Correlations between Mitofusin 2 Expression in Fibroblasts and Pelvic Organ Prolapse: An *In vitro* Study

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Abstract

Background: Both Mitofusin 2 (*Mfn2*) and pelvic organ prolapse (POP) are related to aging. The aim of the present study was to investigate the variations of *Mfn2* expression in the uterosacral ligaments of patients with and/or without POP and their correlations with the expression of procollagen.

Methods: Fibroblasts were cultured using tissue specimens that were harvested from the uterosacral ligaments of POP and non-POP (NPOP) patients ($n = 10$ for each group) from September 2016 to December 2016. The Cell Counting Kit-8 (CCK-8) assay was used to compare the differences in cell proliferation between the two groups. Relative quantitative reverse transcription-polymerase chain reaction and Western blotting assays were employed to assess the differences in the mRNA and protein expression levels of Mfn2 and procollagen 1A1/1A2/3A1 between the two groups. The changes in procollagen expression were assessed following the downregulation of *Mfn2* in the POP group using RNAi. The data were assessed with independent sample *t*-test or general linear model univariate analysis using the SPSS 13.0 software.

Results: The results from CCK-8 assay indicated that cell viability in the POP group was significantly lower compared with that of the NPOP group ($t_{d5, 7, 9, 11} = -5.925, -6.851, -9.129$, and -9.661, respectively, all $P < 0.001$, from D5 to D11). The mRNA and protein expression levels of Mfn2 in the cultured fibroblasts of the POP group were significantly higher compared with those of the NPOP group (mRNA: $t = 2.425$, $P = 0.032$; protein: $t = 2.392$, $P = 0.037$, respectively), whereas only the expression levels of procollagen 1A1/1A2/3A1 were significantly higher in the NPOP group (mRNA: $t = -2.165$, $P_{1A1} = 0.041$; $t = -2.741$, $P_{1A2} = 0.026$; $t = -2.147$, $P_{3A1} = 0.045$, respectively; protein: $t = -2.418$, $P_{1A1} = 0.029$; $t = -2.405$, $P_{1A2} = 0.033$; $t = -2.470$, $P_{3A1} = 0.012$, respectively). The expression levels of procollagen in the POP group increased following the downregulation of *Mfn2*.

Conclusions: The proliferation rate and cell viability of the fibroblasts in the POP group were significantly lower compared with those in the NPOP group. In the POP fibroblasts, *Mfn2* expression was increased, while procollagen expression was decreased.

Key words: Cell Culture; Mitofusin 2; Pelvic Organ Prolapse; Procollagen

INTRODUCTION

The pathophysiological mechanisms of pelvic organ prolapse (POP) remain unknown. Previous studies have focused on the investigation of the extracellular matrix (ECM) as the main cause for the development of POP.[1-3] For example, changes in the content and/or metabolism of collagen fibers have been previously monitored. Although the majority of the studies mainly compared the quantitative and morphological changes of collagen fibers and/or detected the impact of collagenase upon collagen metabolism, the pathogenesis of POP in terms of the variations in the synthesis and secretion of procollagen in fibroblasts have not been explored. Similarly, the proliferation and the induction

of apoptosis of the fibroblasts in POP have not been investigated to date.

Mitofusin 2 (Mfn2) is a transmembrane protein embedded in the mitochondria that mediates mitochondrial fusion and plays an important role in the maintenance of the morphology and function of the mitochondria.[4,5] In addition to the

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aforementioned functions, Mfn2 is a cytoplasmic protein that is involved in the oxidative stress response,[5] signaling transduction,[6,7] the regulation of cell proliferation and apoptosis,[8] and various other biological processes. Therefore, the change in the expression of *Mfn2* in the fibroblasts derived from pelvic floor tissues was used as a marker in the present study to add insight to the pathogenesis of POP.

The previous study (*in vivo* study) conducted by our group demonstrated the isolation of fibroblasts from the uterosacral ligament tissues of POP and non-POP (NPOP) patients by laser capture microdissection.[9] The expression levels of Type I and III procollagen proteins were significantly lower in the POP group compared with the NPOP group, whereas the opposite was noted for the expression of *Mfn2*, and the previous results were published in European Journal of Obstetrics, Gynecology, and Reproductive Biology in 2014. Based on the previous findings, the present study aimed to verify the results derived by the *in vitro* experiments and to further explore the impact of *Mfn2* on the proliferation, induction of apoptosis, and further biological functions of the human uterosacral ligament fibroblasts. The study further aimed to provide conclusive evidence regarding the role of *Mfn2* in the occurrence and progression of POP.

METHODS

Materials

Ethical approval

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the local Ethics Committee of the hospital (No: 2016 [1173]). Informed written consent was obtained from all patients or their guardians for all study participants before their enrollment in this study.

