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Periostin-binding DNA aptamer treatment ameliorates peritoneal dialysis-induced peritoneal fibrosis

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Periostin-binding DNA aptamer treatment ameliorates peritoneal dialysis-induced peritoneal fibrosis

Directed by Professor Shin-Wook Kang

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Young Eun Kwon

June 2016

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YONSEI UNIVERSITY

<TABLE OF CONTENTS>

ABSTRACT 1
Ⅰ. INTRODUCTION 4
Ⅱ. MATERIALS AND METHODS 8
1. Primary culture of HPMCs 8
2. Treatment of HPMCs 8
3. Periostin-binding DNA aptamer 9
4. Animal experiments 9
5. Total RNA extraction 10
6. Reverse transcription 10
7. Quantitative real-time polymerase chain reaction (PCR) 11
8. Western blot analysis 14
9. Immunohistochemical staining and Masson's trichrome staining 14
10. Statistical analysis 15
Ⅲ. RESULTS 16
1. TGF-β1 up-regulates periostin expression and mediates EMT in HPMCs 16
2. Periostin knockdown abrogates TGF-β1-induced EMT in HPMCs 19
3. Confirmation of DNA aptamer binding to periostin in HPMCs 23
4. Periostin-binding DNA aptamer ameliorates TGF-β1-induced EMT in HPMCs 26
5. Periostin-binding DNA aptamer prevents peritoneal fibrosis in PD mouse models 30
Ⅳ. DISCUSSION 35
Ⅴ. CONCLUSION 39
REFERENCES 41

연세대학교
YONSEI UNIVERSITY

ABSTRACT (IN KOREAN).....47

연세대학교
YONSEI UNIVERSITY

LIST OF FIGURES

Figure 1. Periostin and EMT-related molecules expression in HPMCs exposed to TGF-β1 17

Figure 2. Cell morphology, and mRNA and protein expression changes of EMT-related molecules following periostin siRNA transfection 20

Figure 3. Attachment of periostin-binding DNA aptamer specific to periostin expressed in HPMCs..... 24

Figure 4. Cell morphology, and mRNA and protein expression changes of EMT-related molecules following periostin-binding DNA aptamer treatment..... 27

Figure 5. Masson's trichrome staining and immunohistochemical staining of fibronectin, E-cadherin, α-SMA, and Snail in the peritoneum from control, control+periostin aptamer, PD, and PD+periostin aptamer mice..... 31

Figure 6. Periostin and EMT-related molecules expression in the peritoneum from control, control+periostin aptamer, PD, and PD+periostin aptamer 33

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YONSEI UNIVERSITY

LIST OF TABLE

Table 1. Primer sequences..... 12

연세대학교
YONSEI UNIVERSITY

ABSTRACT

Periostin-binding DNA aptamer treatment ameliorates peritoneal dialysis-induced peritoneal fibrosis

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(Directed by Professor Shin-Wook Kang)

Background: Peritoneal fibrosis is a major complication, leading to peritoneal structural changes and ultrafiltration failure in peritoneal dialysis (PD) patients. The epithelial-mesenchymal transition (EMT) of the peritoneal mesothelial cells (PMCs) and peritoneal accumulation of extracellular matrix (ECM) proteins are key features of peritoneal fibrosis in which transforming growth factor (TGF)-β1 is known to play a crucial role. Periostin, an ECM protein increased by the activation of the TGF-β1 pathway, induces the expression of ECM genes, such as collagen and fibronectin, by activating integrin-related intracellular signaling pathways. Accumulating evidence has suggested that periostin expression is related to the development of various fibrotic disorders. Recently, a number of studies have demonstrated that aberrant periostin expression is accompanied in PD-related peritoneal fibrosis. Therefore,

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regulating the expression and function of periostin in human peritoneal mesothelial cells (HPMCs) may have an effect on the development and progression of peritoneal fibrosis in PD patients.

Purpose: This study was undertaken to evaluate the impact of periostin inhibition by a novel aptamer-based inhibitor on TGF-β1-induced EMT in cultured HPMCs. In addition, the effects of the periostin-binding aptamer on EMT and peritoneal fibrosis were also investigated in an animal model of PD.

Methods and Materials: To regulate the functional role of periostin in HPMCs, periostin-binding DNA aptamer that was generated to target human periostin was used. For *in vitro* studies, primary HPMCs were treated with TGF-β1 (2 ng/ml) to induce EMT. The role of periostin in TGF-β1-induced EMT in cultured HPMCs was evaluated by periostin siRNA transfection (100 nM). To validate whether periostin-binding DNA aptamer specifically targets periostin, the fluorescence intensity of Cy3-tagged periostin-binding DNA aptamer was assessed by fluorescence-activated cell sorting analysis. The effect of regulating periostin function through periostin-binding DNA aptamer on EMT and fibrosis was also evaluated by treating TGF-β1-stimulated HPMCs with periostin-binding DNA aptamer. *In vivo*, PD catheters were inserted into 48 C57BL/6 mice, and these mice were infused intraperitoneally for 4 weeks with either saline (C group, n=12), saline with periostin-binding DNA aptamer (C+periostin aptamer group, n=12), 4.25% PD solution (PD group, n=12), or 4.25% PD solution with periostin-binding DNA aptamer (PD+periostin aptamer group, n=12). After 4 weeks, the mice were sacrificed and the peritoneal tissues were removed. The mRNA levels and protein expression of periostin, fibronectin, α-smooth muscle actin (α-SMA), Snail, and E-cadherin in HPMCs and the mouse peritoneum were evaluated by quantitative real-time polymerase chain reaction and western blotting, respectively. Peritoneal fibrosis was assessed by Masson's trichrome staining.

Results: *In vitro*, TGF-β1 treatment significantly up-regulated periostin, fibronectin, α-SMA and Snail expression, while it reduced E-cadherin expression in HPMCs ($P < 0.01$). When Cy3-tagged periostin-binding DNA aptamer was introduced to HPMCs,

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the Cy3 fluorescence activity was significantly increased in TGF-β1-stimulated HPMCs. In contrast, the activity was significantly decreased in periostin-knocked down HPMCs. Periostin siRNA treatment ameliorated TGF-β1-induced periostin, fibronectin, α-SMA, and Snail expression and restored E-cadherin expression in HPMCs ($P < 0.05$). Similarly, periostin-binding DNA aptamer also attenuated fibronectin, α-SMA, and Snail up-regulation and E-cadherin down-regulation in TGF-β1-stimulated HPMCs ($P < 0.05$). *In vivo*, in mice treated with PD solution for 4 weeks, the expression of periostin, fibronectin, α-SMA, and Snail was significantly increased in the peritoneum, while the E-cadherin expression was significantly decreased ($P < 0.05$). The thickness of submesothelial layer and the intensity of Masson's trichrome staining of the PD group were significantly increased compared to the C group. These changes were significantly abrogated by the intraperitoneal administration of periostin-binding DNA aptamer.

Conclusion: These findings suggest that periostin-binding DNA aptamer can be a potential therapeutic strategy for peritoneal fibrosis in PD patients.

Key words: Periostin, transforming growth factor-β1, peritoneal dialysis, peritoneal fibrosis, aptamer, epithelial-mesenchymal transition

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Periostin-binding DNA aptamer treatment ameliorates peritoneal dialysis-induced peritoneal fibrosis

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Ⅰ. INTRODUCTION

Peritoneal dialysis (PD) is a commonly used renal replacement modality to treat end-stage renal disease patients. PD is characterized by its ability to be applied in hemodynamically unstable patients, to remove high-molecular-weight solutes more efficiently than hemodialysis, and to provide home-based and self-care treatment environments. However, long-term PD leads to peritoneal fibrosis in most of the patients receiving PD[1]. The progression of peritoneal fibrosis invariably causes membrane failure, leading to the failure in volume control and loss of dialysis adequacy and consequently resulting in the discontinuation of PD. Several risk factors have been demonstrated to be associated with peritoneal fibrosis; bio-incompatible PD solutions with low pH or lactate buffer, chronic exposure to high glucose-based dialysates, and recurrent peritonitis[2-4]. However, the detailed molecular mechanisms underlying peritoneal fibrosis development and progression have not been fully elucidated. Therefore, strategies to prevent peritoneal fibrosis and to treat established

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peritoneal fibrosis are limited.

The peritoneal membrane is covered by a single layer of peritoneal mesothelial cells (PMCs), which are supported by a submesothelial connective tissue area composed of fibroblasts, mast cells, and macrophages[5]. Chronic mechanical, chemical, and infectious insults lead to the loss of mesothelial cells, angiogenesis, and submesothelial layer thickening of the peritoneum[6,7], and peritoneal fibroblasts and inflammatory cells have been recognized to be involved in the development of peritoneal fibrosis. Recently, however, PMCs have been suggested to play a key role in the development and progression of peritoneal fibrosis by producing type I and type II collagen and fibronectin[8]. The synthesis of these extracellular matrix (ECM) proteins by PMCs is accompanied by a complex mechanism called the epithelial-mesenchymal transition (EMT)[9]. EMT is a reversible process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become myofibroblast-like cells[2]. In addition, EMT-induced myofibroblast-like cells produce ECM proteins in response to various stimuli, contributing to organ fibrosis in multiple disease entities[10,11].

