadherin, α-SMA and miR-30b as determined using western blot or real-time PCR analyses. A – Changes in E-cadherin expression. B – Changes in α-SMA expression. C, D – The bar graphs show the densitometric quantification of E-cadherin and α-SMA under the indicated conditions. β-actin served as an internal control. The expression of E-cadherin significantly decreased in the EMT and MIS groups and significantly increased in the ASO group. α-SMA significantly increased in the EMT and MIS groups and significantly decreased in the ASO group. E – The expression of miR-30b was high in the EMT and MIS groups and significantly decreased in the ASO group. $^{a}p < 0.05$ vs. CTL, $^{b}p < 0.05$ vs. EMT, $^{c}p < 0.05$ vs. MIS (n = 10).

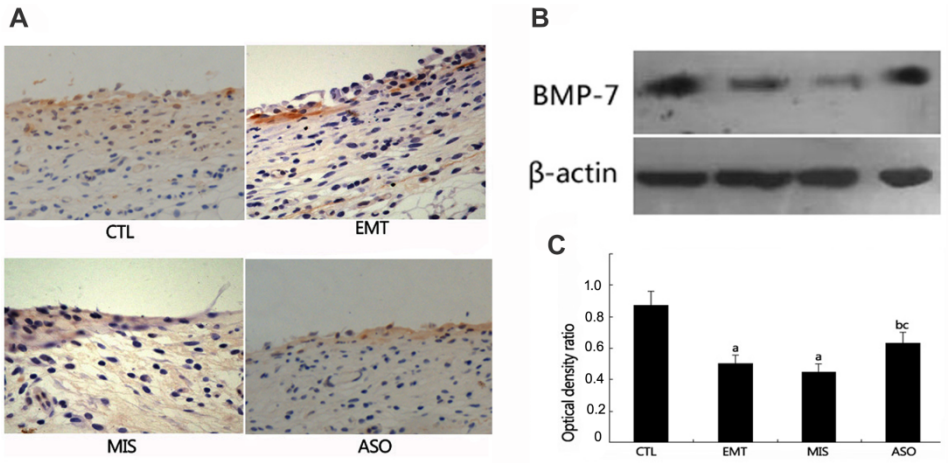

Fig. 4. BMP-7 expression assessed by immunohistochemistry and western blot after week 4. A – The expression levels of BMP-7 were low in the EMT and MIS groups and significantly increased in the ASO group (original magnification × 400). B – The expression of BMP-7 decreased significantly in the EMT and MIS groups and significantly increased in the ASO group. C – The bar graph shows the densitometric quantification of BMP-7 under the indicated conditions. β-actin served as an internal control. $^{a}p < 0.05$ vs. CTL, $^{b}p < 0.05$ vs. EMT, $^{c}p < 0.05$ vs. MIS (n = 10).

