

RhoA/ROCK/ARHGAP26 signaling in the eutopic and ectopic endometrium is involved in clinical characteristics of adenomyosis

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Abstract

Objective: This study aimed to investigate RhoA, RhoA-associated coiled-coil containing protein kinase (ROCK) 1, ROCK2, and Rho GTPase-activating protein 26 (ARHGAP26) expression in the eutopic endometrium (EU) and ectopic endometrium (EC), and examine their relationships with the clinical characteristics of adenomyosis.

Methods: Twenty patients with adenomyosis who underwent laparoscopy were recruited. Protein and mRNA expression of RhoA, ROCK1, ROCK2, and ARHGAP26 in EU and EC of patients with adenomyosis and in control endometrium without adenomyosis (CE) was detected.

Results: ROCK1, ROCK2, and RhoA mRNA expression in EU was significantly higher than that in CE, and was highest in EC. ARHGAP26 mRNA expression in EC and EU was significantly lower than that in CE. ROCK1, ROCK2, and RhoA protein expression in EC and EU was significantly higher than that in CE. ARHGAP26 protein expression in EC and EU was significantly lower than that in CE. ROCK1, ROCK2, and RhoA gene and protein expression was positively associated

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and ARHGAP26 was negatively associated with the severity of menorrhagia and menstrual capacity in adenomyosis.

Conclusions: RhoA, ROCK1, and ROCK2 expression is upregulated, and ARHGAP26 expression is downregulated in adenomyosis. The RhoA/ROCK-mediated signaling pathway is associated with dysmenorrhea and menstrual capacity in adenomyosis.

Keywords

Adenomyosis, rho-associated kinases, Rho GTPase-activating protein 26 (ARHGAP26), dysmenorrhea, menorrhagia, endometrium

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Introduction

Adenomyosis is a common chronic gynecological disease that is defined by the presence of heterotopic endometrial glands and stroma within the myometrium, and this disease produces a diffusely enlarged uterus.¹ The prevalence of adenomyosis ranges between 5% and 70% in hysterectomy samples obtained for gynecological purposes. The risk factors for adenomyosis include changes in marriage and childbirth mode, uterine trauma and repair, genital tract infections, and increased serum estrogen levels.² Adenomyosis is responsible for disabling symptoms, such as dysmenorrhea, menorrhagia, abnormal uterine bleeding, and infertility.³ The exact pathogenesis of adenomyosis remains unclear. Many different signaling pathways are involved in the pathogenic mechanisms of adenomyosis.^{4,5}

RhoA is a member of the Rho family of small (20–40 kDa) guanine nucleotide exchange factors (GTPases), and is activated by guanine nucleotide exchange factors. These guanine nucleotide exchange factors activate RhoA by promoting conversion of an inactive guanine diphosphate-bound form to an active guanine triphosphate (GTP)-bound form.⁶ RhoA-associated coiled-coil containing protein kinase

(ROCK), including the two subtypes ROCK1 and ROCK2, belongs to the serine/threonine protein kinase and is the downstream effector protein of RhoA.⁷ Rho GTPase-activating protein 26 (ARHGAP26, also known as GRAF, GRAF1, and OPHN1L) is a regulator of the Rho family that converts the small G protein RhoA to its inactive guanine diphosphate-bound forms. Through negative regulation of the small G protein RhoA, ARHGAP26 is critical for muscle development. ARHGAP26 is also thought to be a putative tumor suppressor by negatively regulating RhoA in human cancer, and is known for its growth-promoting effects in oncoprotein Ras-mediated malignant transformation.⁸ The RhoA/ROCK signaling pathway is involved in various cellular functions, including cell proliferation, differentiation, migration, apoptosis, and smooth muscle contraction.⁹ Accumulating evidence has shown that RhoA/ROCK overexpression plays an important role in tumor cell metastasis by promoting the invasiveness of some cancers, such as ovarian cancer.¹⁰ Additionally, numerous studies have suggested that the RhoA/ROCK signaling pathway plays a major role in the invasion and growth of the ectopic endometrium

(EC). The RhoA/ROCK signaling pathway is currently recognized as a multifunctional pathway that is involved in a wide range of pathophysiological processes in endometriosis and adenomyosis, or even affects the clinical characteristics of dysmenorrhea.^{11,12} However, the exact mechanism of the RhoA/ROCK signaling pathway and the role of its downstream effectors in the development of adenomyosis remain unclear.

To the best of our knowledge, no study has reported ARHGAP26 expression in adenomyosis. Therefore, the present study aimed to investigate RhoA, ROCK1, ROCK2, and ARHGAP26 expression in eutopic endometrium (EU) and EC from women with and without adenomyosis. We also analyzed the correlations between the expression of these parameters and the clinical characteristics of patients with adenomyosis.

