Mitofusin2 regulates the proliferation and function of fibroblasts: The possible mechanisms underlying pelvic organ prolapse development

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Abstract. The present study aimed to investigate the effects of Mitofusin2 (Mfn2) on the proliferation of human uterosacral ligament fibroblasts and on the expression of procollagen. We also aimed to identify the possible signal transduction pathway involved in the development of pelvic organ prolapse (POP). For this purpose, uterosacral ligaments were harvested from POP and non-pelvic organ prolapse (nPoP) patients for fibroblast culture. Cellular proliferation and the cell cycle were assessed following transduction with lentiviral vectors for the overexpression and suppression of Mfn2. The expression levels of the proteins Mfn2, procollagens, phosphoprotein 21 wild-type p53 activating fragment (p21Waf1), cyclin-dependent kinase 2 (CDK2), extracellular signal-regulated kinase1/2 (ERK1/2) and rapidly accelerated fibrosarcoma-1 (Raf-1) were examined. Overexpression of Mfn2 resulted in the decreased proliferation of cells and the induction of G0/G1 phase arrest. Concomitantly, the relative expression levels of procollagen proteins, CDK2 and the phosphorylation levels of ERK1/2 and Raf-1 proteins were notably decreased, while the levels of the p21waf1 protein were increased in the Mfn2 overexpressing group. Opposing results were reported cells following Mfn2 silencing via RNA interference. The results of the present study indicated that the cell cycle of the fibroblasts, their cellular proliferation and the levels of the procollagen proteins could be inhibited via the Ras-Raf-ERK axis as a result of the increased levels of Mfn2 during the development of POP.

Introduction

The incidence of pelvic organ prolapse (POP) has increased during the last few years (1). This condition notably affects the quality of life of many females worldwide; however, at present, several studies have reported the lack of effective preventative methods for patients with high risk factors of POP (2), and deficiencies in strategies to suppress the development of POP (1-8). Previous studies have notably focused on the clinical diagnosis and treatment of POP, and the mechanism of action associated the extracellular matrix (ECM) (9-13). Few studies have investigated the proliferation and activity of fibroblasts, which are associated with the synthesis and secretion of procollagen (14).

Mfn2 serves an important role in signal transduction, the prevention of sustained extensive mitochondrial elongation, the activation of cellular senescence and the induction of apoptosis (15-18). It is important to note that Mfn2 controls cell metabolism by limiting the production of reactive oxygen species and by modulating the endoplasmic reticulum stress (15). Thus, the mitochondrial fusion protein mitofusin 2 (Mfn2) is a cellular hub that senses the metabolic and hormonal milieu, and drives the control of metabolic homeostasis (15). Abnormal expression of Mfn2 can cause adverse effects. Previous studies have reported that Mfn2 controls cell metabolism by limiting the production of reactive oxygen species and by modulating the endoplasmic reticulum stress (15). Thus, the mitochondrial fusion protein mitofusin 2 (Mfn2) is a cellular hub that senses the metabolic and hormonal milieu, and drives the control of metabolic homeostasis (15). Abnormal expression of Mfn2 can cause adverse effects. Previous studies have reported that Mfn2 is associated with lung diseases (18,19) and the incidence of cancer (19). A limited number of studies have focused on pelvic floor diseases. POP is a common disease in the elderly population, which is associated with the damage and aging of the pelvic floor support structure (1,20). Furthermore, Mfn2 is associated with aging (17). Our previous study (21) demonstrated a correlation between Mfn2 expression levels and POP in vivo. Fibroblasts were isolated from uterosacral ligaments using laser capture microdissection. The results indicated that the expression levels of Mfn2 in patients with POP increased...
and the expression levels of the procollagen protein decreased compared with those of non-pelvic organ prolapse (NPOP) samples (21). The successive study of our group (22) demonstrated in vitro that the proliferation of primary cultured fibroblasts and the expression levels of procollagen protein had decreased, while the expression levels of Mfn2 were increased in POP uterosacral ligaments compared with NPOP samples. Following the inhibition of Mfn2 expression, the levels of procollagen protein were increased. Based on these previous findings, we further examined alterations in the cellular proliferation of fibroblasts, their progression through the various stages of the cell cycle and the expression levels of procollagen proteins following upregulation of Mfn2. The present study aimed to determine the possible mechanism underlying the pathogenesis of POP.

