# MicroRNA-30d and microRNA-181a regulate the expression of HOXA11 in the uterosacral ligaments and are overexpressed in pelvic organ prolapse

Myung Jae Jeon

Department of Medicine

The Graduate School, Yonsei University

# MicroRNA-30d and microRNA-181a regulate the expression of HOXA11 in the uterosacral ligaments and are overexpressed in pelvic organ prolapse

Directed by Professor Sang Wook Bai

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

Myung Jae Jeon

December 2013

This certifies that the Doctoral Dissertation of Myung Jae Jeon is approved.

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Thesis Supervisor: Sang Wook Bai

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Thesis Committee Member: Sei Kwang Kim

_____

Thesis Committee Member: Hoguen Kim

_____

Thesis Committee Member: Jong Rak Choi

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Thesis Committee Member: Hee Dong Chae

The Graduate School
Yonsei University

December 2013

# ACKNOWLEDGEMENTS

It is such a pleasure to complete my dissertation and finally be awarded a Ph.D. This was not an easy task, but I somehow survived and finished it. I feel that I can now venture back into the field of urogynecology as a mature explorer.

In retrospect, Prof. Sang Wook Bai taught me everything I know about urogynecology. He has been, and will continue to be, a perfect model of a scholar, physician and professor to me. I really appreciate his advice, endless efforts, encouragement and sharp comments, not only regarding my academic works but also regarding my academic life.

I would like to thank Prof. Sei Kwang Kim, Prof. Hogeun Kim, Prof. Jong Rak Choi and Prof. Hee Dong Chae for their valuable comments and advice regarding my works. They also showed me how successful scholars behave in teaching, researching, and interacting with others.

My special thanks also go to my family who have always been on my side and supported me.

*Myung Jae Jeon*

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<ABSTRACT>

# MicroRNA-30d and microRNA-181a regulate the expression of HOXA11 in the uterosacral ligaments and are overexpressed in pelvic organ prolapse

Myung Jae Jeon

*Department of Medicine*

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**Objectives:** The balance between the synthesis and degradation of collagen is important in maintaining the structural integrity and tensile strength of pelvic supportive connective tissues. Homeobox A11 (HOXA11) is a key transcriptional factor that regulates collagen metabolism, and its expression is decreased in the uterosacral ligaments (USLs) of women with pelvic organ prolapse (POP). The aim of the present study was to identify specific microRNAs (miRNAs) involved in the regulation of HOXA11 expression in the USLs and to define their biologically functional effects.

**Methods:** miRNA expression was assessed in the USLs of women with and without

POP using microarray and quantitative real-time polymerase chain reaction (RT-PCR) analysis. To determine the role of selected miRNAs in the regulation of HOXA11, 293T cells were transfected with miR mimic, anti-miR or negative controls. Then, quantitative RT-PCR, Western blotting and luciferase reporter assays were performed.

**Results:** MiR-30d and miR-181a were overexpressed in the USLs of women with POP, and the expression of both miRNAs was inversely correlated with HOXA11 mRNA expression. In cultured 293T cells, the overexpression of miR-30d/181a suppressed HOXA11 mRNA and protein levels, whereas knockdown of these miRNAs enhanced HOXA11 levels. Cotransfection of a luciferase reporter plasmid containing the 3′-UTR of HOXA11 with miR-30d/181a mimics resulted in decreased relative luciferase activity. Conversely, cotransfection with anti-miR-30d/181a increased the relative luciferase activity.

**Conclusion:** These results indicate that both miR-30d and miR-181a directly downregulate HOXA11 expression at the posttranscriptional level and that the decreased HOXA11 expression in the USLs of women with POP might be caused by the aberrant expression of these miRNAs. Therefore, therapeutic approaches aimed at decreasing these miRNAs might be useful for restoring the altered collagen metabolism and homeostasis in the USLs that leads to POP.

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**Key words:** pelvic organ prolapse, uterosacral ligament, HOXA11, microRNA-30d, microRNA-181a

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# I. INTRODUCTION

Pelvic organ prolapse (POP) is the downward descent of the pelvic organs that results in a protrusion of the vagina, uterus or both. While not life-threatening, it often causes bladder, bowel and pelvic symptoms that can have an adverse effect on a woman's daily activities and quality of life.[1] POP is also a significant economic burden to women and healthcare systems. It affects almost half of all women over 50 years of age, with a lifetime prevalence of 30-50%,[2] and one in five women will undergo surgery for POP in their lifetime.[3] In the United States, the direct cost of prolapse surgery is greater than 1 billion dollars per year,[2] and as the population is aging, the demand for POP care is estimated to double over the next 40 years.[4]

Nonetheless, relatively little is known about the underlying pathophysiology of POP. Although several factors, including advancing age, vaginal childbirth and obesity, increase the risk of POP,[1] these factors do not fully explain the development of POP. Moreover, the need to improve treatment strategies is highlighted by the high failure rate after surgery. Approximately 13% of women require reoperation for recurrent prolapse within 5 years,[5] and this is related to the use of native tissues that are already attenuated. Therefore, the identification of the critical molecular pathways that lead to the weakness of the pelvic floor supportive tissues is essential for developing effective prevention and treatment strategies for POP.

Homeobox (HOX) genes are evolutionally conserved genes encoding transcriptional factors that regulate embryonic development and adult function of the female reproductive tract.[6] Recently, Connell et al. found that HOXA11 knockout mice lack the uterosacral ligament (USL), the key supportive structure of the uterus and vagina, and that the expression of HOXA11 mRNA was decreased in the USLs of women with POP compared with controls.[7] In a subsequent experiment using short hairpin RNA transfection, they found that the knockdown of HOXA11 decreased collagen and increased the expression of matrix metalloproteinase in the USLs of mice.[8] HOXA11 has also been reported to promote the proliferation of fibroblasts, the major cell type that produces collagen, in vitro.[9] These findings imply that the precise regulation of HOXA11 is crucial for the maintenance of adequate collagen contents

within the USLs and that dysregulation of HOXA11 contributes to alterations in the biochemical strength of the USLs, leading to POP. However, it is unknown how HOXA11 transcripts are regulated in the USLs.