Materials and methods

Ethics statement

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Yangpu Hospital, Tongji University School of Medicine (Shanghai, China). Written informed consent was obtained from all individuals who participated in this study.

Participants

From December 2016 to June 2017, patients who underwent laparoscopic surgery for adenomyosis at the Gynecological Department of Yangpu Hospital, Tongji University School of Medicine were included in this study. The patients' diagnoses were confirmed by histology. Healthy women who underwent intrauterine device (IUD) placement were recruited as the

control group. All patients who were enrolled in this study were in the proliferative phase. Participants with regular menstrual cycles (28–32 days) did not have any hormonal therapy administered, including oral contraceptive pills, progestins, gonadotropin-releasing hormone agonists, or the levonorgestrel intrauterine system, in the most recent 3 months. Individuals with cancer, cardiovascular diseases, autoimmune diseases, endocrine diseases, metabolic diseases, pelvic inflammatory disease, and other infectious diseases were excluded.

The severity of dysmenorrhea was assessed using the visual analogue scale (VAS)¹³ system as follows: 0, no pain; 1–3, minimal pain; 4–6, moderate pain; and 7–10, severe pain. Menstrual blood loss was evaluated using the pictorial blood loss assessment chart (PBAC).¹³ Menorrhagia was defined as excessive menstrual blood loss > 80 mL (PBAC > 100) per period.

Tissue collection

EU and EC samples were collected from patients with adenomyosis during surgery. Using information from preoperative magnetic resonance imaging, EC samples were taken from ectopic lesions that were located in the myometrium during surgery (hysterectomy or partial resection of adenomyosis via laparoscopy), and pathological confirmation of the same area was simultaneously performed. The adenomyosis lesions were diagnosed by their macroscopic appearance according to the published criteria and the final diagnosis was based mainly on histological examination of a biopsy. Under strict asepsis, EU samples were obtained from the uterine cavity of patients with adenomyosis. Control endometrium without adenomyosis (CE) samples were derived from the uterine cavity of healthy women before the IUD was placed. All of the

fresh tissue specimens were frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction.

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from frozen tissue samples using Trizol reagent (Invitrogen, Waltham, MA, USA). Complementary DNA was synthesized by reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) at 42°C for 1 hour and 75°C for 5 minutes. Specific primers for ROCK1, ROCK2, RhoA, and ARHGAP26 are shown in Table 1. Real-time polymerase chain reaction (PCR) was performed with SYBR-Green Master Mix on an ABI7300 platform and related software (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The PCR cycling conditions were as follows: 94°C for 7 minutes, followed by 40 cycles of 15 seconds at 94°C and 60 seconds at 60°C . The change in cycle threshold (ΔCt) was defined as the difference in the cycle threshold between the target gene and internal control, and $\Delta\Delta\text{Ct}$ was defined as the difference between the ΔCt values of the test samples and controls.

The relative expression of the target genes was calculated as $2^{-\Delta\Delta\text{Ct}}$.

Western blot analysis

Protein was extracted with radioimmuno-precipitation assay buffer containing a protease inhibitor cocktail and centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The supernatant protein was quantified by bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA) and stored at -80°C . Total lysates were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were blotted onto a nitrocellulose membrane and incubated at 4°C overnight with one of the following primary antibodies: anti-ROCK1 (1:1000, ab45171), anti-ROCK2 (1:1000, ab71598), anti-RhoA (1:800, ab54835), anti-ARHGAP26 (1:2000, ab137085) (Abcam, Cambridge, MA, USA), and GAPDH (1:2000, 5174; CST, Danvers, MA, USA). Antibodies were diluted in tris-buffered saline containing 1% skimmed milk. The blots were then washed with tris-buffered saline and incubated with the corresponding secondary antibodies (HRP-labeled goat anti-rabbit IgG [H+L], a0208; HRP-labeled donkey anti-goat IgG [H+L], a0181; HRP-labeled goat anti-mouse IgG

Table 1. Primers for real-time PCR

Name	Sequences (5'-3')	Annealing temperature ($^{\circ}\text{C}$)	Product size (bp)
ROCK1	F: AACATGCTGCTGGATAAATCTGG R: TGTATCACATCGTACCATGCCT	59	93
ROCK2	F: TCAGAGGTCTACAGATGAAGGC R: CCAGGGGCTATTGGCAAAGG	60	209
RhoA	F: AGCCTGTGGAAAGACATGCTT R: TCAAACACTGTGGGCACATAC	59	78
ARHGAP26	F: GACTGCTGCCTCGATAGTCC R: CTTCCGCTTCGCTGAAGACAA	60	150
GAPDH	F: CACCCACTCCTCCACCTTTG R: CCACCACCCTGTTGCTGTAG	60	110

bp: base pairs; PCR: polymerase chain reaction; ROCK, RhoA-associated coiled-coil containing protein kinase; ARHGAP26, Rho GTPase-activating protein 26; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

[H+L], a0216) (Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 1 hour. The optical density method of protein signal strength was performed by using the enhanced chemiluminescence western blotting system (BioRad, Richmond, CA, USA). GAPDH served as an internal standard to determine protein levels.