EMT of PMCs and their excessive production of ECM proteins are known to be largely attributed to the activation of the transforming growth factor (TGF)-β1 pathway[12]. Binding of TGF-β1 to its receptor activates SMAD-induced transcription, resulting in the induction of mesenchymal markers, such as α-smooth muscle actin (α-SMA), fibronectin, and Snail, and the suppression of epithelial markers, including E-cadherin[13]. The PD fluid is known to increase the production of TGF-β1 in PMCs[14], and the high glucose in the peritoneal dialysate has been proven to be one of the main causes of this up-regulation of TGF-β1 in PMCs. The activation of the TGF-β1 pathway in PMCs is considered the main denominator leading to peritoneal fibrosis in PD patients[15].

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Periostin is an ECM protein and is known to be up-regulated by the activation of the TGF-β1 pathway in several cell types[16]. It functions as a ligand of α_V/β_3 and α_V/β_5 integrin, activating integrin-related intracellular signaling pathways and resulting in the increased transcription of ECM genes, such as collagen and fibronectin[17]. Periostin has been shown to play an important role in the pathogenesis of developmental diseases[18], wound healing processes[19], and bone differentiation[20]. Notably, periostin expression was found to be linked to cell growth and metastasis through promoting EMT in cancer cells[21]. Recently, moreover, periostin levels in PD fluid were demonstrated to be up-regulated in patients with encapsulated peritoneal sclerosis, proposing a possibility that periostin contributed to peritoneal fibrosis in patients undergoing PD[22].

Aptamers are single-stranded oligonucleotides that bind to specific target molecules. Once bound to specific targets, aptamers inhibit their activities[23]. Monoclonal antibodies have been applied in the clinical field to suppress the activity of specific proteins. However, using antibodies to treat diseases is accompanied by some critical limitations, such as low bio-stability, the induction of undesirable immune responses, and high production costs. In contrast, aptamers are produced chemically and are relatively bio-stable, enabling their production costs to be lower than antibodies and allowing their storage and distribution to be more convenient[23,24]. They are also less immunogenic, and therefore possess fewer risks of developing hypersensitivity reactions. Due to these advantages, aptamers have been proposed as promising drug candidates to inhibit specific target molecules in various diseases. Animal studies as well as clinical research on aptamers targeting human immunodeficiency virus (HIV) trans-acting responsive element, interferon ɣ, and vascular endothelial growth factor are ongoing to evaluate their potential as therapeutics for several diseases, including HIV infection and age-related macular degeneration[25,26]. Furthermore, a periostin-binding DNA aptamer was recently identified to suppress breast cancer growth and metastasis by inhibiting EMT in breast

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cancer cells[27].

Based on these findings, it was hypothesized that periostin may play a critical role in TGF-β1-induced EMT in PD-related peritoneal fibrosis, and periostin-binding DNA aptamer may abrogate PD-induced peritoneal fibrosis by inhibiting the activity of periostin in PMCs. In this study, the effects of periostin-binding DNA aptamer on TGF-β1-induced EMT and ECM accumulation were evaluated using human peritoneal mesothelial cells (HPMCs) and an animal model of PD.

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Ⅱ. MATERIALS AND METHODS

1. Primary culture of HPMCs

Primary HPMCs were isolated in accordance with the process stated by Stylianou et al[28]. Human omenta specimens from patients who had undergone abdominal surgery and agreed to provide consent were obtained. The specimens were washed three times using sterile phosphate-buffered saline (PBS) and incubated in a solution containing 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) for 20 min at 37°C, with continuous shaking. After an incubation period, free HPMC-containing suspension was centrifuged at 100 g for 10 min at 4°C. The supernatant was removed, and the pellet was washed once. Subsequently, the pellet was re-suspended into M199 media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 26 mM $NaHCO_3$, and seeded on cell culture dishes. The cells were cultured in the same media at 37°C in humidified 5% CO_2 in air. The media were exchanged 24 hr after seeding, and then every three days.

2. Treatment of HPMCs

Initially, subconfluent HPMCs were FBS restricted for 24 hr. Next, the media were exchanged to FBS-free M199 media for the control group and media with TGF-β1 (2 ng/ml) (R&D Systems, Minneapolis, MN, USA) for the intervention group. HPMCs were harvested for RNA and protein analyses 72 hr after media change. For periostin knockdown experiments, periostin siRNA was purchased from Dharmacon (Lafayette, CO, USA). Periostin siRNA was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 6 μl of Lipofectamine 2000 was diluted in 1 ml of Opti-MEM I Reduced Serum Medium (Invitrogen), incubated for 15 min at room temperature (RT), and mixed with periostin siRNA. After a 15-min incubation at RT, the mixture was added to each well of HPMCs, which were plated at a density of 5×10^5 cells/well into six-well plates the day before, and the medium was changed after 24 hr. The doses of TGF-β1 and periostin siRNA for this study were determined based on preliminary experimental

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results.

3. Periostin-binding DNA aptamer

Two kinds of periostin-binding DNA aptamers were purchased from Aptamer Sciences Inc. (Cat # 098-60-01; Pohang, South Korea). Cy3-labeled periostin DNA aptamer was used to evaluate the periostin-specific aptamer attachment in *in vitro* studies. Polyethylene glycol (PEG)-conjugated periostin-binding DNA aptamer that was designed to increase the *in vivo* bio-stability was applied for animal studies.

To evaluate the cell surface attachment specificity of periostin-binding DNA aptamer, fluorescence-activated cell sorting (FACS) analysis was performed. HPMCs were centrifuged at 500 g for 5 min and washed twice in an isotonic, calcium and magnesium free-PBS buffer to remove residual growth factors in the medium. A final concentration of 1×10^7 cells/ml was re-suspended and incubated with 200 nmol/l Cy3-labeled periostin DNA aptamer containing binding buffer (PBS including 5 mM $MgCl_2$). Samples of 100 µl were used per assay (1×10^6 cells) into each 1.5 ml tube for staining. Flow cytometric analysis of surface staining intensity was performed using an FACSVERSE system (BD Biosciences, Franklin Lakes, NJ, USA), and analysis of the results was conducted using FACSuite software (BD Biosciences).

4. Animal experiments

The protocols for animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University College of Medicine, Seoul, Korea. All of the animal experiments were conducted in accordance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1985)

Forty-eight male C57BL/6 mice weighing 20~25 g were used. For PD, PD catheter and ports (Cat # MMP-4S-061108A; Access Technologies & Solomon Scientific, Skokie, IL, USA) were inserted, and wounds were observed for one week. One week after catheter insertion, the control group received 2 ml of saline instillation

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once a day, and the PD group was infused intraperitoneally with 2 ml of peritoneal dialysate daily (Physioneal PD solution with 4.25% dextrose, pH 7.4; Baxter International, Deerfield, IL, USA) for 4 weeks. The aptamer group received 500 μg/kg/d of periostin-binding DNA aptamer mixed with PD solution to a final volume of 2 ml through the PD catheter daily. After 4 weeks of PD treatment, all of the mice were sacrificed, and the parietal peritoneum was collected.

5. Total RNA extraction

The RNA extraction methods from HPMCs and the peritoneum were described in a previous study[29]. Whole peritoneal samples were rapidly frozen using liquid nitrogen and homogenized by mortar and pestle thrice with 100 μl of RNA STAT-60 reagent (Tel-Test, Friendswood, TX, USA). For HPMCs' RNA extraction, 700 μl of RNA STAT-60 were added to the cell culture dish, and the suspension was collected. After homogenizing the suspension for 5 min at RT, 160 μl of chloroform was added. Next, the mixture was shaken vigorously for 30 sec, stored for 3 min at RT, and centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase located in the top of three phases was transferred to a fresh tube carefully not to be contaminated with the other phases. Extracted RNA was precipitated by adding 400 μl of isopropanol, and centrifuged at 12,000 g for 30 min at 4°C. The RNA pellet was washed with 70% ethanol, air-dried for 2 min, and dissolved in sterile DEPC-treated distilled water. The quantity and quality of extracted RNA were assessed by spectrophotometric measurements at wavelengths from 260 to 280 nm.

6. Reverse transcription

A Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany) was used to obtain first-strand cDNA. Reverse transcription was conducted using 2 μg of total RNA extracts with 10 μM random hexanuclotide primers, 1 mM dNTPs, 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris-HCl at pH 8.5, 0.2 mM dithiothreithol, 25 U RNAse inhibitor, and 40 U AMV reverse transcriptase. The

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mixture was incubated for 10 min at 30°C, and then for 1 hr at 42°C, followed by incubation for 5 min at 99°C for the inactivation of the enzyme.