MicroRNAs (miRNAs) are small (~22 nucleotides), endogenous RNA molecules that play important regulatory roles by targeting mRNAs for cleavage or translational repression. Although miRNA genes represent nearly 1% of the genome, it is estimated that ~30% of the protein-encoding genes are regulated by at least one miRNA.[10,11] They have been shown to play key roles in diverse regulatory pathways including developmental processes, cell growth, differentiation and apoptosis,[12] and it has been reported that aberrant miRNA expression is associated with various human diseases.[13,14] However, the role of miRNAs in the pathogenesis of POP has not been addressed. The aim of the present study was to identify the specific miRNAs involved in the regulation of HOXA11 expression in the USLs and to define their biologically functional effects.

# II. MATERIALS AND METHODS

## 1. Tissue collection

All experiments were performed following the approval of the review board for human research of Seoul National University Hospital (H-1302-087-466). Samples were collected between July 2010 and July 2012 from women undergoing hysterectomy. Informed consent was obtained from all of the participating women. Prior to surgery, a pelvic examination using the POP-quantification system was performed to evaluate the presence of POP. Data regarding age, vaginal parity, body mass index, and menopausal status were also recorded. Menopause was defined as the cessation of menses for at least one year.

At the time of surgery, USL samples 1 × 1 cm in size were collected from the area of insertion into the cervix, a location where the ligament is consistently identifiable. The samples were immediately snap frozen in liquid nitrogen and kept at -80℃ until RNA extraction was performed.

## 2. Cell culture

293T cells, derived from human embryonic kidney, were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, St Louis, MO, USA), containing 10% fetal bovine serum without antibiotics-antimycotics, at 37℃ in a humidified 5% $CO_2$ incubator until the cells reached 40% to 50% confluence. Culture media were replaced with fresh media every 2-3 days. The cells were used between

passages 5 and 10.

**3. miRNA microarray**

miRNAs were extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocols. Purified miRNAs were labeled using the mirVana miRNA Array Labeling kit and coupled to the Cy5 Post-Labeling Reactive Dye (Amersham, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The labeled samples were washed and hybridized in duplicate to mirVana miRNA Bioarrays (Ambion) using the mirVana miRNA Bioarray Essentials kit. Fluorescence intensities were processed and measured using the GeneChip scanner 3000 7G (Agilent Technologies, Santa Clara, CA, USA). The levels of miRNA hybridization were determined using GenePix Pro 6.0 software as recommended by the manufacturer. The background-adjusted intensity for each miRNA was subjected to a global variance stabilization normalization procedure.[15] miRNAs were considered to be overexpressed only if the differences were determined to be significant by a two-sample t-test ($p<0.05$) and on average showed at least a 1.5-fold increase in patient samples compared with matched controls. Heatmap analysis and hierarchical clustering were performed using R project software.

**4. Transfection experiments**

293T cells were plated at a cellular density of $1 \times 10^6$ per well on 100-mm culture dishes and cultured overnight. The cells were then transfected with double-stranded

RNA oligos comprising the mature miRNA (miR mimic), RNA oligonucleotides complementary to mature miR (anti-miR) or negative controls (miR mimic/anti-miR negative control; GenePharma Co., Ltd, Shanghai, China) at a final concentration of 100 nM with the use of Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, cellular lysates were collected for RNA or protein isolation. The transfection efficiency was ~80%, as measured by the uptake of a FAM-labeled negative control.

**5. Quantitative real-time polymerase chain reaction (RT-PCR) analysis**

Total RNA was extracted from cells or tissue samples using the mirVana miRNA isolation kit (Ambion). cDNA was generated using the GoScript RT system (Promega, Madison, WI, USA) according to the manufacturer's protocol. PCR amplification was carried out with the following primers: HOXA11, forward 5′-GTACTTACTACGTCTCGGGTCCAG-3′ and reverse 5'-AGTCTCTGTGCACGA GCTCCT-3′; and β-actin, forward 5′-CGTACCACTGGCATCT GAT-3′ and reverse 5′-GTGTTGGCGTACAGGTCTTTG-3′. PCR reactions were performed using the SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. Each 20 μL reaction mixture contained 10 μL of 2× SYBR Premix Ex Taq, 0.4 μL of 50× ROX Reference Dye, 4 μL of 10 μM forward and reverse primer mix, 3.6 μL of nuclease-free water and 2 μL of cDNA. The reaction mixtures were preincubated at 95℃ for 5 sec followed by 40 cycles of denaturation at 95℃ for 5 sec, annealing at 60℃ for 34 sec and extension at 95℃ for 15 sec, 60℃ for 1 min and

95℃ for 15 sec. To detect miRNA expression, the RT reaction was performed using TaqMan miRNA assays (Applied Biosystems, Carlsbad, CA, USA). The quantification of miRNAs was carried out using TaqMan miRNA assays (Applied Biosystems). The PCR amplification was conducted using the TaqMan Universal PCR Master Mix according to the protocol supplied by the manufacturer. The samples were analyzed with the 7500 real-time PCR system (Applied Biosystems). All PCRs were performed in triplicate, and the specificity of each reaction was determined by melting curve analysis at the dissociation stage. For relative quantification, the RT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method, where β-actin and U6B were used as internal controls for HOXA11 and miRNAs, respectively.

**6. Western blot analysis**

Cells were lysed in 200 μl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 8.0), 100 mM PMSF) with a protease inhibitor and centrifuged at 14,000 × g for 10 min at 4℃. The proteins in the supernatant were mixed with denaturing sample buffer (1:1) and boiled for 5 min at 94℃. Equal amounts of protein (30 μg) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with TBST containing 5% nonfat dry milk for 1 h at 4℃ and incubated with anti-HOXA11 (1:1,000 Abcam, Cambridge, UK) antibodies overnight at 4℃. An anti-β-actin antibody (1:5,000 Sigma-Aldrich, St. Louis, MO, USA) was used as a control. After

washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam) for 1 h at 4℃ and washed again in TBST. The signal was detected using an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL, USA) and the intensity was quantified using ImageJ software.