Study subjects

Uterosacral ligament tissues were obtained from ten cases of Stage III–IV POP patients (postmenopausal) who underwent hysterectomy at the Department of Obstetrics and Gynecology of Peking University First Hospital from September 2016 to December 2016. NPOP patients who accepted hysterectomy due to a benign gynecological disease (*n* = 10) were enrolled as the control group [Table 1]. The following endpoints were

Table 1: Comparison of clinical data between patients of the POP and NPOP (control) groups

Parameter	NPOP (*n* = 10)	POP (*n* = 10)	*t*	*P*
Age (years)	59.87 ± 7.10	60.19 ± 6.83	0.169	0.886
BMI (kg/m²)	24.62 ± 3.08	25.72 ± 2.46	1.455	0.152
Age of first delivery (years)	25.83 ± 3.49	25.32 ± 2.85	0.584	0.562
Number of deliveries	2.13 ± 0.82	2.26 ± 0.99	0.501	0.618
Postmenopausal duration (years)	8.61 ± 6.79	9.32 ± 7.01	0.374	0.710

BMI: Body mass index; POP: Pelvic organ prolapse; NPOP: Non-POP.

measured in the control group, namely, matched age, body mass index, age of first delivery, number of deliveries, and postmenopausal duration. The POP Quantification System (POP-Q)[10] was used to grade the POPs. None of the patients received hormonal therapy during the 3 months before the surgery, and the participants were free of a history of urogenital tract infections, vaginal surgeries, and diseases that affected collagen metabolism. All the operations were conducted by the same surgical team that consisted of experienced doctors. Specimens were taken from the uterosacral ligament 1 cm beside the cervix.

Main reagents and instruments

The primers for *Mfn2*, the reference gene *β*-actin, and procollagen 1A1/1A2/3A1 were synthesized by Shanghai Sangon Biotech (China). Dulbecco's Modified Eagles Medium with High Glucose (SH30022.01B, HyClone) was purchased from GE Healthcare (USA). Dimethyl sulfoxide (DMSO, A3672) was purchased from APPLICHEM (Germany), whereas Trizol RNA extraction reagent, the reverse transcription kit, and the polymerase chain reaction (PCR) kit were purchased from ABI (USA). The Western Blotting Detection Kit was from GE Healthcare (USA) and the fetal bovine serum (10099-141) was from Gibco (USA). Penicillin/streptomycin mixture (KGY002-100), trypsin digestion solution (KGY001), and trypsin-EDTA digestion solution (KGY0012) were purchased from Nanjing KeyGen BioTECH (China). The anti-Mfn2 monoclonal antibody was purchased from Abcom (USA, ab56889), whereas anti-procollagen 1A1/1A2/3A1 monoclonal antibodies and anti-*β*-actin (TA-09) monoclonal antibody were from Santa Cruz (USA, 1A1: sc-133179; 1A2: sc-166572; 3A1: sc-166333). Mouse antihuman vimentin monoclonal antibody (ZM-0260), mouse antihuman cytokeratin (CK-19) monoclonal antibody, and horseradish peroxidase (HRP)-labeled goat antimouse IgG (ZB-2305) were purchased from Beijing Zhongshan Golden Bridge Biological Technology (China). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Japan). Both double chain DNAoligo and lentiviral expression vector of RNAi were provided by Shanghai Genechem (China).

Primer design

The primers of the target and the internal reference genes were designed based on the primer design principles. The GenBank database was used as a reference. The primers were synthesized by Shanghai Sangon Biotech [Table 2].

The RNAi sequence of *Mfn2* and the negative control sequence were as follows: ACTTTGTCACTGCCAAGAA, GTTCTCCGAACGTGTCACGT.

Primary culture and passage of fibroblasts derived from tissue specimens

During hysterectomy, fresh sterile uterosacral ligament tissues with a size of approximately 0.5 cm × 0.5 cm × 0.5 cm were harvested and then cut into pieces with a diameter smaller than 0.1 cm for the primary culture. Following 1–2 weeks

Table 2: Primers sequences of the study

Name	Sequence	Product length (bp)
Procollagen 1A1	Upstream: 5'-CGAGGGCCAAGACGAAGA-3'	73
	Downstream: 5'-CACGTCTCGGTCATGGTACCT-3'	
Procollagen 1A2	Upstream: 5'-TGGATACGCGGACTTTGTTG-3'	92
	Downstream: 5'-GGCTGGGCCCTTTCTTACAG-3'	
Procollagen 3A1	Upstream: 5'-TCGCCCTCCTAATGGTCAAG-3'	80
	Downstream: 5'-GGTCACCATTTCTCCCAGGAA-3'	
Mitofusin 2	Upstream: 5'-CATCAGCTACACTGGCTCCAACT-3'	65
	Downstream: 5'-GATGAGCAAAGGTCCCAGACA-3'	
β-actin	Upstream: 5'-CACGGCTGCTTCCAGCTC-3'	135
	Downstream: 5'-CACAGGACTCCATGCCCAG-3'	

of culture, the cells were observed under the microscope and passaged every 2–3 days.

Immunohistochemical identification of fibroblasts (SP method)

Following adhesion to the slides, the cells were incubated with primary antibodies (anti-vimentin and anti-CK-19 antibodies) in a 4°C humidified incubator overnight, while phosphate-buffered saline solution was used for the blank control. The following morning, the cells were incubated with the secondary antibodies (biotin-labeled goat antimouse antibodies) and with the HRP-labeled streptavidin conjugate in a 37°C humidified incubator. Subsequently, the cells were stained using diaminobenzidine, restained with hematoxylin, and washed with running tap water for counterstaining. The slides were dehydrated with gradient ethanol, cleared with xylene twice, mounted with neutral gum, and finally observed under the microscope.