7. Quantitative real-time polymerase chain reaction (PCR)

The primer sequences used in this study were described in Table 1. The RNAs used for amplification were 25 ng per reaction tube. Using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), a total volume of 20 µl mixture in each well was used containing 10 µl of SYBR Green PCR Master Mix (Applied Biosystems), 5 µl of cDNA, and 5 pmol sense and antisense primers. The primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. The PCR conditions were as follows: 35 cycles of denaturation for 30 min at 94.5°C, annealing for 30 sec at 60°C, and extension for 1 min at 72°C. Initial heating for 9 min at 95°C and final extension for 7 min at 72°C were performed for all PCR reactions. Each sample was run in triplicate in separate tubes and a control without cDNA was also run in parallel with each assay. After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. The cDNA content of each specimen was determined using a comparative C_T method with $2^{-\Delta\Delta CT}$. The results were given as the relative expression normalized to the expression of 18S rRNA and expressed in arbitrary units.

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Table 1. Primer sequences

		Sequence
Periostin	Sense	5’-GGCACCAAAAAGAAATACT-3’
(mouse and human)	Antisense	5’-GGAAGGTAAGAGTATAT-3’
Fibronectin	Sense	5’-TGACAACTGCCGTAGACCTGG-3’
(mouse)	Antisense	5’-TACTGGTTGTAGGTGTGGCCG-3’
Fibronectin	Sense	5’-AACCTACGGATGACTCGTGC-3’
(human)	Antisense	5’-TGAATCACATCTGAAATGACCAC-3’
E-cadherin	Sense	5’-TTTCTACAGCATCACCGGC-3’
(mouse)	Antisense	5’-CACTTTCAGCCAGCCTGTC-3’
E-cadherin	Sense	5’-CACAGACGCGGACGATGAT-3’
(human)	Antisense	5’-AGGATCTTGGCTGAGGATGGT-3’
α-SMA	Sense	5’-CTGACAGAGGCACCACTGAA-3’
(mouse)	Antisense	5’-CATCTCCAGAGTCCAGCACA-3’
α-SMA	Sense	5’-GCCTTGGTGTGTGACAATGG-3’
(human)	Antisense	5’-AAAACAGCCCTGGGAGCAT-3’

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		Sequence
Snail (mouse)	Sense	5'-ATCCTCACCTCGGGAGCATAC-3'
	Antisense	5'-AGGCCACTGGGTAAAGGAGAGT-3'
Snail (human)	Sense	5'-ATCCTCACCTCGGGAGCATAC-3'
	Antisense	5'-AGGCCACTGGGTAAAGGAGAGT-3'
18S (mouse)	Sense	5'-AACTAAGAACGGCCATGCAC-3'
	Antisense	5'-CCTGCGGCTTAATTTGACTC-3'
18S (human)	Sense	5'-TGGTGCATGGCCGTTCT-3'
	Antisense	5'-CATGCCAGAGTCTCGTTCGTT-3'

Abbreviation: α-SMA, α-smooth muscle actin

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8. Western blot analysis

Harvested cultured cells and the mouse peritoneum were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mM Tris-HCl at pH 6.8, 10% (vol/vol) glycerol]. Lysates were centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was stored at -70°C. Protein concentrations were determined using the Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Laemmli sample buffer was added to aliquots of 50 μg of the protein extracts, which were heated for 5 min at 100°C and electrophoresed in acrylamide denaturating SDS-polyacrylamide gel. A Hybond-ECL membrane was used to transfer protein using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA). After the protein was transferred to the membrane, it was incubated in blocking buffer A (1 x PBS, 0.1% Tween-20, and 5% nonfat milk) for 1 hr at RT, and then incubated overnight at 4°C in a 1:500 dilution of polyclonal antibodies to periostin (Abcam, Cambridge, UK), E-cadherin (BD Bioscience), α-SMA (Abcam), fibronectin (Dako, Glostrup, Denmark), Snail (Abcam), or β-actin (Sigma Chemical Co., Perth, Australia). The membranes were washed thrice for 10 min in 1 x PBS with 0.1% Tween-20 and incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The washing process was repeated thrice, and the membranes were developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL, USA). TINA image software (Raytest, Straubenhardt, Germany) was used to measure the band densities, and the changes in the optical densities of bands from the treated groups relative to control cells or tissues were used for analysis.

9. Immunohistochemical staining and Masson's trichrome staining

To fix the peritoneum samples, 10% neutral-buffered formalin was used. Paraffin-embedded tissues were processed in 5 μm-thick sections for immunohistochemical staining. Tissue sections were deparaffinized, rehydrated in

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ethyl alcohol, and washed in tap water. Antigen retrieval was performed in 10 mM sodium citrate buffer for 20 min using a Black & Decker vegetable steamer. Slides were blocked with 10% donkey serum for 30 min at RT and then washed using PBS. Primary antibodies for fibronectin, E-cadherin, α-SMA, and Snail were diluted to the proper concentrations with 2% casein in bovine serum albumin, added to the slides, and then incubated overnight at 4°C. After washing, the slides were treated with a secondary antibody for 1 hr at RT. Diaminobenzidine was added for 2 min, and hematoxylin was used to counterstain the slides. For Masson's trichrome staining, paraffin-embedded tissues processed in 5 μm-thick sections were deparaffinized, rehydrated in ethyl alcohol, washed in tap water, and re-fixed in Bouin's solution for 1 hr at 56°C. After rinsing in running tap water for 10 min and staining with Weigert's iron hematoxylin working solution for 10 min, the slides were stained with Biebrich scarlet-acid fuchsin solution for 15 min and washed in tap water. The sections were differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min, transferred to aniline blue solution, and stained for 10 min. After rinsing briefly in tap water, the slides were reacted with 1% acetic acid solution for 5 min. A semi-quantitative score for staining intensity was assessed by examining at least 5 fields in each section under x 400 magnification and with digital image analysis (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA, USA).

10. Statistical analysis

Statistical analyses were conducted using SPSS for Windows version 21 (IBM Corporation, Armonk, NY, USA). Data are expressed as means ± standard errors of the mean (S.E.). One-way ANOVA with *post-hoc* test using Bonferonni's correction was used for multiple comparisons. A P value less than 0.05 was considered statistically significant.

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Ⅲ. RESULTS

1. TGF-β1 up-regulates periostin expression and mediates EMT in HPMCs

First, to verify that periostin is expressed during the EMT process of HPMCs, the expression of periostin was evaluated in HPMCs treated with TGF-β1. HPMCs treated with TGF-β1 (2 ng/ml) showed significantly increased expression of periostin mRNA and protein. This increase in periostin mRNA expression was accompanied by the up-regulation of fibronectin, α-SMA, and Snail and the down-regulation of E-cadherin mRNA expression. The protein levels of fibronectin, α-SMA, Snail, and E-cadherin showed a similar expression pattern to the mRNA expression in HPMCs (Fig. 1). These results indicate that TGF-β1 administration induces EMT in HPMCs along with a significant up-regulation of periostin.

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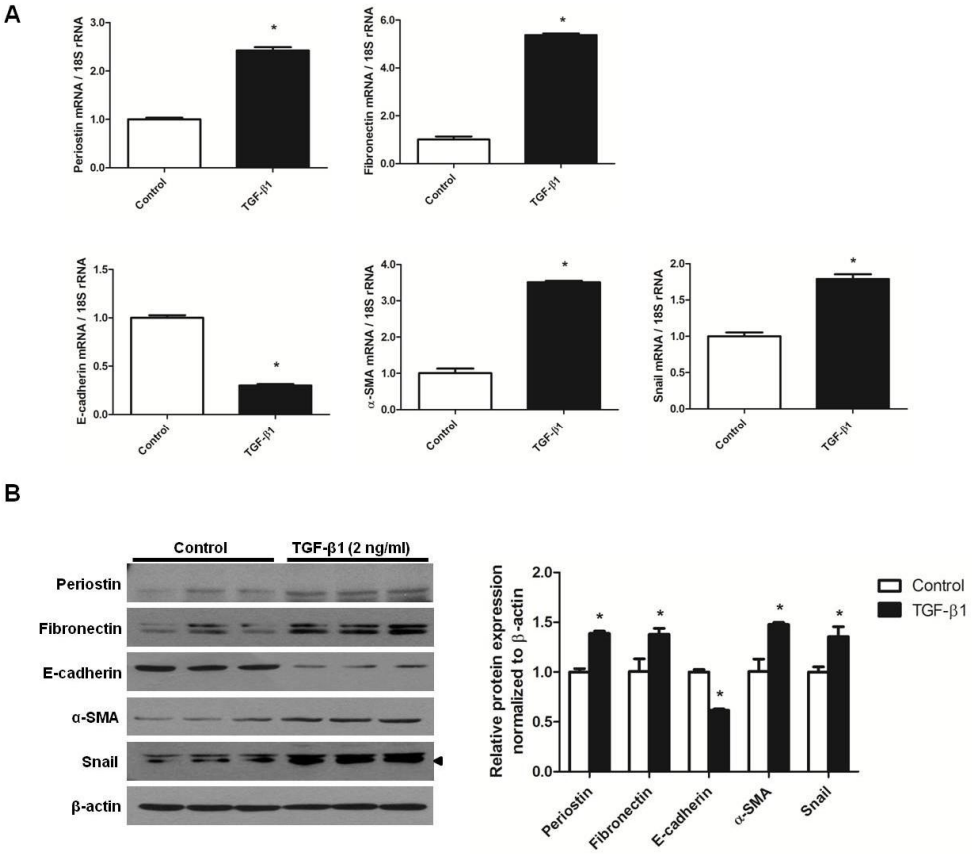