Materials and methods

Study subjects. Uterosacral ligaments were collected from 10 POP and 10 NPOP cases during hysterectomy in the Peking University First Hospital from September 2016 to December 2016. After hysterectomy, fresh uterosacral ligament tissues were cut into pieces with a diameter <0.1 cm, these tissues were cultured in Dulbecco’s Modified Eagle’s medium Nutrient Mixture F-12 (Ham) (DMEM/F12; Gibco; Thermo Fisher Scientific) containing 20% FBS (Gibco; Thermo Fisher Scientific) and 1% antibiotic solution in an incubator at 37°C containing 5% CO₂. Primary culture was conducted until 2-4 colonies were formed, which were used for transfection. The POP group comprised stage III to IV cases (according to the POP-Q score) (23). The NPOP group was matched with patients who required hysterectomy due to benign gynecological diseases, including hysteromyoma and adenomyosis, according to age (years; POP, 55.60±11.33 and NPOP, 50.70±7.90), body mass index (POP, 23.57±3.33 and NPOP, 23.03±2.91), parity (POP, 1.40±0.70 and NPOP, 1.20±0.42) and postmenopausal duration (years; POP, 5.75±9.59 and NPOP, 1.90±0.61) (n=10, P>0.05, all). All patients exhibited no history of urinary tract infection, estrogen drugs, vaginal surgery and/or diseases that could affect the metabolism of collagen during the 3 month period prior to the study. The clinical data of patients is presented in Table I. Samples were collected after informed consent from patients was obtained. The present study was approved by the ethics committee of Peking University First Hospital [approval no. 2016 (1173)].

Main reagents and instruments. Anti-extracellular-signal regulated kinase 1/2 (ERK1/2; diluted 1/200, sc-135900), anti-phosphorylated (p)ERK1/2 (diluted 1/200, sc-377400), anti-Raf-1 (diluted 1/200, sc-52827), anti-pRaf-1 (diluted 1/200, sc-293351), anti-p21waf1 (diluted 1/200, sc-90110), anti-cyclin dependent kinase 2 (CDK2; diluted 1/200, sc-136191), anti-proc collagen 1A1/1A2/3A1 (diluted 1/200, sc-133179, sc-166572, sc-16333, respectively) were purchased from Santa Cruz Biotechnology, Inc. Anti-β-tubulin monoclonal antibody (diluted 1/1,000, TA437064) and anti-β-actin monoclonal antibody (diluted 1/1,000, TA-09) were obtained from ZSGB-BIO. Anti-Mfn2 monoclonal antibody (diluted 1/500, ab56889) was obtained from Abcam. The cell cycle detection kit, myllicin mixture (KGY0023), Pancreatin (KGY001) and Pancreatin-EDTA (KGY0012) were obtained from Nanjing KeyGen Biotech Co., Ltd. The cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. DMSO was obtained from AppliChem GmbH; Enhanced Chemiluminescent Reagent was purchased from GE Healthcare Life Sciences. Fetal bovine serum (cat. no. 10099-141) was purchased from Gibco (Thermo Fisher Scientific, Inc.). The mouse anti-human cytoketarin monoclonal (CK-19), horseradish peroxidase-labeled goat anti-mouse IgG (ZB-2305) antibodies and peroxidase-conjugated goat anti-rabbit IgG (ZB-2301) were purchased from OriGene Technologies, Inc.

Lentiviral vectors. Mfn2-overexpression and RNA interference (RNAi) lentiviral vectors were provided by Shanghai GeneChem Co., Ltd. The lentiviral vectors employed for Mfn2 overexpression and downregulation were Ubi-MCS-3FLAG-SV40-enhanced green fluorescent protein (EGFP) and U6-MCS-Ubi-EGFP, respectively. The RNAi sequences for Mfn2 and the negative control sample were ACTTTGTCACTGGCAAGAA and GTTCTTCGAACGTG CACGT, respectively.

Primary cell culture, subculture, cryopreservation and resuscitation. Uterosacral ligament tissues were harvested during hysterectomy. The tissue fragments possessed dimensions of 0.5x0.5x0.5 cm³. The fibroblasts could be observed under a light microscope (magnification, x4) after 1-2 weeks of primary culture. The cells were collected for subculture (passage 2-4) and/or long term storage at -80°C when the confluence of the cell culture was ~70-80%. The procedure was conducted in a biosafety cabinet. For resuscitation, frozen cells were removed from -80°C and warmed rapidly in water at 37°C. The contents of the tube was added to 10X volume of culture medium and centrifuged at 300 x g for 5 min. The cell pellet was resuspended in medium (containing 20% FBS and 1% antibiotics) and placed in an incubator at 37°C.