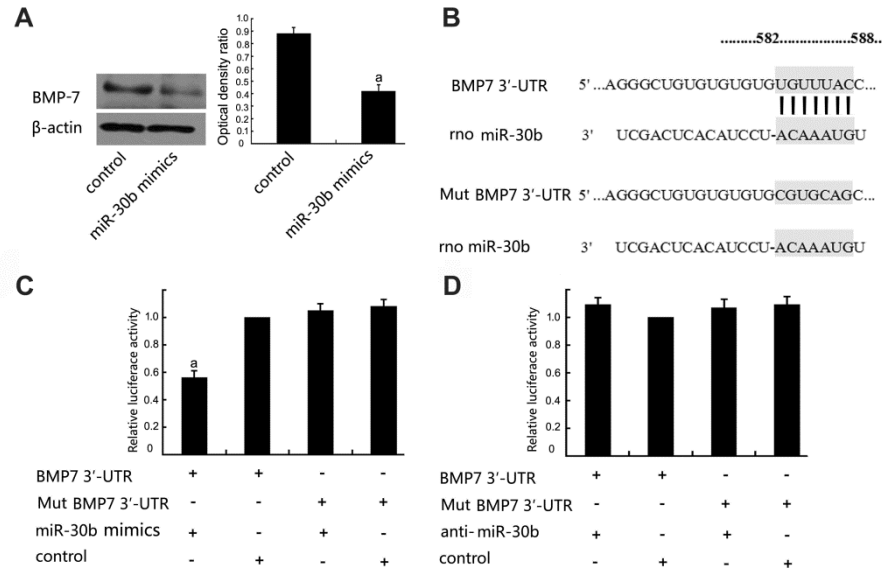

Fig. 5. MiR-30b targets *BMP7.* A – Western blotting analysis of BMP-7 protein in rat PMCs transfected with miR-30b mimics or control mimics showed that BMP-7 was inhibited significantly by miR-30b ($^{a}p < 0.05$). B – Bioinformatic analysis for the target site of miR-30b in rat *BMP7* mRNA. C – The results of luciferase reporter experiments with transient co-transfection of miR-30b mimics or control mimics and pmirGLO vector containing the wild-type or mutant (Mut) binding site of miR-30b from rat *BMP7* mRNA into rat PMCs. D – The results of luciferase reporter experiments with transient co-transfection of anti-miR-30b or control mimics and pmirGLO vector containing the wild-type or mutant (Mut) binding site of miR-30b from rat *BMP7* mRNA into rat PMCs (n = 3).

Transient co-transfection of miR-30b mimics with luciferase expression plasmids resulted in a significant repression of luciferase activity in rat PMCs. However, mutations within the putative binding site to *BMP7* abrogated the effect of miR-30b mimics (Fig. 5C). Furthermore, inhibition of endogenous miR-30b using anti-miR-30b increased luciferase activity after transfection and no significant difference was observed. There were also no apparent changes in the luciferase activities when the 3'-UTR was mutated (Fig. 5D).

DISCUSSION

PD is an accepted form of renal replacement therapy (RRT) for patients with ESRD [15]. Frequently, conventional PDFs with high glucose levels are used to maintain the fluid balance in patients in PD programs. Much evidence implicates repeated exposure to high glucose and GDPs, such as MGO, as the major causes of PM deterioration [16]. Hirahara et al. reported that MGO could induce peritoneal fibrosis thickening with the proliferation of mesenchymal-like mesothelial cells, which might be transdifferentiated from mesothelial cells by

EMT in vivo [13]. In our animal model, which used treatment with 20 mM MGO for 4 weeks, peritoneal expression of E-cadherin decreased significantly whereas α-SMA increased markedly, suggesting that MGO might induce the EMT of PMC in rats. However, the exact mechanisms of the EMT process and the peritoneal fibrosis later induced by MGO remain unclear.

MiRNAs constitute a robust regulatory network with post-transcription regulatory efficiency for almost one half of human coding genes [17]. Alterations in miRNA expression profiles could be useful in the prediction of the individual response to variant injury factors and could even be potential targets for medication. Many previous studies have shown that miRNAs are thought to interact with multiple mRNAs that are involved in the EMT process [18, 19]. However, the molecular mechanism for which miRNA induces the peritoneal EMT response has rarely been reported on. In order to identify key regulatory miRNAs in the EMT of PMCs, we argued that it would be necessary to examine miRNA expression patterns in an unbiased manner. In this study, to explore the functional roles of miRNAs that might correlate with EMT in PMCs, we profiled the differences in peritoneal miRNA expression between rats injected with MGO and control rats. MiRNA microarray analysis revealed that the most notable of the upregulated miRNAs were miR-136, miR-703, miR-30b and miR-107, with the greatest increase shown by miR-30b after weeks 1 and 2.

Many researchers indicated that miRNA ASO could effectively induce target miRNA silencing when administered intraperitoneally in mice [20, 21]. To determine the possible role of miR-30b in the EMT of PMCs, we focused on miR-30b ASO for further study by intraperitoneal injection in MGO-treated rats. After 2 weeks of miR-30b ASO injections, the peritoneal expression of E-cadherin had increased significantly, whereas the α-SMA expression decreased significantly accompanied by peritoneal miR-30b downregulation, which meant that miR-30b might be involved in MGO-induced EMT of PMCs in rats.