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Figure 1. Periostin and EMT-related molecules expression in HPMCs exposed to TGF-β1. (A) Periostin, fibronectin, E-cadherin, α-SMA, and Snail mRNA levels in HPMCs treated with or without TGF-β1 (2 ng/ml) (n=12). TGF-β1 significantly induced fibronectin, α-SMA, and Snail mRNA expression and significantly reduced E-cadherin mRNA level in HPMCs. Periostin mRNA expression was also significantly increased by TGF-β1. (B) A representative western blot analysis of periostin, fibronectin, E-cadherin, α-SMA, Snail, and β-actin protein expression in HPMCs treated with or without TGF-β1 (2 ng/ml) (a representative of 4 blots). The protein expression of periostin, fibronectin, α-SMA, and Snail were significantly increased, while E-cadherin protein expression was significantly decreased in TGF-β1-stimulated cells.

*;$P<0.01$

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2. Periostin knockdown abrogates TGF-β1-induced EMT in HPMCs

To evaluate the role of periostin in the EMT process of HPMCs, periostin expression was knocked down by siRNA transfection in TGF-β1-stimulated HPMCs. As shown in Figure 2A, TGF-β1 (2 ng/ml) provoked fibroblast-like cell morphological changes characterized by thin spindle-like cell shapes, in contrast to the cuboidal shape of unstimulated HPMCs. Interestingly, periostin siRNA (100 nM) administration remarkably ameliorated TGF-β1-induced cell morphology changes. This intriguing result prompted the examination of whether periostin knockdown normalized mesenchymal markers expression in HPMCs triggered by TGF-β1. As expected, periostin knockdown by siRNA effectively inhibited periostin expression under TGF-β1 treatment. Fibronectin, α-SMA, and Snail mRNA expression were also significantly decreased in HPMCs treated by TGF-β1 and periostin siRNA compared to HPMCs with TGF-β1 treatment alone, and decreased E-cadherin mRNA level in TGF-β1-stimulated HPMCs was significantly restored by periostin siRNA transfection. Regarding the protein levels, periostin siRNA significantly attenuated the increases in periostin, fibronectin, α-SMA, and Snail levels and the decrease in E-cadherin expression by TGF-β1 (Fig. 2). These results collectively suggest that periostin up-regulation is essentially involved in the EMT process of TGF-β1-stimulated HPMCs, and periostin inhibition can mitigate the pathologic EMT of HPMCs.

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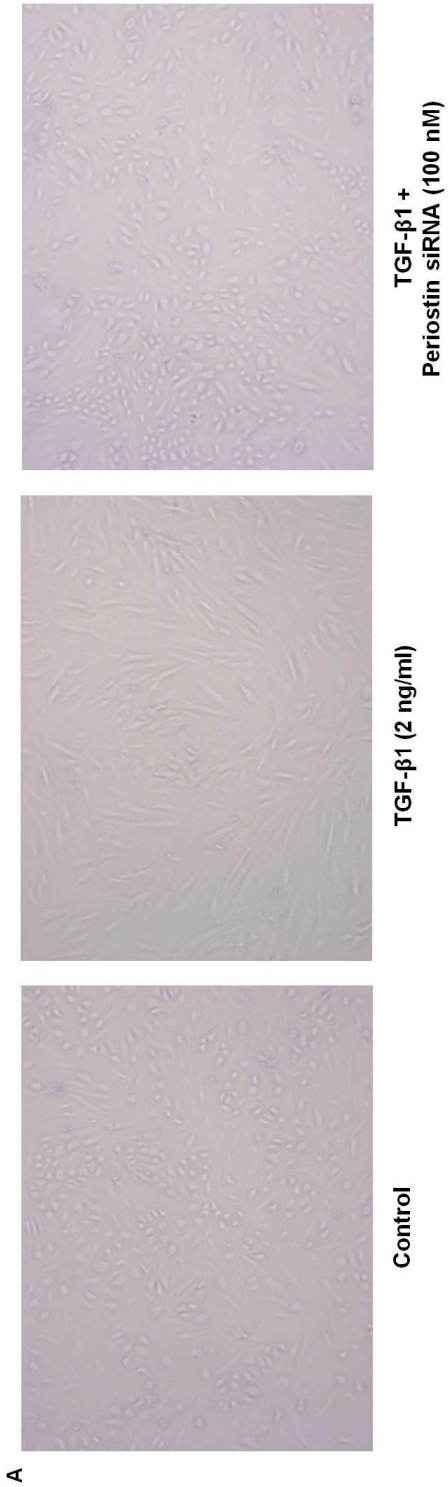

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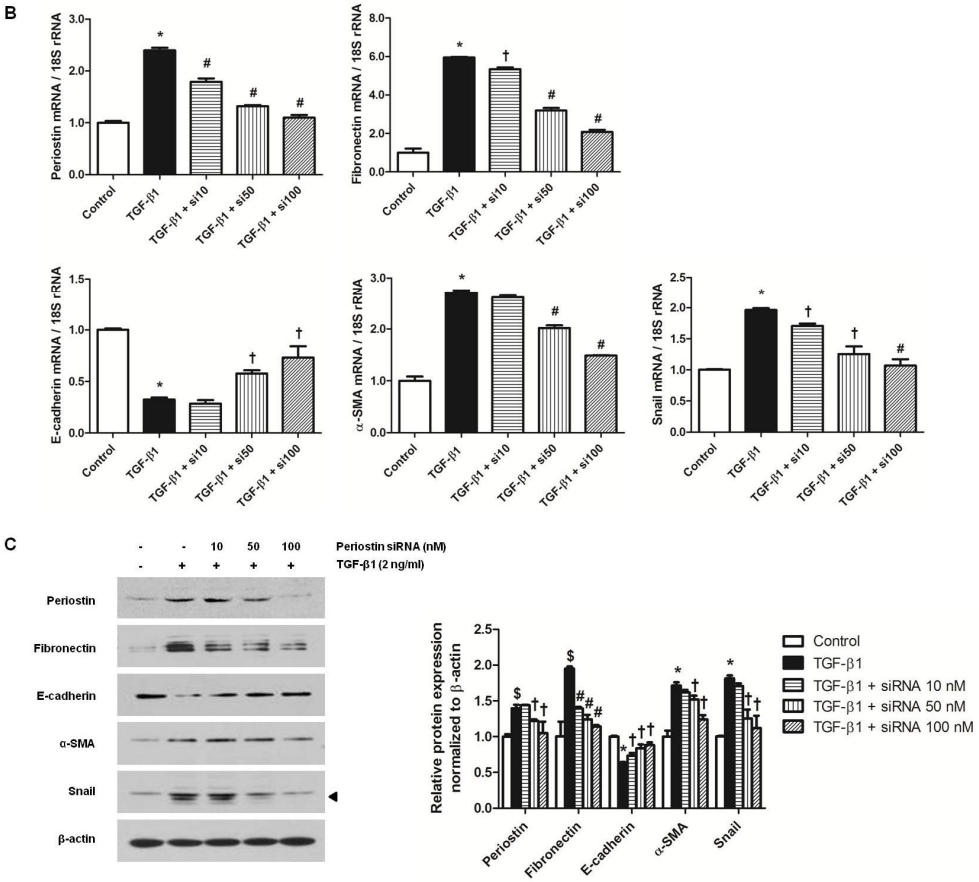

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Figure 2. Cell morphology, and mRNA and protein expression changes of EMT-related molecules following periostin siRNA transfection. (A) Cell morphology of control, and TGF-β1 (2 ng/ml)-stimulated HPMCs with or without periostin siRNA (100 nM) transfection. TGF-β1 treatment provoked fibroblast-like cell morphological changes in HPMCs, and periostin siRNA transfection reversed these TGF-β1-induced cell morphology changes (x 100). (B) Periostin, fibronectin, E-cadherin, α-SMA, and Snail mRNA levels in TGF-β1-stimulated HPMCs treated with or without periostin siRNA (n=12). Periostin siRNA significantly attenuated TGF-β1-induced increases in fibronectin, α-SMA, and Snail mRNA expression and restored the down-regulated E-cadherin mRNA level in HPMCs treated with TGF-β1. (C) A representative western blot analysis of periostin, fibronectin, E-cadherin, α-SMA, Snail, and β-actin protein expression in TGF-β1-stimulated HPMCs with or without periostin siRNA transfection (a representative of 4 blots). Periostin siRNA transfection significantly mitigated the increases in periostin, fibronectin, α-SMA, and Snail protein expression and the decrease in E-cadherin protein level in TGF-β1-stimulated HPMCs in a dose-dependent manner.