Detection of fibroblast proliferation (Cell Counting Kit-8 assay)

A standard curve based on the optical density (OD) value that corresponded to cell number was developed. A total of six wells were inoculated with 100 µl of cell suspension at a concentration of 4×10^4 cell/ml per well. The cells were subsequently cultured under the same culturing conditions utilized in the standard curve experiment and incubated with CCK-8 solution for 2 h. The OD values of the cells were measured using a microplate reader at 450 nm. The cell counts were calculated based on the standard curve. The measurements were conducted in a new 96-well plate and six different time points were recorded as described above.

Real-time, fluorescence-based quantitative polymerase chain reaction for the detection of the mRNA expression levels of mitofusin 2 and procollagen

Total RNA was extracted from the cells that were cultured in the 60 mm dishes on day 11. Following reverse transcription to cDNA, quantitative PCR was conducted to detect the target and internal reference genes using the primers listed in Table 2. The relative quantification of the expression levels of the target and internal reference genes was estimated using the $2^{-\Delta CT}$ method.

Protein expression of mitofusin 2 and procollagen in fibroblasts

Total proteins were extracted from 10^6 cells, and the protein concentration was determined using a protein concentration standard curve. Using a loading volume of 60 µg per well, the proteins were separated by electrophoresis, transferred to a PVDF membrane, stained with Ponceau S and blocked with skimmed milk. Subsequently, the proteins were incubated with anti-Mfn2, anti-procollagen 1A1/1A2/3A1, and anti-*β*-actin monoclonal antibodies. Following washing of the primary antibodies, the membranes were incubated by HRP-labeled goat antimouse IgG antibody for enhanced chemiluminescence Western blotting detection. The AlphaImager™ Gel Imaging System (Alpha Innotech) was used to scan the grayscale levels of the specific ladders and calculate the grayscale ratios of the target proteins to the *β*-actin protein.

Transfection experiments

Mfn2 RNAi was conducted in POP fibroblasts, and the cells were divided into three groups. (1) The short hairpin RNA group (sh group) contained POP fibroblasts that were transfected with short hairpin RNA (shRNA)-positive interference lentiviral sequence; (2) The short hairpin RNA-negative control (shNC) group contained POP fibroblasts that were transfected with shRNA negative control lentiviral sequence; (3) The control group: POP fibroblasts in the absence of lentiviral infection. The expression levels of *Mfn2* and procollagen in each group were measured following successful transfection.

Statistical analysis

The data measured, namely, the expression levels of the Mfn2 and the procollagen proteins were expressed as a mean ± standard deviation (SD). All the data were analyzed using the SPSS 13.0 software (New York, IBM, USA). If the data were in normal distributions, between-group comparisons were assessed with the independent sample *t*-test and general linear model-univariate analysis and/or one-way analysis of variance (ANOVA) test; otherwise, between-group comparisons were assessed with Wilcoxon test. A value of $P < 0.05$ was used to indicate significant differences.

RESULTS

Identification of fibroblasts

The cell growth was monitored by microscopy following 1–2 weeks of inoculation of the tissues in the culture flask. The cells manifested at a growth zone that surrounded the tissue specimens [Figure 1a]. The cells were gradually elongated and formed a long spindle and/or polygonal shape. Following 7 days, local cell fusions that covered the major part of the flask surface could be observed. At the 20-day period, complete cell fusions were observed. Following passage of the cells, the cell growth was rapid, and the cells that exhibited optimal conditions were highly elongated. The fusion was completed on the 7th day [Figure 1b]. However, the cells would accumulate spirally when cultured continuously [Figure 1c]. Following passage to the third generation, the majority of the cells in the flask exhibited a spindle shape.

Cell immunohistochemistry indicated that intracellular expression of vimentin was positive (2A), whereas that of the CK-19 was negative (2B), highlighting that these fibroblasts were interstitially derived [Figure 2].

Determination of proliferation in the fibroblasts of the pelvic organ prolapse and the non-pelvic organ prolapse groups

Following seeding of the fibroblasts that corresponded to the two different groups in separate culture flasks, the differences in the cell proliferation were monitored between a 3- and 5-day time period. The cells were cultured using the same cell density and culturing conditions. The data obtained by microscopical evaluation indicated that fibroblasts in the POP group exhibited lower adhesion numbers, survival rate, and smaller cell size, whereas those in the NPOP group indicated higher cell numbers and faster proliferation rates. The cells in the POP groups were further characterized by a wider, longer, and more elongated morphology with broad intercellular contacts [Figure 3].

The CCK-8 assay was applied to assess the cellular viability daily, for a total period of 11 days following initial seeding. During the period of 5–11 days following seeding, the cells were in the logarithmic phase of proliferation, whereas the viability of the fibroblasts from the POP group was significantly lower compared with that of the NPOP group ($t_{d5,\ 7,\ 9,\ 11}$ = −5.925, −6.851, −9.129, and −9.661, respectively; all $P < 0.001$, from D5 to D11 [Table 3]).

The mRNA and protein expression levels of mitofusin 2 were upregulated, while those of procollagen were downregulated in pelvic organ prolapse fibroblasts

The mRNA and protein expression levels of *Mfn2* in the cultured POP fibroblasts were significantly higher compared with those of the NPOP group (mRNA: $t = 2.425$, $P = 0.032$; protein: $t = 2.392$, $P = 0.037$), whereas the mRNA and protein expression levels of procollagen 1A1/1A2/3A1 were significantly higher in the NPOP compared with the POP groups (mRNA: $t = -2.165$, $P_{1A1} = 0.041$; $t = -2.741$, $P_{1A2} = 0.026$; $t = -2.147$, $P_{3A1} = 0.045$; protein: $t = -2.418$, $P_{1A1} = 0.029$; $t = -2.405$, $P_{1A2} = 0.033$; $t = -2.470$, $P_{3A1} = 0.012$, respectively) [Tables 4, 5 and Figures 4, 5]. These results were consistent with the *in vivo* results reported in the previous study of our group.