To the best of our knowledge, this is the first report that proves treatment with miRNA ASO is likely to contribute to the alleviation of EMT of PMC in vivo and to prevent peritoneal damage induced by MGO. Mechanistically, the miR-30b–ASO-mediated alleviation of EMT might be explained by upregulation of modulator(s) possibly targeted by miR-30b, or alternatively, by the downregulation of cytokine(s) that bring about EMT directly or indirectly. The possible roles of modulators associated with miR-30b in the EMT of PMC need to be explored in depth.

BMP-7, an endogenous factor, is a member of the transforming growth factor-β (TGF-β) superfamily that negatively regulates EMT and prevents fibrosis in the kidney, liver and heart [22–24]. It has also been demonstrated that PMCs constitutively express BMP-7, which is able to block and reverse high glucose-induced EMT of PMCs in vitro [16, 25]. Our study revealed that 4 weeks after MGO injection, peritoneal BMP-7 expression of rats was significantly downregulated accompanied with peritoneal EMT. The role of miRNAs might be a possible mechanism for the precise regulation of peritoneal BMP-7. Our

miRNA microarray analysis revealed that 4 miRNAs (miR-136, miR-703, miR-30b and miR-107) were significantly upregulated during peritoneal EMT. The bioinformatic assessment showed that miR-30b was the predicted miRNA target site in *BMP7* 3'-UTRs according to Targetscan (Release 6.2), suggesting its possible role in the regulation of the gene *BMP7*. The in vitro study showed that miR-30b directly targeted and inhibited *BMP7* in rat PMCs. Thus the influence of miR-30b on peritoneal EMT is likely to be through controlling the expression of the *BMP7* gene.

It is well known that TGF-β1 is one of the key growth factors involved in EMT and the pathological process of peritoneal fibrosis [26]. BMP-7 could antagonize TGF-β1-induced EMT of PMC in vitro and protect against PM worsening in a rat model of PD fluid exposure [16]. In our study, due to peritoneal upregulation of miR-30b, which proved to target *BMP7* in vitro, BMP-7 expression significantly decreased. The consequence was that TGF-β1 cannot be effectively antagonized and offset by BMP-7. This may be one of the mechanisms to explain the link between MGO and the EMT of PMC.

It is known that regulation of miRNAs is affected by many factors. Individual miRNA can pleiotropically regulate the expressions of many genes, whereas one gene can be regulated by multiple miRNAs. There are different viewpoints about the relationship between the miR-30 family and the EMT of PMCs. A recent paper reported that miR-30a negatively regulated TGF-β1-induced EMT and peritoneal fibrosis by targeting Snai1 [27]. MGO in the present study and TGF-β1 are two different regulators that induced changes in the peritoneal membrane [13, 28]. Regarding their roles as two different regulators, they may modify the membrane-inducing changes in miRNA expression or induce changes in miRNA expression, which then affects the membrane. Either way, the assumed target with respect to miR-30 has to be fully investigated.

In our study, miR-30b targets *BMP7*, which could antagonize the effects of TGF-β1. The study mentioned above showed that miR-30a could target Snai1, which is an important transcript factor of EMT, by inhibiting an E-box gene, such as E-cadherin [29, 30]. Thus, the regulatory effects of miRNAs are different for different factors.

Our combined results indicate the potential relationship between miR-30b and peritoneal EMT in MGO-injected rats, which leads to a better understanding of the possible mechanisms underlying peritoneal fibrosis. Mechanistically, binding of miR-30b to the coding region of *BMP7* acts as negative regulator and causes a lack in BMP-7 that might be available for the antagonizer of TGF-β1-induced EMT. In summary, we provide evidence about the feasibility of considering the miRNAs in EMT of PMC as a therapeutic target to ameliorate PM deterioration in PD patients.

Conflicts of interest. The authors declare that there are no conflicts of interest.