Statistical analysis

All statistical analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Normality testing of the measurement data was conducted by using the kurtosis and skewness coefficients and the Shapiro–Wilks test. Normally distributed data are presented as means \pm standard deviations, and intragroup differences were investigated using analysis of variance or the Student's *t* test when necessary. Non-normally distributed data are shown as the median (quartiles), and intragroup differences were examined using the Kruskal–Wallis or Mann–Whitney test when necessary. Categorical variables are expressed as the number of cases and percentages. A value of $P \leq 0.05$ (two-sided test) was considered statistically significant. Statistics diagrams were performed with GraphPad Prism 5 (La Jolla, CA, USA).

Results

General data of patients in the adenomyosis and control groups

There were 20 patients with adenomyosis and 20 women who were controls. The 20 patients in the adenomyosis group included 2 nulliparas and 18 multiparas, who ranged in age from 23 to 53 years, with a mean age of 41.00 ± 7.11 years. These patients had a mean body mass index of 21.61 ± 3.08 kg/m² (16.84–27.34 kg/m²). The 20 controls subjects were all multiparas who

ranged in age from 25 to 52 years with a mean age of 39.25 ± 6.92 years and a mean body mass index of 20.29 ± 2.67 kg/m² (15.24–27.22 kg/m²). There were no significant differences in age, body mass index, fertility, or history of prior surgery between the groups.

Clinical characteristics of the adenomyosis group

The mean history of the patients with adenomyosis was 11.37 ± 7.24 (1–156) months. According to the VAS system, there were five (25.0%) cases of minimal dysmenorrhea, six (30.0%) cases of moderate dysmenorrhea, and nine (45.0%) cases of severe dysmenorrhea. According to the PBAC, there were eight (40.00%) cases of normal menstrual capacity and 12 (60.0%) cases of menorrhagia.

ROCK1, ROCK2, RhoA, and ARHGAP26 mRNA expression in CE, EU, and EC

RT-PCR analysis showed that ROCK1, ROCK2, and RhoA mRNA expression in EC and EU was significantly higher than that in CE (all $P < 0.01$), and their expression in EC was significantly higher than that in EU (all $P < 0.01$). In contrast, ARHGAP26 mRNA expression in EC and EU was significantly lower than that in CE (both $P < 0.01$), but there was no significant difference between EC and EU (Figure 1).

ROCK1, ROCK2, RhoA, and ARHGAP26 protein expression in CE, EU, and EC

Western blot analysis showed that ROCK1, ROCK2, and RhoA protein expression in EC and EU was significantly higher than that in CE (all $P < 0.01$). Only ROCK1 protein expression in EC was significantly higher than that in EU ($P < 0.05$). In contrast, ARHGAP26 protein expression in

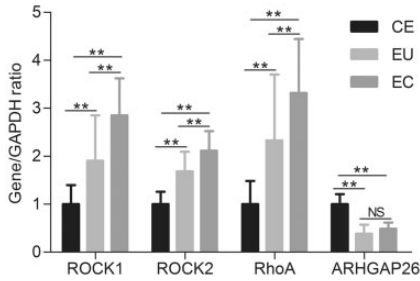


Figure 1. ROCK1, ROCK2, RhoA, and ARHGAP26 mRNA expression in CE, EU, and EC
 ROCK: RhoA-associated coiled-coil containing protein kinase; ARHGAP26: Rho GTPase-activating protein 26; CE: control endometrium without adenomyosis; EU: eutopic endometrium; EC: ectopic endometrium; NS: nonsignificant ($P > 0.05$). * $P < 0.05$; ** $P < 0.01$

EC and EU was significantly lower than that in CE (both $P < 0.01$) (Figure 2).

Associations between ROCK1, ROCK2, RhoA, and ARHGAP26 expression and the severity of dysmenorrhea

In this study, the patients were divided into three subgroups according to the severity of dysmenorrhea. Gene expression of ROCK1, ROCK2, and RhoA in the severe and moderate dysmenorrhea groups was significantly higher than that in the minimal dysmenorrhea group in EU and EC (all $P < 0.05$). Furthermore, ROCK1, ROCK2, and RhoA gene expression in the severe dysmenorrhea group was significantly