The expression of procollagen was upregulated in pelvic organ prolapse fibroblasts through inhibition of the expression of mitofusin 2

Following 96 h of the initial transfection with *Mfn2* lentiviral RNAi in POP fibroblasts, the protein expression levels of Mfn2 were significantly lower than those in the shNC and control groups although no significant difference between negative control (shNC) and control groups ($F_{Mfn2(sh-shNC-control)}$ = 26.661, $P_{Mfn2(sh:shNC)} < 0.001$, $P_{Mfn2(sh:control)} < 0.001$, and $P_{Mfn2(shNC:control)} = 0.838$, respectively) was noted. Furthermore, the protein expression levels of the procollagen 1A1/1A2/3A1 in the sh group were significantly higher compared with those of the shNC and the control groups ($F_{1A1(sh-shNC-control)} = 18.277$, $F_{1A2(sh-shNC-control)} = 6.606$, and $F_{3A1(sh-shNC-control)} = 36.178$, respectively, $P_{1A1(sh:\ shNC)} < 0.001$, $P_{1A1(sh:control)} < 0.001$, and $P_{1A1(shNC:control)} = 0.445$, respectively; $P_{1A2(sh:shNC)} = 0.017$, $P_{1A2(sh:control)} = 0.007$, and $P_{1A2(shNC:control)} = 0.928$, respectively; $P_{3A1(sh:shNC)} < 0.001$, $P_{3A1(sh:control)} < 0.001$, and $P_{3A1(shNC:control)} = 0.897$, respectively). No significant difference was noted between the two control groups. That indicates that the expression levels of procollagen can be improved by the downregulation of the expression levels of *Mfn2* in POP fibroblasts [Table 6 and Figure 6].
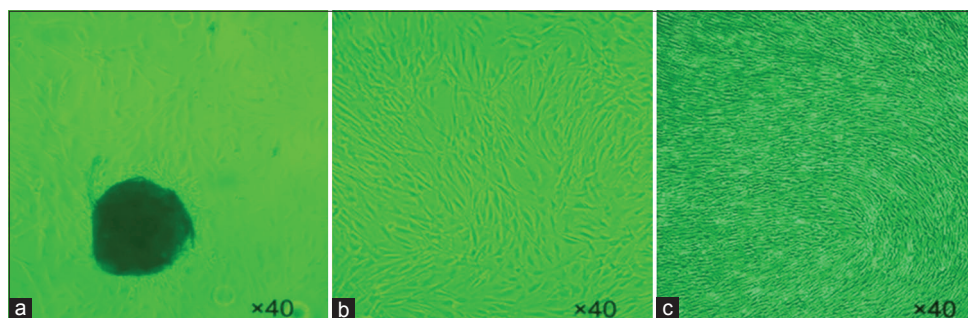

Figure 1: Primary culture of the fibroblasts and their growth and morphology (Fibroblasts observed under ×40 light microscope with no staining). Structures colored in black (a) indicate the tissue specimens; (b) the fusion was completed on the 7th days; (c) the cells would accumulate spirally when cultured continuously.

Table 3: Comparisons of cell viability of fibroblasts (CCK-8) between POP and NPOP groups at different culturing time points

Group	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11
POP ($n = 10$)	0.183 ± 0.003	0.216 ± 0.014	0.264 ± 0.015	0.410 ± 0.044	0.552 ± 0.189	0.792 ± 0.278
NPOP ($n = 10$)	0.182 ± 0.003	0.221 ± 0.022	0.332 ± 0.087	0.552 ± 0.154	1.047 ± 0.375	1.404 ± 0.404
t	1.347	−1.411	−5.925	−6.851	−9.129	−9.661
P	0.181	0.161	<0.001	<0.001	<0.001	<0.001

During the period of 5–11 days following seeding the cells were in the logarithmic phase of proliferation, whereas the viability of the fibroblasts from the POP group was significantly lower compared with that of the NPOP group. POP: Pelvic organ prolapse; NPOP: Non-POP; CCK-8: Cell counting kit-8.
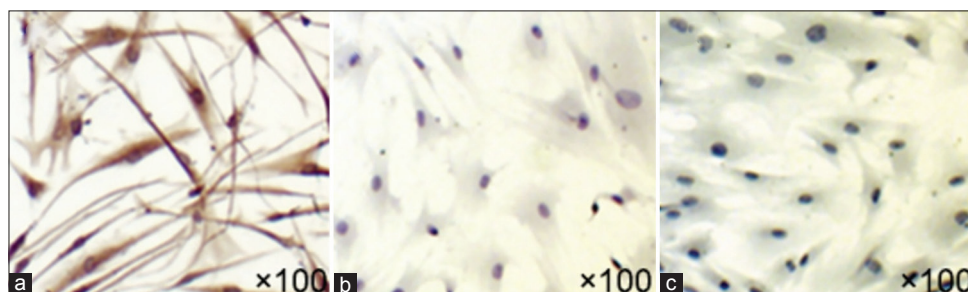