**7. Luciferase reporter assay**

To validate the HOXA11 3′-untranslated region (3′-UTR) as a target of miR-30d and miR-181a, in vitro assays using the miTarget miRNA 3′-UTR target clones (HmiT008983-MT01, Genecopoeia, Rockville, MD, USA) were performed. These miRNA target clones consisted of the pEZX-MT01 vector containing the coding sequences of both firefly and Renilla luciferase and the full 3′-UTR of the HOXA11 transcript (accession number: NM_005523.5) inserted downstream of the firefly luciferase sequence. According to TargetScan (www.targetscan.org), the binding sites of miR-30d and miR-181a are predicted to be located at positions 1173 to 1180 and 1219 to 1226, respectively. For mutagenesis assays, these two miRNA-binding sites within the 3′-UTR of the HOXA11 transcript were deleted. After heat-shock transformation in competent *Escherichia coli* cells (One Shot TOP 10 competent cells; Invitrogen), the plasmids were amplified in Luria-Bertani medium supplemented with 50 μg/ml kanamycin (Bio Basic Inc., Markham, ON, Canada). Plasmid DNA was prepared on columns (NucleoBond PC 500; Macherey-Nagel, Düren, Germany). The identity of the amplified plasmids was confirmed by capillary

sequencing (ABI 3730XL, Applied Biosystems) using the sequencing primers 5′-GATCCGCGAGATCCT GAT-3′ (forward) and 5′-CCTATTGGCGTTACTATG -3′ (reverse).

293T cells were plated ($1 \times 10^4$/well) in 96-well plates. A total of 100 ng of plasmid DNA was cotransfected with miRNA mimic, anti-miR or negative controls as described above. Luciferase assays were performed 48 hours after transfection using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase expression for each sample. Each experiment was conducted in triplicate.

**8. Statistical analysis**

Statistical analyses were performed using SPSS 19.0 for Windows (SPSS, Chicago, IL, USA). The normality of the data was assessed using the Shapiro-Wilk test. Comparisons between two groups were performed using the two-sample t-test or the Mann-Whitney U test for continuous variables and the chi-square test for categorical variables. For comparisons among >2 groups, one-way analysis of variance was performed, and Dunnett's procedure was used for multiple comparisons. Correlation analyses were performed using Pearson's correlation analyses. All of the statistical tests were two-tailed, and p-values less than 0.05 were considered statistically significant.

# III. RESULTS

**1. The expression of miR-30d and miR-181a is significantly increased in the USLs of POP patients and is inversely correlated with HOXA11 expression.**

To assess miRNA expression profiles, surgical USL specimens taken from women with and without POP were used. The patient group consisted of 38 women who underwent hysterectomy with sacrocolpopexy for symptomatic POP that was stage II or greater. The control group consisted of 38 age-matched women with POP-quantification stage 0 or I who received operations for benign gynecologic indications. There were no differences in mean age, vaginal parity, body mass index or menopausal status between the two groups (Table 1). None of the postmenopausal women in either group were undergoing hormone replacement therapy. From each group, eight samples were used for microarray, and the remaining 30 samples were used for quantitative RT- PCR validation.

Table 1. Clinical characteristics of enrolled women with and without POP

| | Patient (n=38) | Control (n=38) | P-value |
|---|---|---|---|
| Age (yr), mean ± SD | 56.0 ± 9.5 | 54.1 ± 7.9 | 0.35 |
| Vaginal parity, median (IQR) | 2 (1) | 2 (1) | 0.21 |
| BMI (kg/m$^2$), mean ± SD | 24.8 ± 3.0 | 24.4 ± 3.3 | 0.66 |
| Menopause, n (%) | 26 (68.4) | 21 (55.3) | 0.24 |
| POP-Q stage, n (%) | | | <0.01 |
| 0-I | 0 | 38 (100) | |
| II | 10 (26.3) | 0 | |
| III | 20 (52.6) | 0 | |
| IV | 8 (21.1) | 0 | |

POP, pelvic organ prolapse; BMI, body mass index; POP-Q, pelvic organ prolapse-quantification; SD, standard deviation; IQR, interquartile range

In an attempt to identify the miRNAs involved in the pathogenesis of POP, miRNA expression profiles in the USLs of POP patients and controls were compared using miRNA microarray. The miRNAs that were overexpressed differentially in POP patients included miR-222, miR-720, miR-1260a, miR-1260b, miR-99b, miR-3653, miR-92a, miR-423, miR-30d, miR-130b, miR-3607, miR-4286, miR-140, miR-181c, miR-1274a, miR-342, miR-151a and miR-181a (Table 2). The expression of miR-30d and miR-181a was verified by quantitative RT-PCR analysis and paralleled the expression pattern observed by microarray (Figure 1).

Table 2. Highly overexpressed miRNAs in the USLs of women with POP

| miRNA | Patients (mean ± SD) | Controls (mean ± SD) | Fold change | P-value |
|---|---|---|---|---|
| miR-222 | 8.8 ± 0.7 | 7.9 ± 0.5 | 1.9 | 0.01 |
| miR-720 | 15.1 ± 0.6 | 14.2 ± 0.5 | 1.9 | <0.01 |
| miR-1260b | 11.9 ± 0.4 | 11.0 ± 0.4 | 1.9 | <0.01 |
| miR-1260a | 12.8 ± 0.6 | 12.0 ± 0.4 | 1.8 | <0.01 |
| miR-99b | 9.4 ± 0.5 | 8.7 ± 0.6 | 1.7 | <0.01 |
| miR-3653 | 9.1 ± 0.6 | 8.3 ± 0.4 | 1.7 | <0.01 |
| miR-92a | 10.6 ± 0.5 | 9.9 ± 0.4 | 1.6 | <0.01 |
| miR-423 | 8.4 ± 0.6 | 7.7 ± 0.3 | 1.6 | 0.01 |
| miR-30d | 10.4 ± 0.3 | 9.7 ± 0.6 | 1.6 | 0.01 |
| miR-130b | 7.1 ± 0.4 | 6.4 ± 0.6 | 1.6 | 0.02 |
| miR-3607 | 6.4 ± 0.3 | 5.7 ± 0.5 | 1.6 | 0.01 |
| miR-4286 | 13.4 ± 0.4 | 12.7 ± 0.5 | 1.6 | 0.02 |
| miR-140 | 11.2 ± 0.4 | 10.6 ± 0.6 | 1.6 | 0.02 |
| miR-181c | 6.5 ± 0.4 | 5.8 ± 0.5 | 1.6 | 0.02 |
| miR-1274a | 11.6 ± 0.4 | 11.0 ± 0.5 | 1.6 | 0.01 |
| miR-342 | 10.1 ± 0.4 | 9.5 ± 0.5 | 1.5 | 0.01 |
| miR-151a | 8.7 ± 0.3 | 8.1 ± 0.5 | 1.5 | <0.01 |
| miR-181a | 9.1 ± 0.3 | 8.5 ± 0.5 | 1.5 | <0.01 |