*;$P<0.01$ vs. control, $^{\#}$;$P<0.01$ vs. TGF-β1, †;$P<0.05$ vs. TGF-β1, $^{\$}$;$P<0.05$ vs. control

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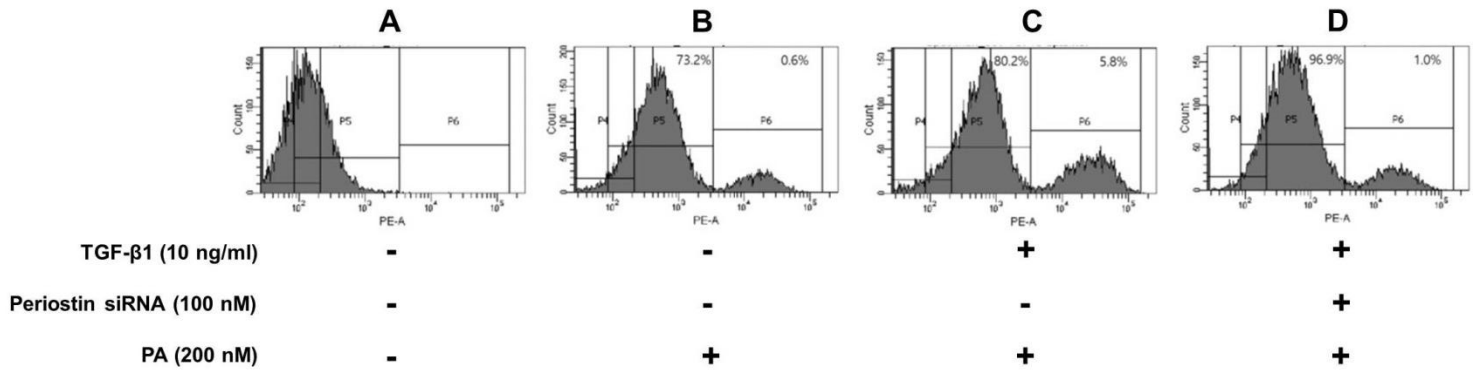
3. Confirmation of DNA aptamer binding to periostin in HPMCs

After confirming that periostin played a role in EMT induction in HPMCs, whether periostin-binding DNA aptamer could affect the EMT process in HPMCs was validated. First, FACS analysis was conducted to prove that periostin-binding DNA aptamer attaches specifically to periostin in HPMCs. Cy3-tagged periostin-binding DNA aptamer was used to check the amount of periostin-binding DNA aptamer attached to the periostin expressed in HPMCs. Compared to the negative control, Cy3-tagged periostin-binding DNA aptamer-treated cells showed an increased Cy3-positive fraction of the total HPMC count (0.6%). The Cy3-positive fraction was further increased to 5.8% in TGF-β1 (10 ng/ml)-stimulated HPMCs treated with Cy3-tagged periostin-binding DNA aptamer (200 nM), which was significantly abrogated by periostin siRNA (100 nM) transfection (1.0%). These findings suggest that the periostin-binding DNA aptamer specifically binds to periostin expressed in HPMCs (Fig. 3).


A
B
C
D
Count
PE-A
P4
P5
P6
73.2%
0.6%
80.2%
5.8%
96.9%
1.0%
TGF-β1 (10 ng/ml) - - + +
Periostin siRNA (100 nM) - - - +
PA (200 nM) - + + +

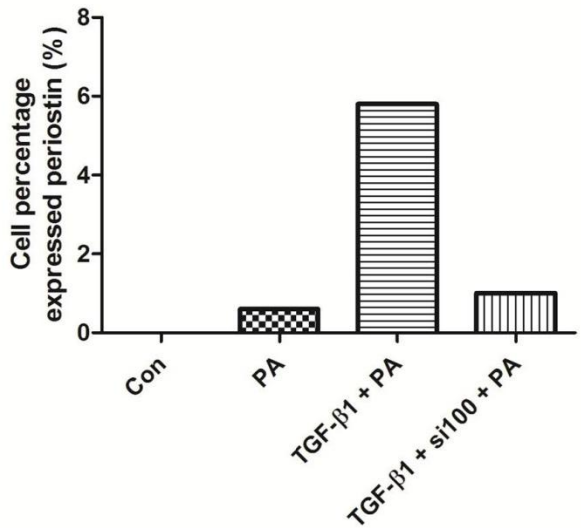

Cell percentage expressed periostin (%)
0
2
4
6
8
Con
PA
TGF-β1 + PA
TGF-β1 + si100 + PA

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Figure 3. Attachment of periostin-binding DNA aptamer specific to periostin expressed in HPMCs. FACS analysis was performed in HPMCs treated with or without TGF-β1 (10 ng/ml), periostin siRNA (100 nM), or Cy3-tagged periostin-binding DNA aptamer (200 nM). The Cy3-positive fraction was significantly increased in TGF-β1-stimulated HPMCs treated with Cy3-tagged periostin-binding DNA aptamer, which was significantly abrogated by periostin siRNA transfection.

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4. Periostin-binding DNA aptamer ameliorates TGF-β1-induced EMT in HPMCs

To investigate whether blocking periostin signaling with periostin-binding DNA aptamer could affect the EMT process, the changes in morphology and the expression of EMT-related molecules were explored after treatment with periostin-binding DNA aptamer in TGF-β1-stimulated HPMCs. A spindle shape feature of HPMCs exposed to TGF-β1 (2 ng/ml) was significantly alleviated by periostin-binding DNA aptamer treatment (Fig. 4), which was similar to the findings observed in TGF-β1-stimulated HPMCs transfected with periostin siRNA (Fig. 2). When the cells were examined for periostin expression, the increases in periostin mRNA and protein expression upon TGF-β1 stimulation were not significantly changed by the treatment with periostin-binding DNA aptamer. In contrast, the up-regulated fibronectin, α-SMA, and Snail expression and the down-regulated E-cadherin expression in TGF-β1-stimulated HPMCs were significantly attenuated by periostin-binding DNA aptamer treatment. These results propose that periostin-binding DNA aptamer can effectively mitigate the TGF-β1-induced EMT process in HPMCs by inhibiting periostin-related pathway activation rather than periostin expression *per se*.

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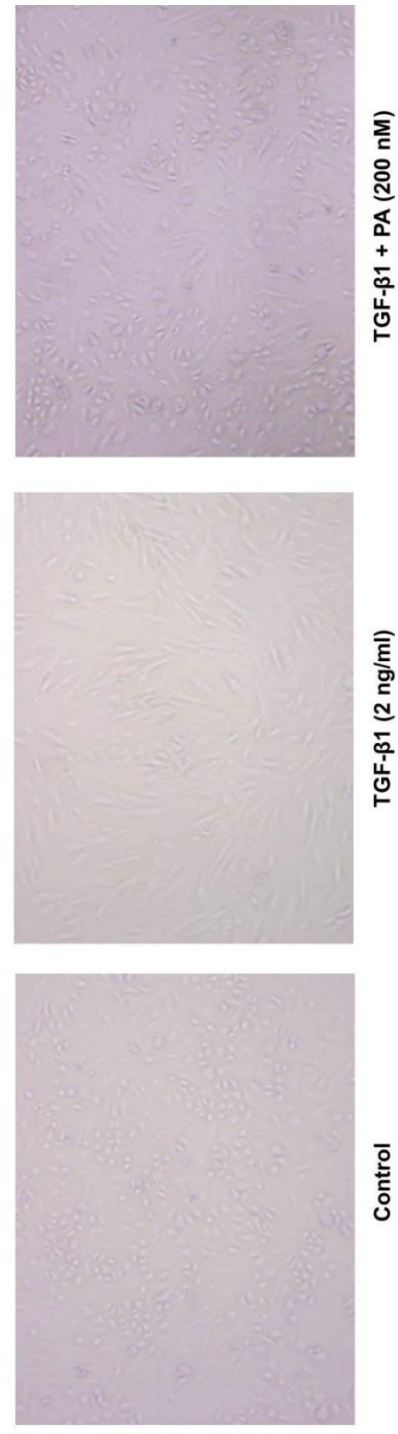