Cell groups. Fibroblasts in the NPOP group were used for the Mfn2-overexpression experiments. The cells were divided into three groups as follows: Mfn2+ group, comprising NPOP fibroblasts transduced with lentiviral vector containing the open reading frame of Mfn2; Mfn2- group, NPOP fibroblasts were transduced with an empty lentiviral vector and the control group, comprising non-transduced NPOP fibroblasts. Concomitantly, POP fibroblasts were used for Mfn2 knockdown experiments according to the following three groups: Short hairpin RNA group (sh-Mfn2 group), comprising POP fibroblasts transduced with lentiviral vector containing the shRNA interference sequence; short hairpin RNA negative control (sh-Mfn2-NC) group that included POP fibroblasts transduced with lentiviral vector containing a shRNA negative control (NC) sequence and the control group, comprising non-transduced POP fibroblasts.

Transduction. Prior to infection, the cells in the logarithm growth phase that were grown in the presence of antibiotic-free medium were prepared into a cell suspension at a concentration of 2-3x10⁴ cells/ml with pancreatin-EDTA. The cell suspension was added to each well and cultured at 37°C, in the presence of 5% CO₂. Adherent cells that
attained 20-30% confluence were observed under an inverted microscope at a magnification of x4 (5 fields per view) the following day. The virus was incubated with the cells in 10% FBS + DMEM/F12 in the absence of antibiotics. The ratio of lentiviral particles to the number of cells (multiplicity of infection) was 20:1. The final concentration of the viral particles was 5 g/ml and Polybrene was employed for transduction. During viral transfection, the cells were washed two times with PBS and 10-12 h later, the complete culture medium was used to replace the medium containing the virus. The expression of GFP was observed under fluorescence microscope (magnification, x40; 5 fields per view) following 96 h of incubation.

Detection of fibroblast proliferation by a CCK-8 assay. A CCK-8 assay was conducted to detect cell proliferation in each group 96 h following transduction with the lentivirus. The steps were as follows: The cell suspension was diluted to the specific cell densities, including 8x10⁴, 4x10⁴, 2x10⁴ and 1x10⁴ cells/ml, and the cells were incubated in a 96-well plate at a volume of 100 µl/well at 37°C. Medium was added to the edge of wells. A total of 100 µl of CCK-8 containing medium (10% FBS: CCK-8=9:1) was added 24 h later. Following 2 h of incubation at 37°C, the absorbance was measured with a microplate reader at the wavelength of 450 nm (OD). Similarly, a standard curve was generated using the number of cells as the abscissa (X axis) and the OD value for the vertical axis (Y axis). A new 96-well plate was used every day and measurements were recorded on days 1, 3, 5, 7, 9 and 11. The cell proliferation curves were plotted according to the data obtained from these six time points.

Cell cycle analysis. The cells were collected using 0.25% no-EDTA trypsin for 1-2 min at 37°C following 96 h of viral infection and subsequently washed with PBS. The samples were centrifuged at 300 x g at room temperature for 5 min and the supernatant was discarded. The cells were fixed at 4°C for 1 h. The fixed cells were washed two times with 75% ethanol, centrifuged at 500 x g for 5 min at room temperature and incubated with 100 µl RNase A (100 µg/ml) in a 37°C water bath for 30 min in the presence of ethidium bromide (Sigma-Aldrich; Merck KGaA). Flow cytometry (FCM) was performed within 1 h with a flow cytometer. Each group analysis was repeated three times with the mean value was obtained. The results were analyzed with ModFit LT 4.1 software (Verity Software House, Inc.).

Table I. Comparison of clinical data between POP and NPOP patients.