Figure 2: Fibroblasts which were observed after cell IHC. Fibroblasts observed under ×100 light microscope after IHC, IHC indicated that intracellular expression of vimentin was positive (a), while that of the CK-19 was negative (b), highlighting that these fibroblasts were interstitially derived. (a) Anti-vimentin antibody (primary antibody); (b) anti-CK-19 antibody (primary antibody); (c) blank control (PBS). IHC: Immunohistochemistry.
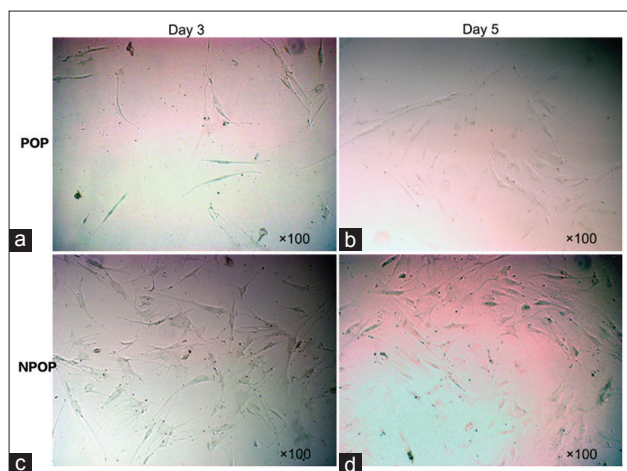

Figure 3: Growth and morphological differences of fibroblasts between POP and NPOP groups. Growth and morphological differences of fibroblasts observed under ×100 light microscope without staining. Fibroblasts in the POP group exhibited lower adhesion numbers, survival rate, and smaller cell size, while those in the NPOP group indicated higher cell numbers and faster proliferation rates. (a) POP cells observed on the 3rd day, (b) POP-cells observed on the 5th day, (c) NPOP-cells observed on the 3rd day, (d) NPOP-cells observed on the 5th day. POP: Pelvic organ prolapse; NPOP: Non-pelvic organ prolapse.

DISCUSSION

Previous studies that examined the POP mainly focused on the investigation of clinical research[11-14] or the ECM as the main cause for the development of POP. A limited number of studies have addressed the morphology and cell proliferation of the fibroblasts that are secreted to the ECM. A previous study conducted by our group demonstrated the successful isolation of fibroblasts from the uterosacral ligament tissues of POP and NPOP patients using laser capture microdissection.[9] Abnormal mRNA and protein expression levels of Mfn2 and procollagen (upregulated *Mfn2* and downregulated procollagen in the POP group) were noted.[9] In the present study, an *in vitro* cultured fibroblast model was used to add insight to the pathogenesis of POP. The cells were derived from the uterosacral ligament tissues of POP and NPOP patients and the differences in the expression of *Mfn2* and procollagen as well as the differences in the cell proliferation and apoptosis between the two groups were compared. Consistency in the *Mfn2* and procollagen expression (POP and NPOP groups) was noted between the *in vivo* study conducted previously and the *in vitro* results produced in the current study. The *in vitro* data further indicated that altered *Mfn2* expression resulted in abnormal fibroblast proliferation and function, which might be one of the key factors that contribute to the occurrence and progression of POP.

The comparison between the *in vivo* and *in vitro* studies was achieved by the selection of appropriate patients that exhibited the same inclusion criteria. All participants enrolled were postmenopausal females, who complied with the inclusion criteria reported in the *in vivo* study, whereas the diagnostic criteria for POP were based on the current internationally recognized POP-Q system. In addition, in terms of the enrollment criteria for the control group, only NPOP patients with a matched age, BMI, age of first delivery, number of deliveries, and postmenopausal duration who accepted hysterectomy due to a benign gynecologic disease were enrolled. None of the patients received any estrogenic treatment during the 3 months period before the surgery, and the participants were free of a history of urogenital tract infections, vaginal surgeries, and diseases that affect collagen metabolism. The aforementioned selection criteria

eliminated the impact of female hormones and other factors that could interfere with the validation of the *in vitro* method

Group	*Mfn2*	Procollagen		
		1A1	1A2	3A1
POP ($n = 10$)	0.76 ± 0.21	0.70 ± 0.19	0.82 ± 0.22	0.55 ± 0.13
NPOP ($n = 10$)	0.53 ± 0.17	0.95 ± 0.25	1.26 ± 0.34	0.78 ± 0.23
t	2.425	−2.165	−2.741	−2.147
P	0.032	0.041	0.026	0.045

Compared to the NPOP, in the POP fibroblasts, the mRNA of *Mfn2* was increased, while mRNA of procollagen was decreased. POP: Pelvic organ prolapse; NPOP: Non-POP; *Mfn2*: Mitofusin 2.

Group	Mfn2	Procollagen		
		1A1	1A2	3A1
POP ($n = 10$)	0.84 ± 0.17	0.88 ± 0.21	0.93 ± 0.29	0.54 ± 0.13
NPOP ($n = 10$)	0.53 ± 0.15	1.20 ± 0.29	1.31 ± 0.38	0.91 ± 0.23
t	2.392	−2.418	−2.405	−2.470
P	0.037	0.029	0.033	0.012

Compared to the NPOP, in the POP fibroblasts, the protein expression of Mfn2 was increased, while procollagen expression was decreased. POP: Pelvic organ prolapse; NPOP: Non-POP; Mfn2: Mitofusin 2.

that utilized hosting fibroblasts. The same surgeon with the same experienced surgical team further ensured the homogeneity of the tissue/cell origin.