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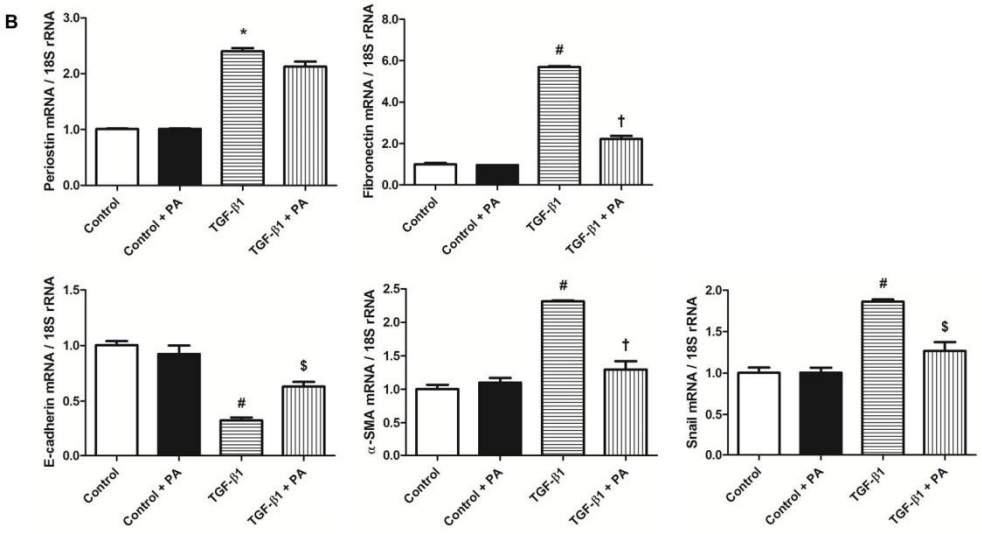

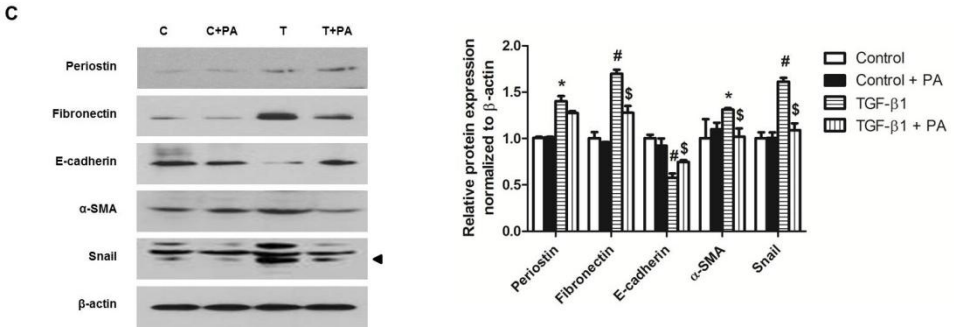

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Figure 4. Cell morphology, and mRNA and protein expression changes of EMT-related molecules following periostin-binding DNA aptamer treatment. (A) Morphologic features of control, and TGF-β1 (2 ng/ml)-stimulated HPMCs with or without periostin-binding DNA aptamer (200 nM). Periostin-binding DNA aptamer inhibited fibroblast-like cell morphological change in HPMCs exposed to TGF-β1 (x 100). (B) Periostin, fibronectin, E-cadherin, α-SMA, and Snail mRNA levels in TGF-β1-stimulated HPMCs treated with or without periostin-binding DNA aptamer (n=12). Periostin-binding DNA aptamer significantly attenuated TGF-β1-induced fibronectin, α-SMA, and Snail mRNA expression and significantly restored the decrease in E-cadherin mRNA levels in HPMCs stimulated by TGF-β1. (C) A representative western blot analysis of periostin, fibronectin, E-cadherin, α-SMA, Snail, and β-actin protein expression in HPMCs treated with or without periostin-binding DNA aptamer (a representative of 4 blots). Periostin-binding DNA aptamer significantly mitigated the increases in fibronectin, α-SMA, and Snail protein expression and the decrease in E-cadherin protein level in TGF-β1-stimulated HPMCs.

*;$P<0.05$ vs. control, $^{\#}$;$P<0.01$ vs. control, †;$P<0.01$ vs. TGF-β1, $^{\$}$;$P<0.05$ vs. TGF-β1

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5. Periostin-binding DNA aptamer prevents peritoneal fibrosis in PD mouse models

To investigate the *in vivo* effect of periostin-binding DNA aptamer on the development of peritoneal fibrosis, periostin-binding DNA aptamer was administered to a mouse model of PD. The peritoneal morphology and EMT-related molecules were examined in mice treated with PD solutions for 4 weeks with or without intraperitoneal periostin-binding DNA aptamer administration. Masson's trichrome staining and immunohistochemical staining of the peritoneum revealed that ECM accumulation was significantly severer and periostin expression was significantly higher in mice treated with PD solution relative to control mice, and periostin-binding DNA aptamer treatment significantly abrogated ECM accumulation but not periostin expression in PD mice. The changes in the expression of EMT-related molecules in the peritoneum of PD mice were also significantly ameliorated by the administration of periostin-binding DNA aptamer (Fig. 5). Furthermore, fibronectin, α-SMA, and Snail mRNA and protein expression were significantly higher, while E-cadherin expression was significantly lower in mice treated with PD solution compared to control mice, and these changes were significantly attenuated by periostin-binding DNA aptamer treatment (Fig. 6). These findings suggest that periostin-binding DNA aptamer can be effective in preventing PD-induced peritoneal fibrosis.

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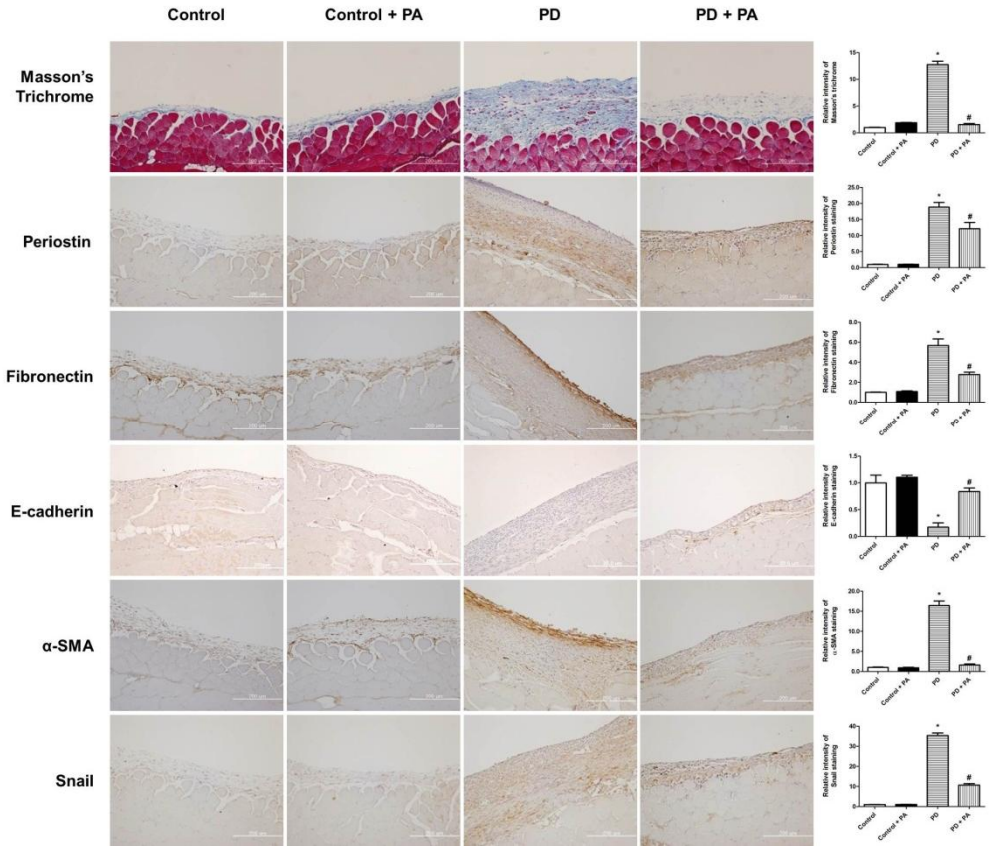

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Figure 5. Masson's trichrome staining and immunohistochemical staining of periostin, fibronectin, E-cadherin, α-SMA, and Snail in the peritoneum from control, control+periostin aptamer, PD, and PD+periostin aptamer mice. Masson's trichrome staining and immunohistochemical staining of the peritoneum revealed that ECM accumulation was significantly severer and periostin expression was significantly higher in mice treated with PD solution relative to control mice, and periostin-binding DNA aptamer treatment significantly abrogated ECM accumulation but not periostin expression in PD mice. The changes in the expression of EMT-related molecules in the peritoneum of PD mice were also significantly ameliorated by the administration of periostin-binding DNA aptamer (x 200).