USL, uterosacral ligaments; POP, pelvic organ prolapse

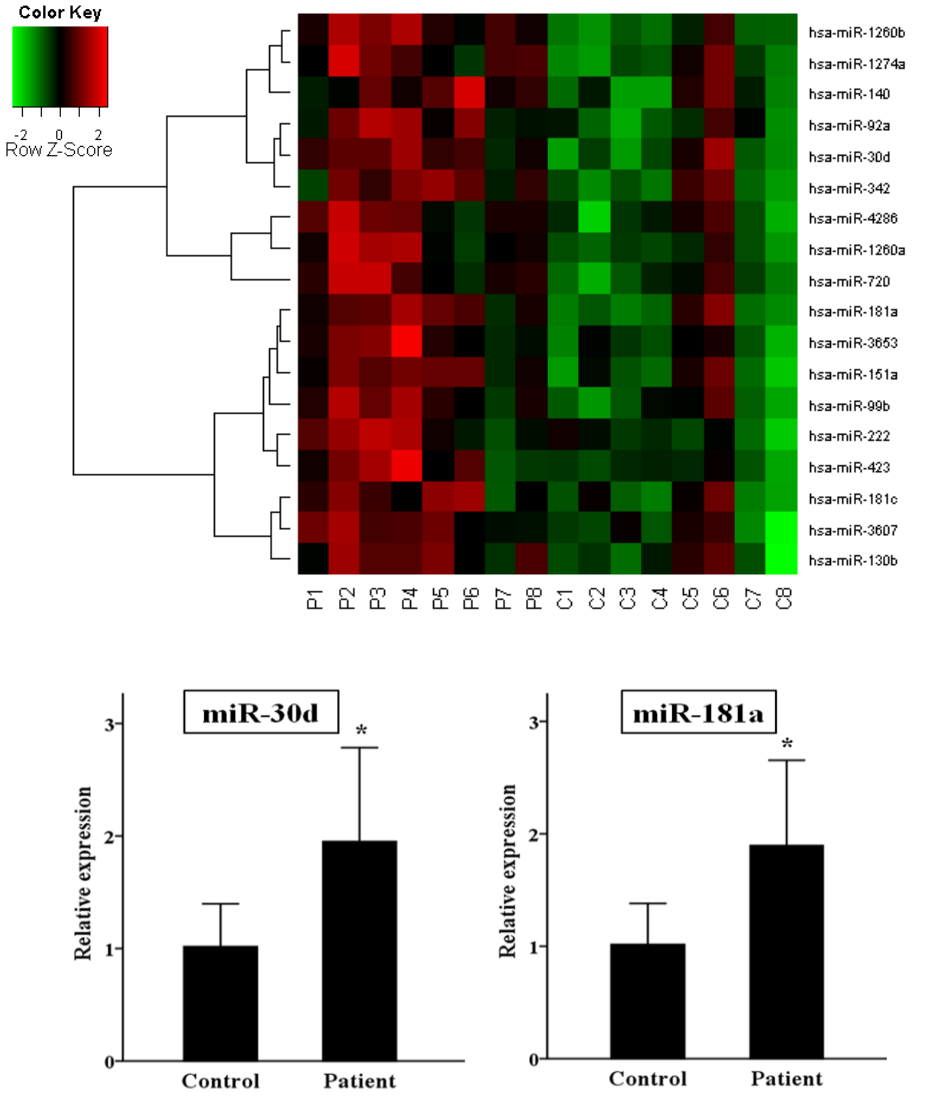


Figure 1. Differential miRNA expression profiles in the USLs of women with POP (P) and controls (C), as shown in the form of heatmaps (upper panel). Eighteen miRNAs were overexpressed in the USLs of women with POP compared with controls. The expression levels of miR-30d and miR-181a were confirmed by quantitative RT-PCR (lower panel). Quantitative data representing the mean and standard deviation are presented in the bar graph. $^{*}P<0.01$ compared with expression in the USLs from the controls.

To identify specific miRNAs that could regulate HOXA11, biocomputational prediction algorithms from three different programs (miRanda, TargetScan and PicTar) were used to provide a good balance of sensitivity and specificity and reduce the risk of predicting false positive targets.[16] Approximately 10 miRNAs were identified as potentially targeting the HOXA11 mRNA. The two most notable miRNAs were miR-30d and miR-181a, which were also significantly overexpressed in the microarray. The alignment of the 3′-UTR of HOXA11 revealed that the putative target sequences for miR-30d and miR-181a are highly conserved across species (Figure 2).

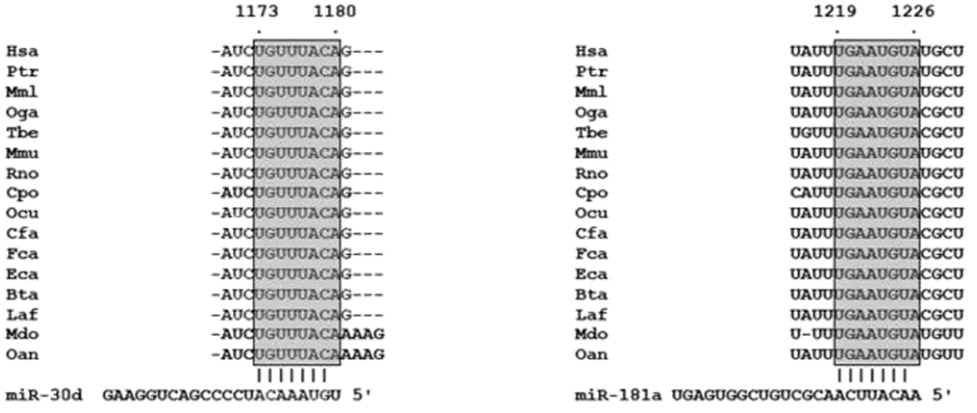


Figure 2. Sequence alignment between miR-30d/181a and the 3′-UTR of HOXA11 in several species. The seed sequences of HOXA11 3′-UTR targeted by miR-30d and miR-181a are highly conserved across species (from TargetScan). The number represents the position of the "seed region" matched to miR-30d and miR-181a within the UTR sequences. *Hsa*, human; *Ptr*, chimpanzee; *Mml*, rhesus; *Oga*, bushbaby; *Tbe*, treeshrew, *Mmu*, mouse; *Rno*, rat; *Cpo*, guinea pig; *Ocu*, rabbit; *Cfa*, dog; *Fca*, cat; *Eca*, horse; *Bta*, cow; *Laf*, elephant; *Mdo*, opossum; *Oan*, platypus.