REFERENCES

1. Williams, J.D., Craig, K.J., Topley, N., Von Ruhland, C., Fallon, M., Newman, G.R., Mackenzie, R.K. and Williams, G.T. Morphologic changes in the peritoneal membrane of patients with renal disease. **J. Am. Soc. Nephrol.** 13 (2002) 470–479.
2. Plum, J., Hermann, S., Fusshöller, A., Schoenicke, G., Donner, A., Röhrborn, A. and Grabensee, B. Peritoneal sclerosis in peritoneal dialysis patients related to dialysis settings and peritoneal transport properties. **Kidney. Int.** Suppl 78 (2001) S42–S47.
3. Kihm, L.P., Wibisono, D., Müller-Krebs, S., Pfisterer, F., Morath, C., Gross, M.L., Morcos, M., Seregin, Y., Bierhaus, A., Nawroth, P.P., Zeier, M. and Schwenger, V. RAGE expression in the human peritoneal membrane. **Nephrol. Dial. Transplant.** 23 (2008) 3302–3306.
4. Hirahara, I., Kusano, E., Yanagiba, S., Miyata, Y., Ando, Y., Muto, S. and Asano, Y. Peritoneal injury by methylglyoxal in peritoneal dialysis. **Perit. Dial. Int.** 26 (2006) 380–392.
5. Oh, E.J., Ryu, H.M., Choi, S.Y., Yook, J.M., Kim, C.D., Park, S.H., Chung, H.Y., Kim, I.S., Yu, M.A., Kang, D.H. and Kim, Y.L. Impact of low glucose degradation product bicarbonate/lactate-buffered dialysis solution on the epithelial-mesenchymal transition of peritoneum. **Am. J. Nephrol.** 31 (2010) 58–67.
6. Li, Y., Yang, J., Dai, C., Wu, C. and Liu, Y. Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. **J. Clin. Invest.** 112 (2003) 503–516.
7. Yáñez-Mó, M., Lara-Pezzi, E., Selgas, R., Ramírez-Huesca, M., Domínguez-Jiménez, C., Jiménez-Heffernan, J.A., Aguilera, A., Sánchez-Tomero, J.A., Bajo, M.A., Alvarez, V., Castro, M.A., del Peso, G., Cirujeda, A., Gamallo, C., Sánchez-Madrid, F. and López-Cabrera, M. Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. **N. Engl. J. Med.** 48 (2003) 403–413.
8. Yang, A.H., Chen, J.Y. and Lin, J.K. Myofibroblastic conversion of mesothelial cells. **Kidney Int.** 63 (2003) 1530–1539.
9. Pillai, R.S., Bhattacharyya, S.N. and Filipowicz, W. Repression of protein synthesis by miRNAs: How many mechanisms? **Trends Cell. Biol.** 17 (2007) 118–126.
10. Khvorova, A., Reynolds, A. and Jayasena, S.D. Functional siRNAs and miRNAs exhibit strand bias. **Cell** 115 (2003) 209–216.
11. Zhang, K., Zhang, H., Zhou, X., Tang, W.B., Xiao, L., Liu, Y.H., Liu, H., Peng, Y.M., Sun, L. and Liu, F.Y. miRNA589 regulates epithelial-mesenchymal transition in human peritoneal mesothelial cells. **J. Biomed. Biotechnol.** 2012;2012:673096. doi: 10.1155/2012/673096. Epub 2012 Oct 3
12. Tang, O., Chen, X.M., Shen, S., Hahn, M. and Pollock, C.A. miRNA-200b represses transforming growth factor beta1-induced EMT and fibronectin