Abnormal procollagen synthesis and secretion in the fibroblasts might be an important pathogenetic mechanism of POP. Currently, it is reported that total collagen content is reduced in the pelvic floor tissues in the POP patients including the parametrium and vaginal apex,[3,15] periurethral ligaments, or sacral ligaments,[16] indicating that variations in the ECM collagen content significantly affect the supporting effect caused by these proteins to the pelvic floor tissues. Type I collagen is characterized by potent plasticity and anti-tension ability that enables it to counter continuous tension. Type III collagen notably increases the elasticity and ductility, contributing to the responses to periodic tension. Microfibers that comprised certain packing arrangements of Type I and Type III collagens exhibited an effect upon the tensile properties of connective tissues.[17,18] The present study indicated that the protein expression levels of Type I and Type III procollagens in the fibroblasts obtained from the uterosacral ligament tissues of the POP patients were significantly reduced, which might lead to a declined Type I/III collagen secretion into the ECM. In contrast to these observations, the protein expression levels of both Type I and Type III procollagens in the *in vitro* cultured POP fibroblasts were lower compared with those of the NPOP fibroblasts, which was consistent with the results observed in the tissue study. Based on the synthetic process of collagens,
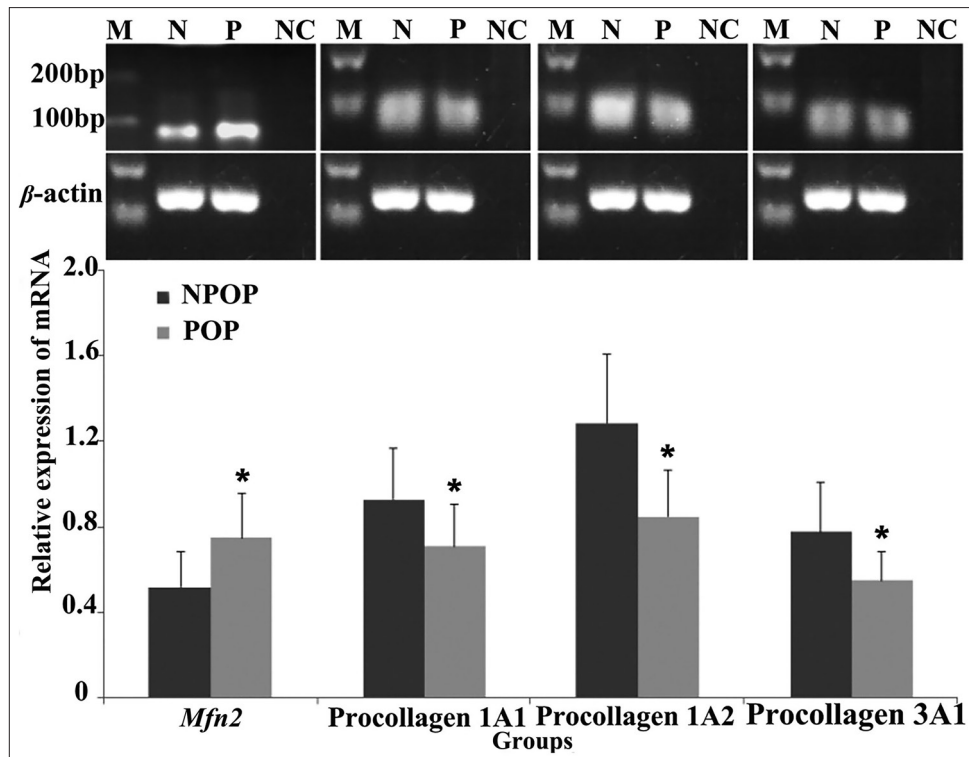