Next, further quantitative RT-PCR analyses for the remaining samples not used in the microarray analysis were performed to investigate the levels of miR-30d/181a and HOXA11 mRNA expression and the correlation between these miRNAs and HOXA11 expression. Compared to controls, the expression levels of miR-30d and miR-181a were significantly increased in POP patients, whereas HOXA11 mRNA expression was significantly decreased (Figure 3). There were significant inverse correlations between the expression of miR-30d/181a and HOXA11 mRNA expression (Pearson correlation coefficient= -0.78 and -0.73, respectively; $p<0.01$).

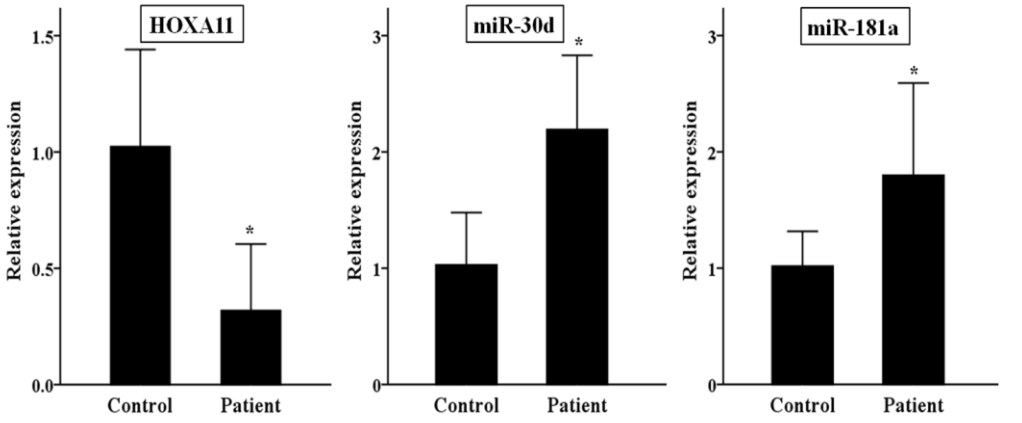


Figure 3. Quantitative RT-PCR analyses of two miRNAs and HOXA11 expression in the USLs of 30 women with POP and 30 controls. The expression of miR-30d and miR-181a was significantly increased in women with POP compared to controls, whereas HOXA11 mRNA expression was significantly decreased in POP patients versus controls. Quantitative data representing the mean and standard deviation are presented in the bar graph. $^{*}P<0.01$ compared with expression in the USLs from the controls.

**2. Both miR-30d and miR-181a regulate HOXA11 mRNA and protein levels.**

Next, a series of functional studies was performed to determine the role of miR-30d and miR-181a in the regulation of HOXA11. First, whether the overexpression of miR-30d or miR-181a was sufficient to repress HOXA11 levels was tested. These miRNAs were overexpressed using specific miR mimics. Quantitative RT-PCR showed that the expression of miR-30d and miR-181a was significantly increased (Figure 4), and this repressed endogenous HOXA11 mRNA and protein levels in 293T cells (Figure 5). Next, cultured 293T cells were transfected with anti-miR-30d, anti-miR-181a or a negative control. The successful knockdown of these miRNAs in the 293T cells was confirmed (Figure 4) and demonstrated that knockdown of miR-30d or miR-181a enhances HOXA11 mRNA and protein levels (Figure 5).