expression in kidney proximal tubular cells. **Am. J. Physiol. Renal. Physiol.** 304 (2013) F1266–F1273.
13. Hirahara, I., Ishibashi, Y., Kaname, S., Kusano, E. and Fujita, T. Methylglyoxal induces peritoneal thickening by mesenchymal-like mesothelial cells in rats. **Nephrol. Dial. Transplant.** 24 (2009) 437–447.
14. Yang, X., Ye, R., Kong, Q., Yang, Q., Dong, X. And Yu , X. CD40 is expressed on rat peritoneal mesothelial cells and upregulates ICAM-1 production. **Nephrol. Dial. Transplant.** 19 (2004) 1378–1784.
15. Grassmann, A., Gioberge, S., Moeller, S. and Brown G. ESRD patients in 2004: Global overview of patient numbers, treatment modalities and associated trends. **Nephrol. Dial. Transplant.** 20 (2005) 2587–2593.
16. Loureiro, J., Schilte, M., Aguilera, A., Albar-Vizcaíno, P., Ramírez-Huesca, M., Pérez-Lozano, M.L., González-Mateo, G., Aroeira, L.S., Selgas, R., Mendoza, L., Ortiz, A., Ruíz-Ortega, M., van den Born, J., Beelen, R.H. and López-Cabrera, M. BMP-7 blocks mesenchymal conversion of mesothelial cells and prevents peritoneal damage induced by dialysis fluid exposure. **Nephrol. Dial. Transplant.** 25 (2010) 1098–1108.
17. Slaby, O., Svoboda, M., Michalek, J. and Vyzula, R. Micrornas in colorectal cancer: Translation of molecular biology into clinical application. **Mol. Cancer** 8 (2009) 102.
18. Acloque, H., Thiery, J.P. and Nieto, M.A. The physiology and pathology of the EMT. Meeting on the epithelial-mesenchymal transition. **EMBO Rep.** 9 (2008) 322–326.
19. Korpal, M. and Kang, Y. The emerging role of miR-200 family of microRNAs in epithelial-mesenchymal transition and cancer metastasis. **RNA Biol.** 5 (2008) 115–119.
20. Long, J., Wang, Y., Wang, W., Chang, B.H. and Danesh, F.R. MicroRNA-29c is a signature microRNA under high glucose conditions that targets Sprouty homolog 1, and its in vivo knockdown prevents progression of diabetic nephropathy. **J. Biol. Chem.** 286 (2011) 11837–11848.
21. Gebeshuber, C.A., Kornauth, C., Dong, L., Sierig, R., Seibler, J., Reiss, M., Tauber, S., Bilban, M., Wang, S., Kain, R., Böhmig, G.A., Moeller, M.J., Gröne, H.J., Englert, C., Martinez, J. and Kerjaschki, D. Focal segmental glomerulosclerosis is induced by microRNA-193a and its downregulation of WT1. **Nat. Med.** 19 (2013) 481–487.
22. Kalluri, R. and Neilson, E.G. Epithelial-mesenchymal transition and its implications for fibrosis. **J. Clin. Invest.** 112 (2003) 1776–1784.
23. Wang, S., Chen, Q., Simon, T.C., Strebeck, F., Chaudhary, L., Morrissey, J., Liapis, H., Klahr, S. and Hruska, K.A. Bone morphogenic protein-7 (BMP-7), a novel therapy for diabetic nephropathy. **Kidney Int.** 63 (2003) 2037–2049.
24. Zeisberg, M., Hanai, J., Sugimoto, H., Mammoto, T., Charytan, D. Strutz, F., and Kalluri, R. BMP-7 counteracts TGF-beta1- induced epithelial-to-mesenchymal transition and reverses chronic renal injury. **Nat. Med.** 9 (2003) 964–968.

25. Yu, M.A., Shin, K.S., Kim, J.H., Kim,Y.I., Chung, S.S., Park, S.H., Kim, Y.L. And Kang, D.H. HGF and BMP-7 ameliorate high glucose- induced epithelial-to-mesenchymal transition of peritoneal mesothelium. **J. Am. Soc. Nephrol.** 20 (2009) 567–581.
26. Margetts, P.J. and Bonniaud, P. Basic mechanisms and clinical implications of peritoneal fibrosis. **Perit. Dial. Int.** 23 (2003) 530–541.
27. Zhou, Q., Yang, M., Lan, H. and Yu, X. miR-30a negatively regulates TGF-β1-induced epithelial-mesenchymal transition and peritoneal fibrosis by targeting Snai1. **Am. J. Pathol.** 183 (2013) 808–819.
28. Margetts, P.J., Kolb, M., Galt, T., Hoff, C.M., Shockley, T.R. and Gauldie, J. Gene transfer of transforming growth factor-beta1 to the rat peritoneum: effects on membrane function. **J. Am. Soc. Nephrol.** 12 (2001) 2029–2039.
29. Vesuna, F., van Diest, P., Chen, J.H. and Raman, V. Twist is a transcriptional repressor of E-cadherin gene expression in breast cancer. **Biochem. Biophys. Res. Commun.** 367 (2008) 235–241.
30. Barrallo-Gimeno, A. and Nieto, M.A. The Snail genes as inducers of cell movement and survival: implications in development and cancer. **Development** 132 (2005) 3151–3161.