Figure 4: mRNA expression levels of *Mfn2* and procollagen in the POP and NPOP fibroblasts. Compared to the NPOP, the mRNA levels of *Mfn2* in POP fibroblasts were upregulated, while mRNA levels of procollagen were downregulated. $t_{Mfn2(P:N)} = 2.425$, $t_{1A1(P:N)} = −2.165$, $t_{1A2(P:N)} = −2.741$, $t_{3A1(P:N)} = −2.147$, respectively; $P_{Mfn2(P:N)} = 0.032$, $P_{1A1(P:N)} = 0.041$, $P_{1A2(P:N)} = 0.026$, $P_{3A1(P:N)} = 0.045$, respectively. The significance was presented as $*P < 0.05$. M: Marker; N(NPOP): Non-pelvic organ prolapse; P(POP): Pelvic organ prolapse; NC: Negative Control; *Mfn2*: Mitofusin 2.
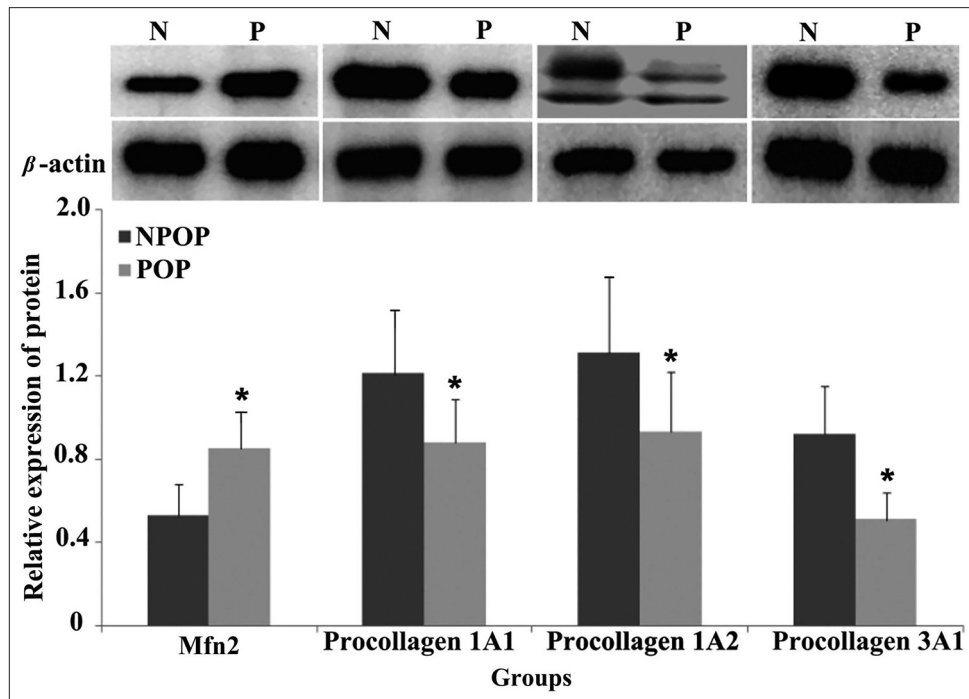
Figure 5: Protein expression levels of Mfn2 and procollagen in the POP and NPOP fibroblasts. Compared to the NPOP, the protein expression levels of Mfn2 in POP fibroblasts were upregulated, while procollagen protein expression levels were downregulated. $t_{Mfn2(P:N)} = 2.392$, $t_{1A1(P:N)} = -2.418$, $t_{1A2(P:N)} = -2.405$, $t_{3A1(P:N)} = -2.470$, respectively; $P_{Mfn2(P:N)} = 0.037$; $P_{1A1(P:N)} = 0.029$; $P_{1A2(P:N)} = 0.033$; $P_{3A1(P:N)} = 0.012$, respectively. The significance was presented as $*P < 0.05$. N(NPOP): Non-pelvic organ prolapse; P(POP): Pelvic organ prolapse; Mfn2: Mitofusin 2.

Table 6: Protein expression levels of procollagen and Mfn2 in POP fibroblasts via Mfn2 inhibition

Group	Mfn2	Procollagen		
		1A1	**1A2**	**3A1**
Sh (n = 10)	0.39 ± 0.07	0.99 ± 0.17	1.23 ± 0.32	1.06 ± 0.19
shNC (n = 10)	0.70 ± 0.14	0.67 ± 0.16	0.92 ± 0.14	0.58 ± 0.12
Control (n = 10)	0.68 ± 0.09	0.57 ± 0.16	0.88 ± 0.18	0.55 ± 0.13
F	26.661	18.277	6.606	36.178
$P_{sh-shNC}$	<0.001	<0.001	0.017	<0.001
$P_{sh-control}$	<0.001	<0.001	0.007	<0.001
$P_{shNC-control}$	0.838	0.445	0.928	0.897

$P<0.05$ indicates significant difference, one-way ANOVA test and univariate analysis. The expression levels of Mfn2 were downregulated in sh group, but the expression levels of procollagen were increased in sh group. POP: Pelvic organ prolapse; Mfn2: Mitofusin 2; sh: POP fibroblasts that were transfected with shRNA positive interference lentiviral sequence; shNC: POP fibroblasts that were transfected with shRNA negative control lentiviral sequence; Control: POP fibroblasts in the absence of lentiviral infection; ANOVA: Analysis of variance.

the declined procollagen production in the fibroblasts will theoretically reduce the syntheses of collagens and collagen fibers in the ECM and result in decreased supporting strength in the pelvic floor tissues.

Mfn2 is a novel gene characterized by Chen *et al.*,[19] which was previously known as hyperplasia suppressor gene. *Mfn2* is a vascular smooth muscle cell (VSMC) proliferation suppressor as demonstrated by studies that