<table>
<thead>
<tr>
<th>Parameters (mean ± standard error of the mean)</th>
<th>NPOP (n=10)</th>
<th>POP (n=10)</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>50.70±7.90</td>
<td>55.60±11.33</td>
<td>1.122</td>
<td>0.277</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.03±2.91</td>
<td>23.57±3.33</td>
<td>0.348</td>
<td>0.732</td>
</tr>
<tr>
<td>Age of first delivery (years)</td>
<td>27.14±3.80</td>
<td>24.75±3.45</td>
<td>1.277</td>
<td>0.224</td>
</tr>
<tr>
<td>Parity (n)</td>
<td>1.20±0.42</td>
<td>1.40±0.70</td>
<td>0.775</td>
<td>0.449</td>
</tr>
<tr>
<td>Postmenopausal duration (years)</td>
<td>1.90±6.01</td>
<td>5.75±9.59</td>
<td>1.043</td>
<td>0.312</td>
</tr>
</tbody>
</table>

Data were acquired via an independent samples t-test. BMI, body mass index; POP, pelvic organ prolapse; NPOP, non-pelvic organ prolapse. No significant differences were observed between the two groups.

Statistical analysis. Experiments were repeated three times and the results were expressed as the mean ± standard deviation. SPSS 23 (IBM, Corp.) was used for analysis. Independent samples t-test and general linear model-univariate analysis and/or one way ANOVA were used for comparison between different groups when the data followed normal distributions. Bonferroni correction was used when the variance was equal and Dunnett’s T3 post hoc test was used if the variance was not equal. For non-normal distributions, between group comparisons were assessed using Wilcoxon and/or Kruskal-Wallis tests, and the rank sum statistic χ² was used. P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitory effects of Mfn2 on fibroblast proliferation. The primary fibroblasts observed via light microscopy were presented in Fig. 1. The data verified the identification of the fibroblasts as reported in our previous study (22). Following 11 days of the initial cell seeding, the cell activity was significantly reduced in the Mfn2-overexpressing model compared
With that of the control and the Mfn2- groups. The absorbance of the Mfn2+, Mfn2- and control groups, respectively on day 1 were determined to be 0.138±0.003, 0.147±0.002 and 0.144±0.003, respectively. Similarly, the corresponding cell proliferative activities were analyzed for the remaining time periods and were as follows: Day 3, 0.206±0.003; 0.211±0.003 and 0.210±0.004; day 5, 0.251±0.003; 0.372±0.004 and 0.425±0.004; day 7, 0.347±0.004; 0.767±0.003 and 0.832±0.004; day 9, 0.513±0.004; 1.406±0.003 and 1.353±0.005 and day 11, 0.606±0.004; 1.696±0.005 and 1.781±0.005 for the respective Mfn2+, Mfn2- and control groups. No significant differences were noted between the two control groups (P>0.05). From days 5-11, the proliferation of Mfn2+ fibroblasts was significantly reduced compared with the control groups (Fig. 2; P<0.001).

The activity of the sh-Mfn2 group fibroblasts was significantly increased in the sh-Mfn2 cells compared with that of the sh-Mfn2-NC and control groups (P<0.001, from days 5-11). The absorbance of the sh-Mfn2, sh-Mfn2-NC and control groups, respectively at the different time periods were as follows: Day 1, 0.142±0.003, 0.144±0.003 and 0.142±0.002; day 3, 0.206±0.004, 0.212±0.004 and 0.210±0.004; day 5, 0.401±0.004, 0.305±0.021 and 0.234±0.004; day 7, 0.729±0.004, 0.338±0.004 and 0.331±0.004; day 9, 1.067±0.004, 0.508±0.003 and 0.418±0.004, and day 11, 1.483±0.005, 0.596±0.004 and 0.530±0.004 for the respective sh-Mfn2, sh-Mfn2-NC and control groups. The proliferation of sh-Mfn2 fibroblast significantly increased from days 5-11 (P<0.001); however, no significant difference was observed between the two control groups (P>0.05) (Fig. 2).

**Inhibitory effects of Mfn2 on fibroblast cell cycle.** The ratio of the percentage of fibroblasts at the G0/G1 phase in the Mfn2+ group (74.939±0.562%) was markedly increased compared with that of the Mfn2- (36.526±0.549%) and the control groups (44.159±0.461%; n=10). Compared with the population of the cells in the G1 phase, the proportion of cells in the S and G2/M phases in the Mfn2+ group was markedly lower compared with that of the control group. No differences were noted between the two control groups (Fig. 3A).