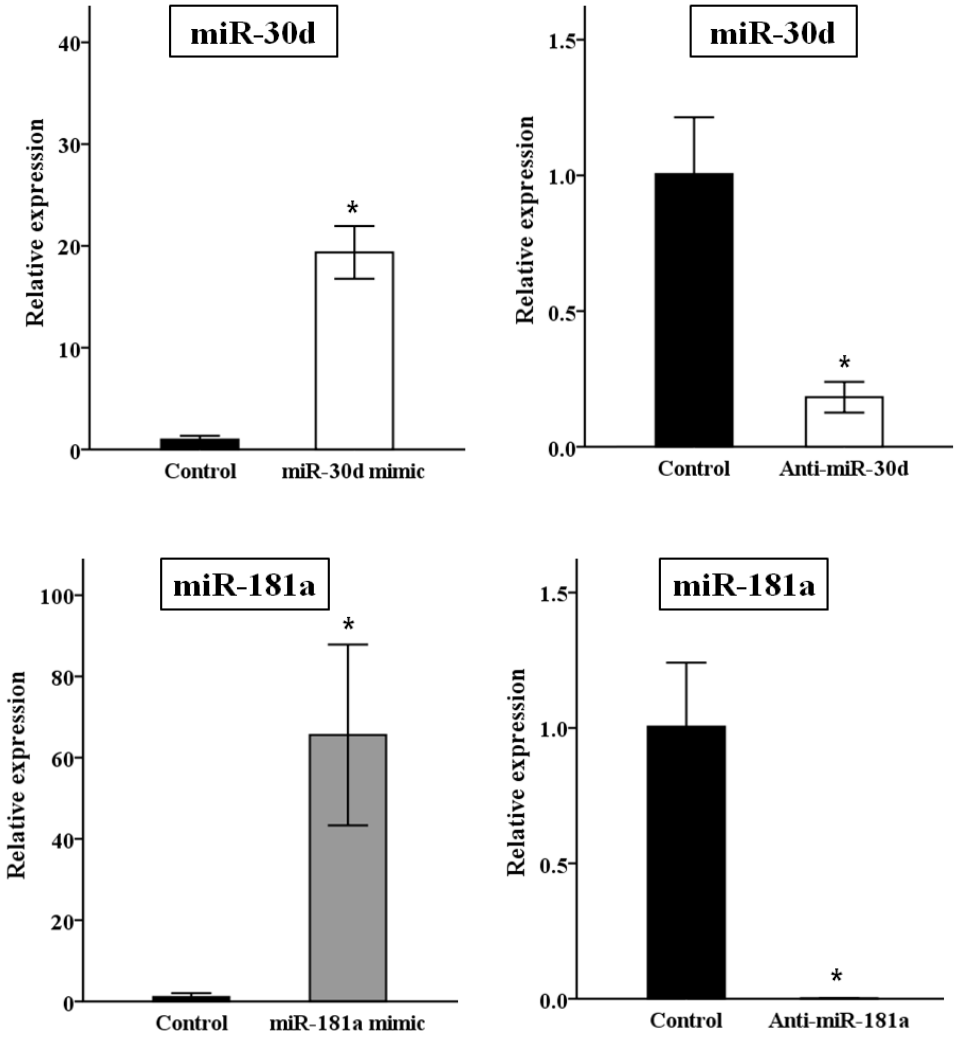


Figure 4. Levels of overexpression and knockdown of miRNAs. Quantitative RT-PCR analysis for miR-30d (upper panel) or miR-181a (lower panel) in 293T cells transfected with a specific miR-mimic or anti-miR, indicating the successful overexpression or knockdown. Quantitative data representing the mean and standard deviation are presented in the bar graph. $^{*}P<0.01$ compared with negative controls.

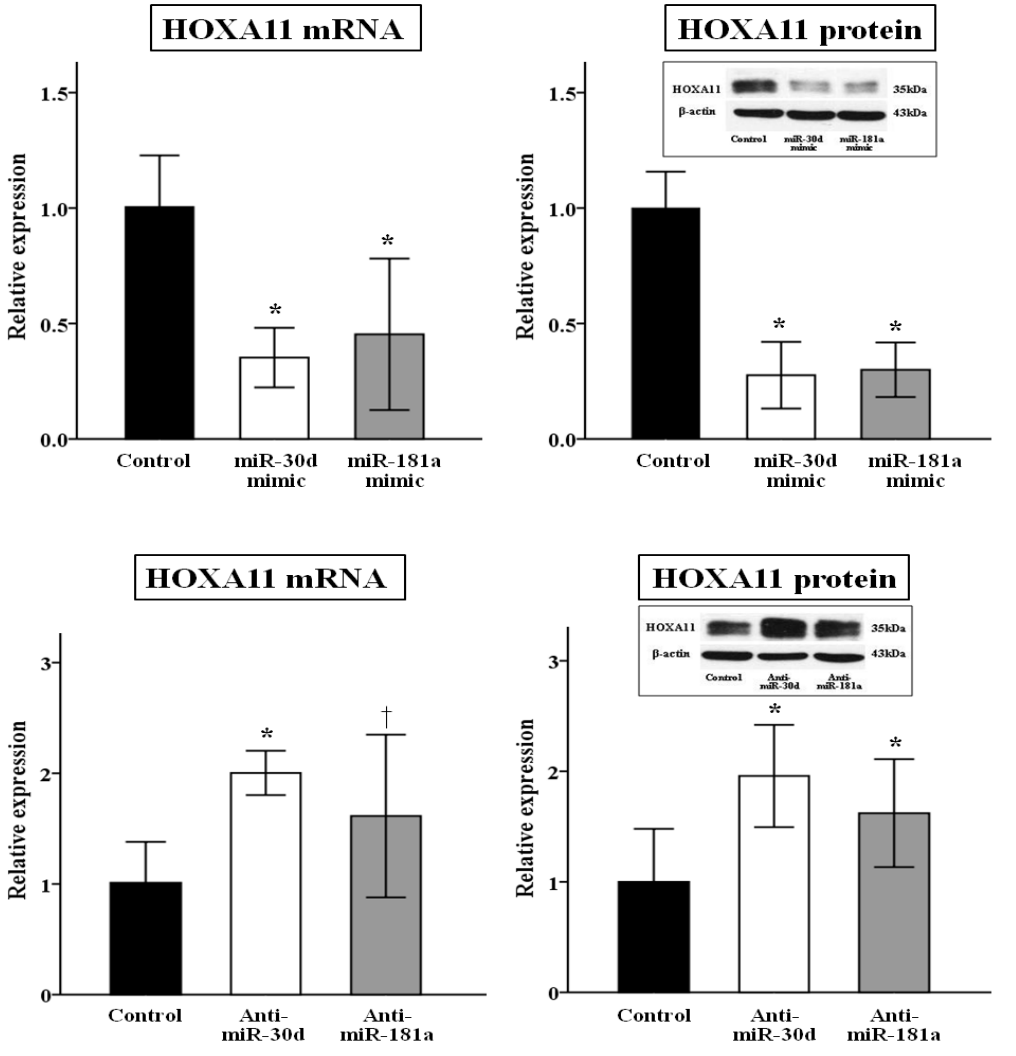


Figure 5. Effects of the overexpression and knockdown of miR-30d/miR-181a on the expression of HOXA11. Transfection with miR-30d/181a mimic decreased HOXA11 mRNA and protein levels in 293T cells (upper panel). Conversely, transfection with anti-miR-30d/181a resulted in an increase in HOXA11 mRNA and protein levels (lower panel). mRNA and protein expression levels were analyzed by quantitative RT-PCR and Western blot analysis, respectively. Quantitative data representing the mean and standard deviation are presented in the bar graph. $^{*}P<0.01$, $^{\dagger}P<0.05$ compared with negative controls.

**3. HOXA11 is a direct target of miR-30d and miR-181a.**

The above findings suggest that HOXA11 is regulated by miR-30d and miR-181a in the USLs. However, the regulation of HOXA11 by these miRNAs may be indirect. To assess whether miR-30d and miR-181a can directly alter the expression of HOXA11, a luciferase expression plasmid containing the full-length 3′-UTR of the HOXA11 transcript was transfected into 293T cells. Cotransfection of this luciferase expression plasmid with miR-30d or miR-181a mimic, but not a negative control, resulted in a significant decrease in relative luciferase activity. Additionally, the deletion of these two miRNA-binding sites completely abolished miR-30d- and miR-181a-mediated repression, demonstrating the specificity of repression (Figure 6).

Because miR-30d and miR-181a mimics could inhibit HOXA11 3'-UTR luciferase activity, inhibitors to these two miRNAs were assessed to determine if they could exert the opposite effect. Not surprisingly, cotransfection with anti-miR-30d or anti-miR-181a significantly increased the relative luciferase activity (Figure 6). Taken together, these results show that both miR-30d and miR-181a can directly influence HOXA11 through specific binding to its 3′-UTR.