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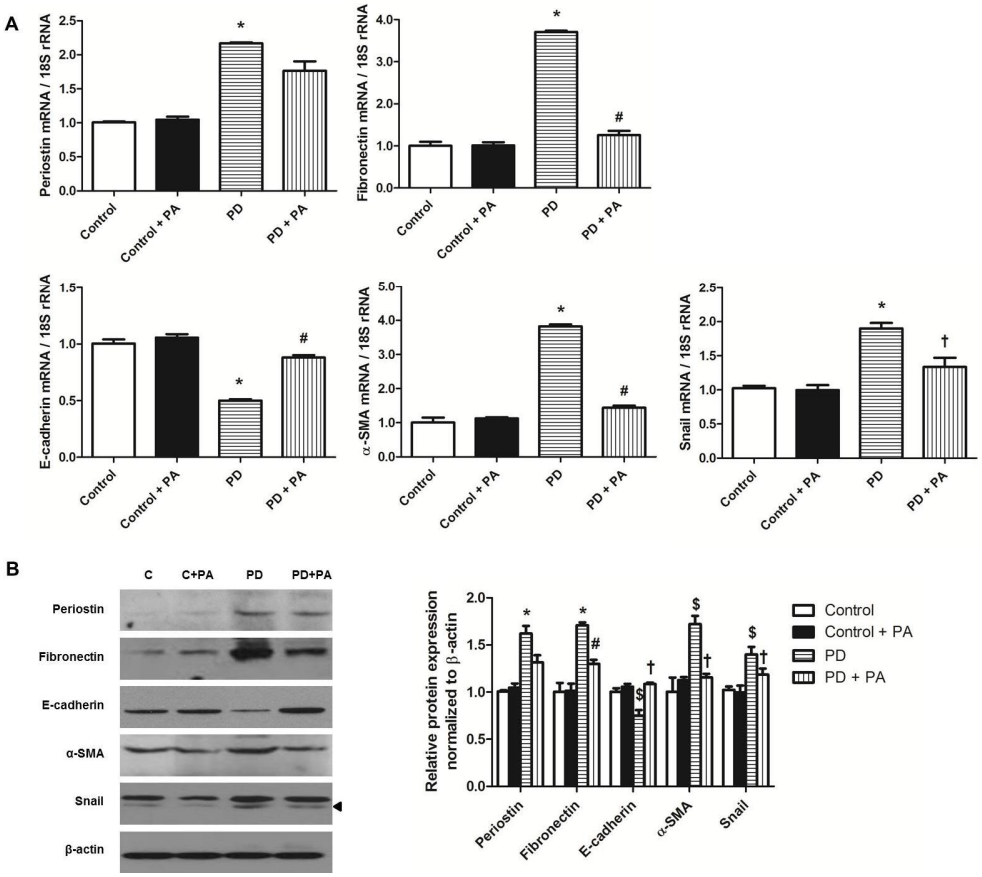

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Figure 6. Periostin and EMT-related molecules expression in the peritoneum from control, control+periostin aptamer, PD, and PD+periostin aptamer mice. (A) Fibronectin, α-SMA, and Snail mRNA expression were significantly higher, while E-cadherin expression was significantly lower in mice treated with PD solution compared to control mice, and these changes were significantly attenuated by periostin-binding DNA aptamer treatment. (B) A representative western blot analysis of periostin, fibronectin, E-cadherin, α-SMA, Snail, and β-actin protein expression in the peritoneum (a representative of 4 blots). The protein expression of EMT-related molecules showed a similar pattern to the mRNA expression.
*;P<0.01 vs. control, $^{\#}$;P<0.01 vs. PD, †;P<0.05 vs. PD, $^{\$}$;P<0.05 vs. control

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Ⅳ. DISCUSSION

In the present study, it was shown that periostin played a role in TGF-β1-induced EMT in cultured HPMCs. The inhibition of periostin expression and function by periostin siRNA or periostin-binding DNA aptamer, respectively, resulted in the abrogation of TGF-β1-induced EMT. In addition, intraperitoneal administration of periostin-binding DNA aptamer successfully inhibited peritoneal fibrosis in an animal model of PD.

In PD, uremic toxins and water removal are maintained by the concentration and osmotic gradient between the high glucose-based dialysate and intravascular space of peritoneal capillaries[30]. Over time, dialysate exposure results in fibrotic changes in the peritoneum, decreasing the efficiency of solute diffusion and salt removal from the peritoneal capillary vessels to the peritoneal space, a condition termed ultrafiltration failure[6,30]. This ultrafiltration failure is one of the major factors that limit PD to be widely used as a long-term renal replacement modality. The results of this study suggest possibilities of intraperitoneal administration of periostin-binding DNA aptamer in PD patients to prevent ultrafiltration failure. Moreover, due to the advantages of aptamers compared to protein- or small-molecule-based treatment options, such as a low manufacturing cost and fewer side effects, the current findings propose a practical and effective strategy to inhibit PD-induced peritoneal fibrosis and have potential clinical application.

Continual exposure to dialysate solutions as well as recurrent peritonitis episodes causes peritoneal inflammation and injury, eventually leading to peritoneal fibrosis[31]. PMCs have been implicated as one of the major cellular elements in the pathogenesis of this peritoneal fibrosis. In the past, resident peritoneal fibroblasts and infiltrating inflammatory cells have been considered the key players promoting peritoneal fibrosis[32]. However, recent studies have shown that the EMT of

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mesothelial cells is an important process in the induction of fibrosis and subsequent deterioration of peritoneal function[8,31,33]. EMT is a complex process by which epithelial cells lose their polarity and cell-cell adhesiveness and gain migratory and invasive characteristics to become myofibroblast-like cells[34]. It is also demonstrated to be a key process in embryonic development, malignant cell metastasis, and the promotion of organ fibrosis[35]. In the case of peritoneal fibrosis, PMCs have been found to lose their epithelial cytokeratins and gain the expression of mesenchymal cellular molecules upon exposure to dialysate[36]. In the present study, TGF-β1 treatment significantly down-regulated E-cadherin expression along with clearly up-regulated α-SMA and Snail expression which was accompanied by a significant increase in fibronectin expression in cultured HPMCs. A similar finding was also found in the peritoneal tissue of PD mice. These findings suggest that EMT is involved in the course of PD-induced peritoneal fibrosis and that PMCs play a significant role in this process, which is in concordance with the results of previous studies[37,38].

TGF-β1 is a well-known profibrotic cytokine that plays key pathological roles, including EMT promotion, in peritoneal fibrosis in patients undergoing PD[39]. TGF-β1 activates Smad2 and Smad3, which consequently results in molecular transition, down-regulating intracellular adhesion molecule E-cadherin and up-regulating mesenchymal-associated molecules, such as Snail, α-SMA, and fibronectin. Clinical studies found that the levels of TGF-β1 detected in the PD fluid correlated with the risk of technical failure in PD patients[40,41]. Furthermore, overexpression of TGF-β1 by the transfection of adenovirus vectors into the peritoneal cavity led to peritoneal fibrosis, angiogenesis, and ultrafiltration failure in an animal model of PD[42]. In addition, stimulation of PMCs *in vitro* with dialysate-containing culture media resulted in an increase in TGF-β1 mRNA and protein expression. Based on these findings, TGF-β1 has been considered a key inducer of fibrotic changes in the peritoneum of patients undergoing PD, and therefore, to

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simulate the PD environment associated with EMT and fibrosis, cultured HPMCs were treated with TGF-β1 for the *in vitro* experiments in this study.

Previously, TGF-β1-inhibiting efforts have been made to prevent organ fibrosis in various disease conditions[43]. The administration of TGF-β1 antibodies has been considered as an option for TGF-β1 inhibition and has shown promising anti-fibrotic results in some animal models[44-46]. Moreover, substances having inhibitory effect of TGF-β1, such as decorin and pirfenidone [5-methyl-1-phenyl-2(1H)-pyridone], have been tested for their efficacies in various diseases, such as systemic sclerosis and glomerulosclerosis[47,48]. Regarding peritoneal fibrosis, *in vitro* studies with PMCs transfected with TGF-β1 shRNA-expressing vectors have demonstrated to ameliorate ECM protein synthesis in these cells[49]. However, in addition to promoting pathological fibrotic changes in disease states, TGF-β1 is also involved in various physiologic functions, such as wound healing and immune reactions[50]. Furthermore, since TGF-β1 is located upstream of the TGF-β1-induced fibrosis signaling cascade, inhibition of TGF-β1 blocks numerous downstream cell signaling pathways critical for cell survival[39]. For these reasons, the clinical applications of anti-TGF-β1 therapies have been limited. Therefore, recent investigations have focused on molecular targets more downstream of the cell signaling cascade for anti-fibrotic effects. The fact that the inhibition of periostin, a terminally located protein of the TGF-β1-induced fibrosis pathway, successfully attenuated PD-related fibrosis process in the peritoneum should be taken into account in this regard.