In the Mfn2 knockdown group, the number of cells in the G0/G1 phase was markedly lower (41.469±0.797%) compared with the sh-Mfn2-NC (61.606±0.711%) and the control groups (57.649±0.795%). By contrast, the proportion of the cells in S and G2/M phases of the sh-Mfn2 group was markedly higher than that of the control group. The cell percentages of the two control groups indicated no difference (Fig. 3).

**Relative expression of Mfn2 and procollagen proteins.** The relative expression of each protein was detected by western blot analysis. The expression of the proteins following the knockdown of Mfn2 in POP fibroblasts has been demonstrated in our previous study (21). In NPPOPOP fibroblasts infected with lentivirus for the overexpression of Mfn2, the relative expression of Mfn2 was 3.850±0.038 (F=4029.096),
which was significantly increased compared with the control (1.081±0.016) and Mfn2- (1.056±0.014) groups (P<0.001). No significant difference was noted between the two control groups (P>0.05). In addition, the relative expression levels of procollagen 1a1/1a2/3a1 in the Mfn2+ group (0.384±0.005, 0.303±0.010, 0.486±0.009, respectively) were significantly decreased compared with those in the Mfn2- (1.676±0.034, 0.652±0.009, 1.003±0.014, respectively) and the control groups (1.755±0.048, 0.630±0.015, 1.059±0.023, respectively) (P<0.001). No significant difference was noted between the two control groups (P>0.05) (Fig. 4).

Phosphorylation of ERK1/2 and Raf-1 proteins following Mfn2 overexpression. Following 96 h of lentiviral transfection, total cellular protein was extracted from fibroblasts. The expression levels of total and phosphorylated levels of ERK1/2 and Raf-1 proteins were detected by western blot analysis (Fig. 5). The levels of total ERK1/2 protein expression were notably similar in the Mfn2+, Mfn2- and control groups (1656.461±88.422, 1642.728±61.549, 1674.064±44.134, respectively). However, the levels of pERK1/2 proteins in the Mfn2+ group (0.120±0.014) were markedly decreased compared with the Mfn2- (0.38±0.027) and control groups (0.353±0.021). The expression profile of total Raf-1 expression was similar to that of ERK.

Following the inhibition of Mfn2 expression in POP fibroblasts, the expression levels of total ERK1/2 protein in the sh-Mfn2, sh-Mfn2-NC and control groups markedly varied (1491.477±37.892, 1491.639±49.668, 1482.802±34.900, respectively, P>0.05). The levels of pERK1/2 in the sh-Mfn2 group (0.805±0.027) were significantly increased compared with the sh-Mfn2-NC (0.3305±0.001) and the control groups (0.5123±0.036) (P<0.05). The trend in expression of total Raf and pRaf was similar to that of ERK1/2 and pERK1/2, respectively (Fig. 5B).

Inhibitory effects of Mfn2 on the expression of p21waf1 and CDK-2. Following lentiviral transfection for 96 h, total protein was extracted from fibroblasts. The relative expression levels of p21waf1 and CDK2 were semi-quantitatively analyzed by western blotting (Figs. 6 and 7).

In NPOP fibroblasts, the expression levels of p21waf1 in the Mfn2+ group (0.322±0.026) were significantly upregulated than in the Mfn2- (0.110±0.001) and control groups (0.107±0.001) (P<0.001). No significant difference was noted between the two control groups. In addition, the relative expression levels of CDK2 in the Mfn2+ group (0.108±0.014) were significantly decreased compared with the Mfn2- (0.166±0.005) and control groups (0.168±0.006) (χ²=15.768, P<0.01). No significant difference was noted between the two control groups (P>0.05) (Fig. 6).

The expression levels of the p21waf1 protein in the sh-Mfn2 group (0.170±0.015) were significantly down-regulated than in the sh-Mfn2-NC (0.328±0.003) and control groups (0.333±0.004) following transfection of RNAi lentiviral particles in POP fibroblasts (χ²=59.874, P<0.001) (Fig. 7). No significant difference was reported between the sh-Mfn2-NC and control groups (P>0.05). In addition, the...
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The relative expression levels of CDK2 in the sh-Mfn2 group (0.228±0.016) were significantly upregulated compared with the sh-Mfn2-NC (0.084±0.001) and control groups (0.082±0.002) (χ²=49.541, P<0.001). No significant difference was noted between the two control groups (P>0.05).