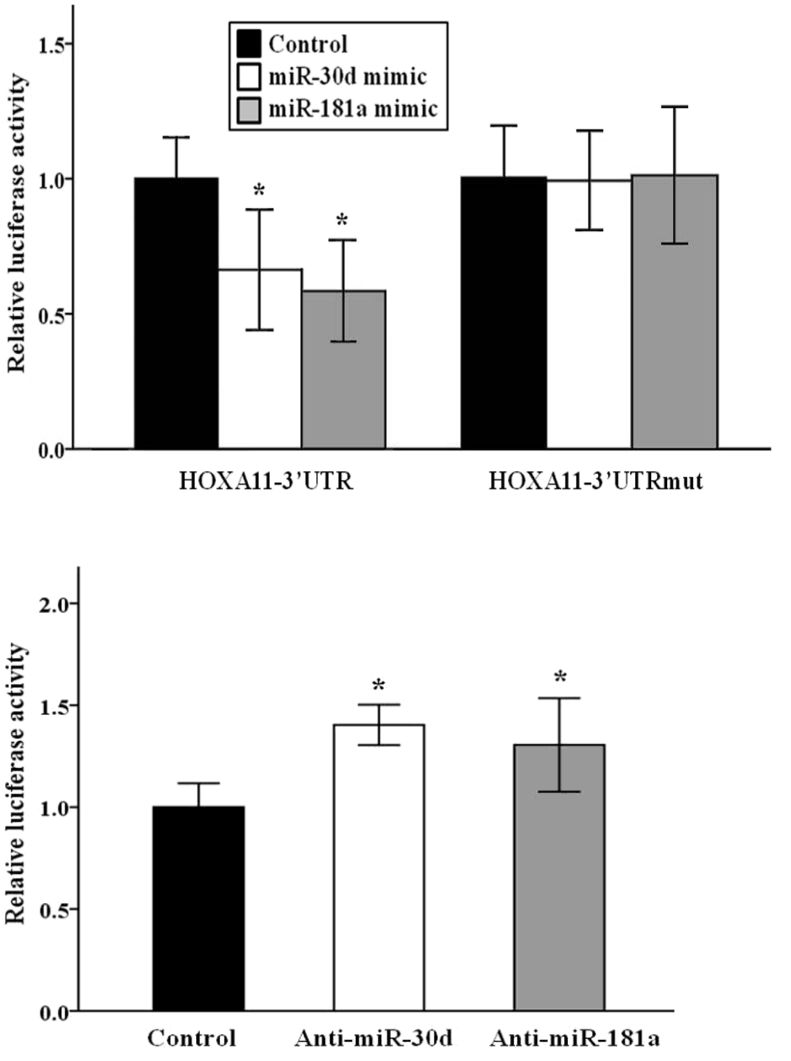


Figure 6. Direct regulation of HOXA11 by miR-30d and miR-181a. 293T cells were transfected with luciferase reporter plasmid containing 3′-UTR of HOXA11, together with miR mimic, anti-miR or negative controls. miR-30d or miR-181a overexpression significantly decreased the relative luciferase activity of the wild-type 3′-UTR but not the mutant 3′-UTR in which these two miRNA-binding sites were deleted (upper panel). Conversely, knockdown of miR-30d/181a significantly increased the relative luciferase activity (lower panel). Quantitative data representing the mean and standard deviation are presented in the bar graph. *P<0.01 compared with negative controls.

# IV. DISCUSSION

Despite its high incidence and significant clinical impacts, the molecular pathogenesis of POP remains unclear. The present study provides evidence that aberrant miRNA expression is involved in a critical pathway that leads to the attenuation of pelvic floor supportive tissues. Specifically, decreased HOXA11 expression in the USLs of women with POP was associated with the overexpression of miR-30d and miR-180a.

Pelvic organ support is provided by the levator ani muscles and the connective tissue (endopelvic fascia and ligaments) attached to the pelvic organs. Therefore, the disruption or dysfunction of these components may lead to the loss of support and, eventually, POP.[1] Pelvic supportive connective tissues are composed of abundant extracellular matrix, which is made up largely of collagen and elastin. While elastin provides elasticity to the tissues, collagen is responsible for conferring structural integrity and tensile strength.[17] Tissue remodeling is a dynamic process, and the balanced turnover of collagen is necessary to maintain the mechanical strength of the tissue. Previous studies have demonstrated a decrease in collagen content in the pelvic supportive connective tissues of women with POP compared to women without POP, and this is related to decreased synthesis and increased breakdown by matrix metalloproteinase as well as decreased cellularity, specifically, of fibroblasts.[9,18-21]

HOXA11 is considered to be a key regulator of collagen metabolism in the USLs and may serve as a therapeutic target for POP.[7-9] However, the regulation of HOXA11

expression has not yet been described. The present study provides several lines of evidence to show that the expression of HOXA11 in USLs is regulated by two miRNAs, miR-30d and miR-181a. First, miR-30d and miR-181a were over-expressed in women with POP compared to women without POP, as determined by microarray expression profiles and quantitative RT-PCR analyses. Furthermore, the expression of both miRNAs was inversely correlated with HOXA11 mRNA expression. Second, in cultured fibroblasts, the overexpression of miR-30d or miR-181a suppressed HOXA11 mRNA and protein levels, whereas the knockdown of these miRNAs enhanced HOXA11 levels. Third, HOXA11 is a direct target of these miRNAs, as shown by their direct interaction with the 3′-UTR of HOXA11. Taken together, these results indicate that both miR-30d and 181a are important posttranscriptional regulators of HOXA11 and could be major determinants of HOXA11 expression in the USLs.

Because each miRNA can bind and regulate multiple mRNA targets,[11] the aberrant expression of these miRNAs may also contribute to the pathogenesis of POP through pathways other than the dysregulation of HOXA11. For example, miR-30d overexpression has been shown to suppress cell proliferation and induce apoptosis,[22] and miR-181a has been shown to directly regulate the expression of Bcl-2, an anti-apoptotic protein that interacts with mitochondria,[23,24] thereby promoting cellular apoptosis. Notably, increased mitochondrial apoptosis and decreased Bcl-2 expression have been reported in the pelvic supportive tissues of POP patients.[25,26] The

overexpression of miR-30d and miR-181a may underlie these changes. Based on the encouraging results from miRNA-based therapeutic approaches in animal studies,[27-29] the knowledge of specific processes that are regulated in a miRNA-dependent manner and the identification of critical targets for individual miRNAs may be useful in the development of effective prevention and treatment strategies for POP. The results of the present and previous studies indicate that a therapeutic approach to decrease miR-30d and miR-181a may potentially be useful in restoring the altered extracellular matrix metabolism and homeostasis of the USLs that leads to POP. Further studies are required to validate the efficacy of this strategy.