investigated the proliferation of rat VSMC. The human Mfn2 protein comprises 757 amino acids and possesses two transmembrane domains that span the outer mitochondrial membrane and are scattered along the mitochondrial networks.[20-22] Mitochondria are considered the main sites for intracellular oxidative phosphorylation and synthesis of adenosine triphosphate, providing energy supplies for various cellular activities. Previous studies have demonstrated that the main function of *Mfn2* was to mediate mitochondrial fusion[4] and regulate mitochondrial morphology.[4,23] This renders mitochondria function as a dynamic network that constantly undergoes fusion and fission.[4,23] However, *Mfn2* overexpression affects mitochondrial fusion,[4,24] causing mitochondrial dysfunction that acts as one of the main pro-aging factors.[25] Mitochondria have an independent genetic system, and the accumulation of mitochondrial DNA mutations promotes tissue aging. Therefore, mitochondrial fusion plays a key role in the prevention of mitochondrial DNA mutations.[26,27] Thus, the examination of the expression levels of *Mfn2* in the pelvic floor tissues is considered a crucial factor in the investigation of the pathogenesis of POP. The majority of studies to date have focused on tissues such as the skeletal muscle, myocardium, vascular endothelium, and/or smooth muscle cell, while a limited number of studies have reported the roles of fibroblasts in the pelvic floor tissues. Since POP is a disease associated with aging,[28] abnormal *Mfn2* expression possibly correlates with the pathogenesis of POP. In the present and the previous studies conducted by our group, the difference in the expression levels of *Mfn2* between the POP and NPOP fibroblasts
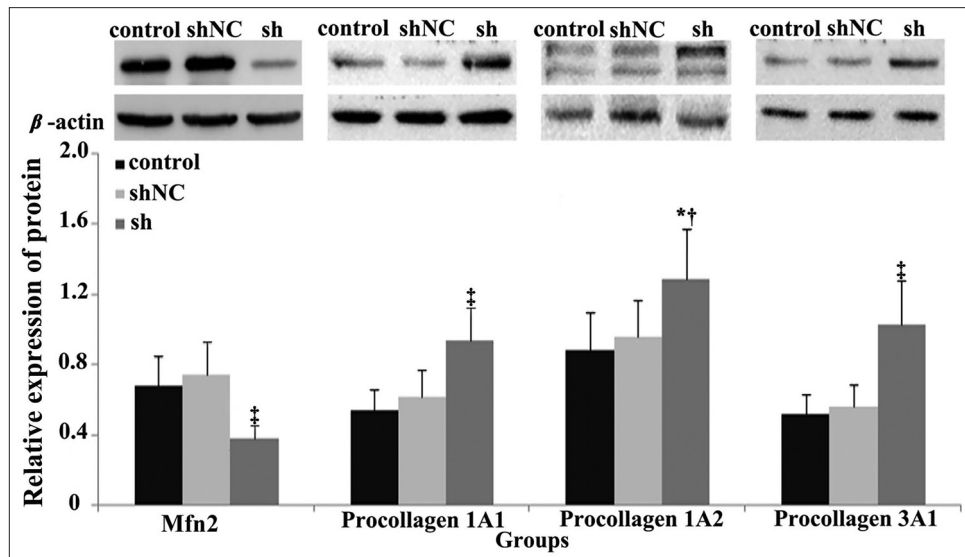
Figure 6: Protein expression levels of Mfn2 and procollagen in RNAi-transfected fibroblasts. Compared to the control and shNC groups, the protein expression levels of Mfn2 were downregulated in sh group, but the protein expression levels of pro-collagen were upregulated. $F_{Mfn2(sh-shNC-control)} = 26.661$; $F_{1A1(sh-shNC-control)} = 18.277$; $F_{1A2(sh-shNC-control)} = 6.606$; $F_{3A1(sh-shNC-control)} = 36.178$, respectively. $P_{Mfn2(sh:shNC)} < 0.001$, $P_{Mfn2(sh:control)} < 0.001$, $P_{Mfn2(shNC:control)} = 0.838$, respectively; $P_{1A1(sh:shNC)} < 0.001$, $P_{1A1(sh:control)} < 0.001$, $P_{1A1(shNC:control)} = 0.445$, respectively; $P_{1A2(sh:shNC)} = 0.017$; $P_{1A2(sh:control)} = 0.007$; $P_{1A2(shNC:control)} = 0.928$, respectively; $P_{3A1(sh:shNC)} < 0.001$; $P_{3A1(sh:control)} < 0.001$; $P_{3A1(shNC:control)} = 0.897$, respectively. $*P < 0.05$, $†P < 0.01$, $‡P < 0.001$. sh: POP fibroblasts that were transfected with shRNA positive interference lentiviral sequence; shNC: POP fibroblasts that were transfected with shRNA negative control lentiviral sequence; control: POP fibroblasts in the absence of lentiviral infection. Mfn2: Mitofusin 2; POP: Pelvic organ prolapse.

was initially compared. The data indicated an upregulated expression of *Mfn2* in the POP fibroblasts that were directly obtained from the uterosacral ligament tissues (previous *in vivo* study). The same pattern was noted in the cultured POP fibroblasts that originated from the uterosacral ligament tissues (present *in vitro* study). The increase in the expression of the *Mfn2* levels indicated a significant negative correlation with the expression of procollagen. The expression of *Mfn2* was downregulated in RNAi-transfected fibroblasts, but the expression of pro-collagen increased. The results indicate that *Mfn2* might inhibit the procollagen expression in the fibroblasts, whereas *Mfn2* overexpression promotes aging, which might also be responsible for the slow growth of POP fibroblasts.

There were some limitations in the study. For example, this research did not focus on the mitochondrial energy metabolism and mitophagy. The link between *Mfn2* and procollagen remains unknown and can be investigated in the future.

In summary, we concluded that lower proliferation rate and viability were observed in the *in vitro* cultured POP fibroblasts compared with the NPOP fibroblasts. In the POP fibroblasts, *Mfn2* expression was upregulated while procollagen expression was downregulated, which was consistent with our previous *in vivo* results. The results indicate that the genetic properties of the cultured fibroblasts remain unchanged and that this *in vitro* model is suitable for subsequent studies.

In the present study, when the expression of *Mfn2* was downregulated, the expression levels of the procollagen forms were upregulated, and the support of the pelvic floor

was enhanced. This might provide the basis for a new therapy of POP.

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Conflicts of interest

There are no conflicts of interest.

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