Discussion

To examine the effects of Mfn2 on fibroblasts, we transduced fibroblasts with lentiviral vectors for the overexpression and knockdown of Mfn2. These vectors are suitable for achieving stable expression of sh-Mfn2 RNA and can significantly improve the transduction efficiency of specific cells that are difficult to transfect, such as primary cells and stem cells (24). In the present study, the infection efficiencies of Mfn2 overexpressing and RNAi lentiviral vectors were >70%, indicating that they could be used successfully for transfection.

The CCK-8 assay is a colorimetric assay used to measure cell proliferation. The formation of the color is directly
proportional to the proliferation of cells. The use of a microplate reader to determine the OD indirectly reflects the number of living cells. The present study indicated that the cell activity in the Mfn2+ group was significantly reduced compared with that noted in the two control groups, whereas in the sh-Mfn2 group, the activity of the sh-Mfn2 fibroblasts was significantly higher compared with that of the sh-Mfn2-nc and control groups. These results were consistent with a previous study (19); however, in POP patients, increased expression levels of Mfn2 may not be beneficial, as decreased proliferation of fibroblasts may be associated with their dysfunction, which could be the cause of POP.

The present study indicated that the Mfn2 gene could cause growth arrest by blocking the cell cycle at the G0/G1 phase (25). Three major classes of proteins are involved in the regulation of the cell cycle: Cyclins, CDK enzymes and cyclin-dependent kinase inhibitors (CDKI) (26). CDK2 is a key kinase that initiates DNA replication required for the G2 phase transition. CDKI inhibits CDK enzymes that prevent cells from passing through the restriction point. p21 is mainly involved in the regulation of the activity of the CDK proteins (26). The present study indicated that overexpression of Mfn2 in NPOP fibroblasts caused a decrease in the expression levels of CDK2, whereas the expression levels of the p21waf1 protein were significantly increased, which is associated with G0/G1 phase arrest. Similarly, a higher percentage of cells in the sh-Mfn2 group were able to enter S phase required for DNA synthesis, and G2/M phase required for mitotic division, which is characterized by increased CDK2 and decreased p21waf1 levels (25).

This conclusion is consistent with our previous results obtained from cell cycle studies (21,22).

The results of the current study indicated that Mfn2 notably inhibited cell proliferation through the ERK signaling pathway (27), which regulates a number of important cellular biological processes, including cell proliferation and differentiation (14). The Ras/Raf/MEK/ERK pathway transmits various downstream signals in order to activate nuclear proteins involved in the aforementioned biological processes (27). Activated ERK can promote the phosphorylation of its cytoplasmic target protein and/or regulate the activity of other protein kinases (14). Our results suggested that the upregulation of Mfn2 in POP fibroblasts inhibited the phosphorylation of these proteins by affecting Ras and Raf-1 proteins, two upstream effectors of ERK1/2, thereby inhibiting cell proliferation.

In conclusion, the present study demonstrated that the Mfn2-overexpression group, the expression levels of the procollagen proteins and cell proliferation were markedly decreased compared with the controls. This indicated that the increase in the levels of Mfn2 in POP fibroblasts inhibited the levels of procollagen proteins, resulting in the decrease of synthesized collagen secreted into the ECM. This could in turn lead to the weakness of the pelvic floor support structure based on the uterosacral ligament. These effects favor the occurrence and development of POP. Furthermore, we also proposed that Mfn2 may affect cell proliferation and the cell cycle via the Ras-Raf-ERK signaling pathway, and CDK2 and p21waf1 proteins. With regards to the causal association between the occurrence of POP and increased Mfn2 expression, Mfn2 was upregulated in the uterosacral ligaments in patients with POP. Our findings may provide insight into the pathogenesis, clinical prediction, individual diagnosis and treatment of POP; however, further investigation is required.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
YL contributed to the conception and design of the study, and obtained funding. CP and YZ communicated with patients, collected samples and revised the manuscript. HC was responsible for the acquisition of data. XqW and XxQ were responsible for the analysis and interpretation of data. XqW drafted the manuscript.

Ethics approval and consent to participate
The samples were obtained following informed consent provided by the patients and the ethics committee of the Peking University First Hospital [2016(1173)] approved the study protocol.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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18. Zheng L, Li S, Zhao S and Fa X: Upregulated miR-17 regulates possible for the acquisition of data. XqW and XxQ were responsible for the analysis and interpretation of data. XqW drafted the manuscript.