Although the present study suggests key roles for miR-30d and miR-181a in the control of HOXA11 expression, the overexpression of these miRNAs may not fully explain the deficient HOXA11 signaling that is observed in the USLs of women with POP. It is important to note that a direct one-to-one stoichiometric relationship between the levels of miR-30d/miR-181a and HOXA11 expression does not exist. In the experiments involving the overexpression and knockdown of miR-30d/miR-181a, the magnitude of the changes in HOXA11 expression was modest compared with the changes in the expression of these miRNAs, suggesting that they are not the sole determinants of HOXA11 mRNA expression. A plausible mechanism is that HOXA11 can be controlled by other miRNAs, and this needs to be evaluated in future studies. In addition, pelvic floor fibroblasts express estrogen receptors[30], and the estrogen-estrogen receptor complex is known to directly bind to regulatory elements

of HOXA11 and induce its expression.[31] Therefore, the overexpression of miRNAs that target the estrogen receptor may also contribute to the decreased HOXA11 expression in the USLs of POP patients. It has been shown that ERα protein expression is reduced in both premenopausal and postmenopausal patients with POP.[32-34] Among the 18 miRNAs that were overexpressed in the miRNA microarray described in the present study, miR-222 is known to regulate ERα expression at the posttranslational level.[35] While this work was in progress, Shi et al. reported that miR-222 expression is significantly increased in the USLs of women with POP compared to women without POP and that there is a significant inverse correlation between ERα protein and miR-222 expression. These findings suggest that the knockdown of miR-222 might restore ERα expression and estrogen sensitivity.[34] Therefore, the combined treatment of estradiol and anti-miR-222 together with anti-miR-30d/181a may further increase the expression of HOXA11 protein compared with anti-miR-30d/181a alone. This also needs to be evaluated in future studies. Finally, biomechanical weakness of the endopelvic fascia has also been proposed as a cause of POP. Like USLs, the mechanical property of the endopelvic fascia is dependent on the quantity and quality of extracellular matrix in these structures.[17] It has been reported that HOXA13 regulates many genes involved in extracellular matrix homeostasis, and its expression is decreased in the vagina, including the endopelvic fascia, of POP patients.[36,37] Given the results of the present study, the aberrant expression of miRNAs might be involved in the decreased expression of HOXA13. Thus, the identification of specific miRNAs regulating HOXA13 will also

help further the understanding of the role of miRNAs in the pathogenesis of POP.

# V. CONCLUSION

In conclusion, the present study shows a novel aspect of the complex regulatory network of extracellular matrix homeostasis in pelvic floor supportive tissues. This study demonstrated that both miR-30d and miR-181a directly regulate HOXA11, an upstream transcriptional factor that controls collagen synthesis and degradation. Furthermore, these miRNAs are overexpressed in the USLs of women with POP, indicating that elevated levels of miR-30d and miR-181a might be responsible for the defective HOXA11 signaling that is observed in the USLs of women with POP. Therefore, therapeutic approaches aimed at decreasing these miRNAs might be useful for restoring the altered collagen metabolism and homeostasis in the USLs that leads to POP.

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<ABSTRACT (IN KOREAN)>

# MicroRNA 30d와 181a에 의한 자궁엉치인대 내 HOXA11 발현조절 및 골반장기탈출증 환자에서의 과발현 양상 규명

<지도교수 배상욱>

연세대학교 대학원 의학과

전 명 재

**연구목적:** 골반지지 결체조직의 구조 안정과 장력 유지에는 콜라겐 합성과 분해의 균형이 중요하다. HOXA11은 콜라겐 대사조절의 핵심 전사인자로서 골반장기탈출증 환자의 자궁엉치인대에서 발현이 저하되어 있다고 알려져 있다. 본 연구의 목적은 자궁엉치인대 내 HOXA11 발현조절에 관여하는 microRNA (miRNA)를 찾아내고, 그 기능을 규명하고자 함에 있다.

**연구방법:** 우선 microarray와 quantitative real-time polymerase chain reaction (RT-PCR) 분석을 통해 골반장기탈출증을 가진 여성과 그렇지 않은 여성의 자궁엉치인대 내 miRNA 발현양상을 살펴보았다. 그 다음 miRNA의 HOXA11 조절 양상을 규명하기 위해 miRNA mimic, anti-miR 또는 negative

controls를 293T 세포에 transfection 시킨 후 quantitative RT-PCR, Western 분석 및 luciferase reporter assay를 시행하였다.

**결과:** 골반장기탈출증을 가진 여성의 자궁엉치인대 조직에서 miR-30d와 181a 발현이 증가되어 있었고, 이들과 HOXA11 mRNA 발현 사이에는 음의 상관관계를 보였다. 293T 세포를 이용한 transfection 실험에서 두 miRNA를 과발현시켰을 때 HOXA11 mRNA와 단백이 감소한 반면, knockdown 시켰을 때는 HOXA11 발현이 증가하는 것을 관찰할 수 있었다. 또한 HOXA11 3′-UTR을 포함한 luciferase reporter plasmid를 두 miRNA mimic과 함께 cotransfection 시켰을 때 상대적 luciferase activity가 감소한 반면, 두 miRNA에 대한 inhibitor와 함께 cotransfection 시켰을 때는 luciferase activity가 증가하는 것을 관찰할 수 있었다.

**결론:** 본 연구결과는 miR-30d와 181a가 전사 후 단계에서 HOXA11 발현을 직접 억제하며, 이들 miRNA 과발현이 골반장기탈출증을 가진 여성의 자궁엉치인대 내 HOXA11 발현저하와 연관되어 있음을 시사한다. 따라서, 이들 miRNA를 감소시키는 것은 골반장기탈출증을 야기하는 콜라겐 대사 및 항상성에 있어서의 변질을 복원시키기 위한 유용한 치료방법이 될 수 있을 것으로 사료된다.

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**핵심되는 말:** 골반장기탈출증, 자궁엉치인대. HOXA11, microRNA-30d, microRNA-181a

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