Treatment with the periostin-binding DNA aptamer resulted in significant abrogation of EMT and fibrosis. Previous studies have found that periostin acts as a ligand and exerts intracellular signaling through the activation of integrin αV/β3 and αV/β5[21]. The activation of integrin is known to involve key cell signaling pathways that contribute to EMT and fibrosis, such as Akt and p38[51]. The periostin-specific

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binding property of periostin-binding DNA aptamer could inhibit the periostin-integrin interaction, thereby suppressing the integrin signaling pathways. This is supported by the periostin protein levels, which were not changed following periostin-binding DNA aptamer treatment despite its anti-fibrotic effects. Further investigations on the cellular pathways that are affected by periostin-binding DNA aptamers are needed to confirm this notion. In addition, since periostin expression *per se* is known to be regulated by integrin activation[21], a higher dose of periostin-binding DNA aptamer could ultimately down-regulate the expression of periostin. Although not a significant change, a down-regulating trend of periostin levels after treatment with periostin-binding DNA aptamer supports this assumption.

In summary, periostin expression induced by TGF-β1 in cultured HPMCs was involved in PD-related EMT and ECM accumulation. Inhibiting periostin function with periostin-binding DNA aptamer significantly ameliorated this PD-induced peritoneal fibrosis. These findings suggest that periostin acts as a key mediator of peritoneal fibrosis in PD patients, and treatment with periostin-binding DNA aptamer can be considered a successful treatment modality of peritoneal fibrosis in these patients.

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Ⅴ. CONCLUSION

In this study, the role of TGF-β1-induced periostin in EMT and ECM accumulation was investigated. Additionally, the effect of periostin-binding DNA aptamer treatment on PD-related peritoneal fibrosis was evaluated in cultured HPMCs and an animal model of PD.

1. TGF-β1 significantly up-regulated periostin mRNA and protein expression in HPMCs. This increase in periostin expression was accompanied by significant increases in the expression of fibronectin, α-SMA, and Snail and a significant decrease in E-cadherin expression in cultured HPMCs.

2. TGF-β1 provoked fibroblast-like cell morphological changes in cultured HPMCs, and periostin siRNA transfection reversed these cell morphological changes. In addition, periostin knockdown by periostin siRNA transfection attenuated the increases in fibronectin, α-SMA, and Snail expression and restored the down-regulated E-cadherin levels in TGF-β1-stimulated HPMCs.

3. FACS analysis of Cy3-tagged periostin-binding DNA aptamer revealed that the Cy3-positive fraction was significantly increased in cultured HPMCs treated with TGF-β1, and this increased Cy3-positive fraction was significantly abrogated in periostin siRNA-transfected cultured HPMCs exposed to TGF-β1.

4. Treatment with periostin-binding DNA aptamer reversed the TGF-β1-induced fibroblast-like cell morphological changes in cultured HPMCs. Moreover, periostin-binding DNA aptamer treatment ameliorated the TGF-β1-induced increases in fibronectin, α-SMA, and Snail expression and restored the down-regulated E-cadherin levels in cultured HPMCs exposed to TGF-β1.

5. In an animal model of PD, fibronectin, α-SMA, and Snail mRNA and protein expression were significantly higher, while E-cadherin mRNA and protein expression were significantly lower in mice treated with PD solution for 4 weeks compared to control mice, and these changes were significantly attenuated by intraperitoneal periostin-binding DNA aptamer treatment.

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6. Peritoneal fibrosis as assessed by Masson's trichrome staining was significantly severer in mice treated with PD solution for 4 weeks relative to control mice, and the extent of peritoneal fibrosis was significantly abrogated by intraperitoneal administration of periostin-binding DNA aptamer.

In conclusion, periostin is involved in PD-related EMT and ECM accumulation, and inhibiting periostin function with periostin-binding DNA aptamer treatment significantly ameliorates PD-induced peritoneal fibrosis.

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연세대학교
YONSEI UNIVERSITY

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연세대학교
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ABSTRACT (IN KOREAN)

Periostin-특이 aptamer 투여가 복막투석에 의한 복막 섬유화에 미치는 영향

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권 영 은

배경: 복막 섬유화는 복막투석을 시행받고 있는 환자에서 복막의 구조 변화 및 초여과 장애를 유발하는 중요한 합병증이다. 복막 중피세포의 상피-중간엽 이행과 복막 내 세포 외 기질의 축적이 복막 섬유화의 특징적인 소견이며, 이러한 변화 과정에 transforming growth factor (TGF)-β1이 중요한 역할을 하는 것으로 알려져 있다. 한편, periostin은 TGF-β1에 의하여 증가되는 세포 외 기질 단백 중 하나로, integrin과 관련된 세포 내 신호전달체계의 활성화를 통하여 collagen과 fibronectin을 포함한 세포 외 기질 단백의 발현을 증가시킨다. 최근에는 periostin 발현이 섬유화와 관련된 각종 질환들의 발생과 밀접한 관련이 있다는 연구 결과들이 발표되었으며, 복막투석과 연관된 복막 섬유화에서도 비정상적인 periostin의 발현 증가가 관찰되었다. 따라서, 복막 중피세포에서 periostin의 발현 및 기능을 조절하는 것이 복막 섬유화의 발생 및 진행에 영향을 미칠 수 있을 것으로 생각되나, 아직 이에 대한 연구는 전무한 상태이다.

연세대학교
YONSEI UNIVERSITY

목적: 본 연구에서는 사람 복막 중피 배양세포에서 periostin-특이 DNA aptamer가 TGF-β1에 의한 상피-중간엽 이행 및 세포 외 기질의 생성에 미치는 영향을 알아보고자 하였다. 또한, periostin-특이 DNA aptamer의 효과를 동물 복막투석 모델에서도 조사하였다.

방법: 생체 외 실험으로는 사람 복막 중피 배양세포를 이용하여 TGF-β1 (2 ng/ml)을 처리한 후 상피-중간엽 이행 및 세포 외 기질의 생성 변화를 확인하였다. Periostin의 역할을 규명하기 위하여 periostin siRNA (100 nM)와 Cy3-tagged periostin-binding DNA aptamer (200 nM)를 이용하였다. 또한, periostin-특이 DNA aptamer가 periostin과 특이적으로 결합하는 지 여부를 확인하기 위하여 Cy3가 결합된 periostin-특이 DNA aptamer의 형광 발현량을 형광-활성 세포 분류법 (fluorescence-activated cell sorting)으로 측정하였다. 생체 내 실험으로는 C57BL/6 마우스 48마리에 복막투석 도관을 삽입한 후 대조군 (N=24)과 복막투석군 (N=24)로 나누었으며, 대조군에는 1일 1회 생리식염수 2 ml를, 그리고 복막투석군에는 1일 1회 4.25% 복막투석액 2 ml를 투석관을 통하여 4주간 매일 주입하였다. 대조군과 복막투석군 각각 12 마리씩에는 periostin-특이 DNA aptamer (500 μg/kg/d)를 생리식염수 또는 복막투석액 주입 시에 함께 투여하였다. 4주 후 동물을 희생하여 복막 조직을 채취하였다. 복막 중피 배양세포와 마우스의 복막에서 periostin, fibronectin, α-smooth muscle actin (α-SMA), Snail, 그리고 E-cadherin의 단백 발현은 western blot을 이용하여 분석하였고, 각각의 mRNA 발현은 real-time PCR로 평가하였다. 마우스 복막 조직의 섬유화 정도는 Masson's trichrome 염색으로 관찰하였으며, 면역조직화학 염색도 시행하였다.

연세대학교
YONSEI UNIVERSITY

결과: 사람 복막 중피 배양세포에서 TGF-β1을 처리한 결과 periostin, fibronectin, α-SMA, 그리고 Snail의 발현은 의미있게 증가된 반면, E-cadherin의 발현은 의의있게 감소되었다. Cy3가 결합된 periostin-특이 DNA aptamer를 사람 복막 중피 배양세포에 처리하였을 때, Cy3 형광 발현량은 TGF-β1 처리 군에서 유의하게 증가되었으며, 이러한 증가는 periostin siRNA 처치로 의미있게 감소되었다. Periostin siRNA뿐만 아니라 periostin-특이 DNA aptamer는 TGF-β1에 의한 fibronectin, α-SMA, 그리고 Snail의 발현 증가와 E-cadherin의 발현 감소를 의의있게 완화시켰다. 그러나, periostin의 발현은 periostin siRNA에 의해서는 유의하게 감소되었으나 periostin-특이 DNA aptamer에 의해서는 의미있는 변화가 없었다. 생체 내 실험상 4주간 복막투석액을 투여한 마우스의 복막에서 fibronectin, α-SMA, 그리고 Snail의 발현은 의의있게 증가되었던 반면, E-cadherin의 발현은 유의하게 감소되었다. 대조군에 비하여 복막투석군에서 복막의 중피세포 하층의 두께는 의미있게 두꺼웠으며, 섬유화 정도도 의의있게 심하였다. 복막투석군에서의 이러한 변화는 복강 내 periostin-특이 DNA aptamer 투여로 유의하게 반전되었다.

결론: 이상의 결과를 종합하여 볼 때, periostin-특이 DNA aptamer는 복막투석 환자에서 복막 섬유화의 예방 및 치료에 유용할 것으로 생각된다.

핵심되는 말: Periostin, transforming growth factor-β1, 복막투석, 복막 섬유화, aptamer, 상피-중간엽 이행