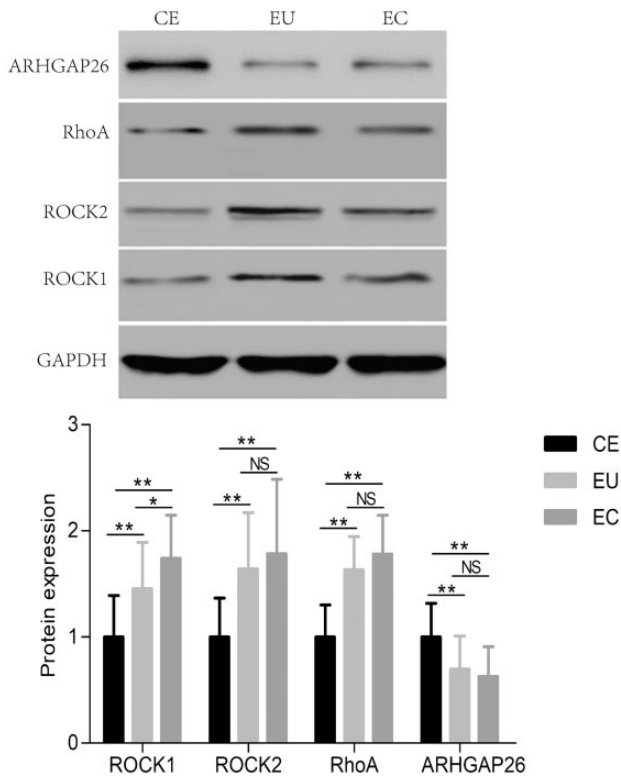


Figure 2. ROCK1, ROCK2, RhoA, and ARHGAP26 protein expression in CE, EU, and EC
 ROCK: RhoA-associated coiled-coil containing protein kinase; ARHGAP26: Rho GTPase-activating protein 26; CE: control endometrium without adenomyosis; EU: eutopic endometrium; EC: ectopic endometrium; NS: nonsignificant ($P > 0.05$). * $P < 0.05$; ** $P < 0.01$

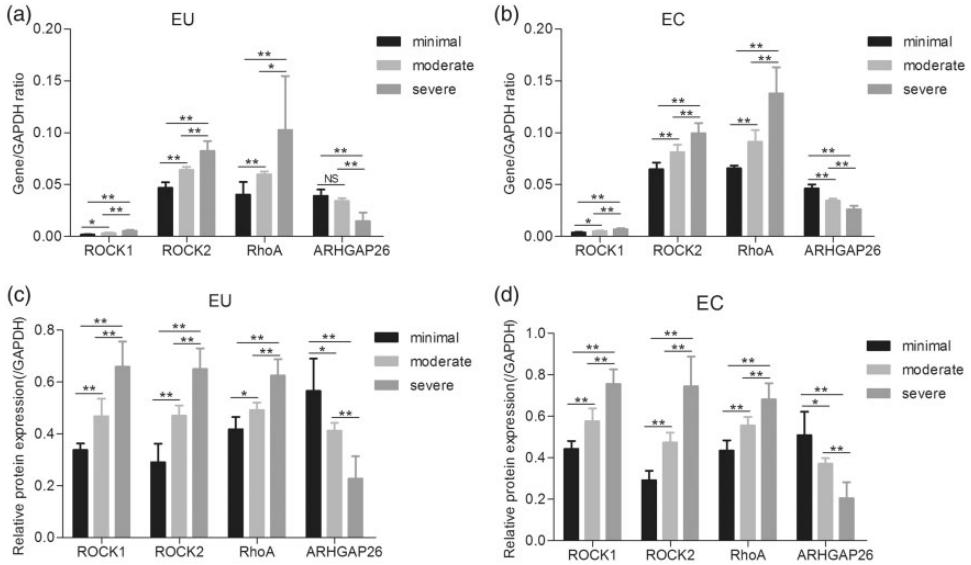


Figure 3. Associations between ROCK1, ROCK2, RhoA, and ARHGAP26 expression and the severity of dysmenorrhea
 ROCK: RhoA-associated coiled-coil containing protein kinase; ARHGAP26: Rho GTPase-activating protein 26; CE: control endometrium without adenomyosis; EU: eutopic endometrium; EC: ectopic endometrium; NS: nonsignificant ($P > 0.05$). * $P < 0.05$; ** $P < 0.01$

higher than that in the moderate dysmenorrhea group (all $P < 0.05$). In contrast, ARHGAP26 gene expression in the severe dysmenorrhea group was significantly lower than that in the moderate and minimal dysmenorrhea groups in EU (both $P < 0.01$), with no significant difference between the moderate and minimal dysmenorrhea groups. ARHGAP26 gene expression was also significantly lower in the moderate dysmenorrhea group than in minimal dysmenorrhea group, and was lowest in the severe dysmenorrhea group in EC (all $P < 0.01$) (Figure 3a, b).

Similar results were obtained at the protein level. ROCK1, ROCK2, and RhoA protein expression in the moderate dysmenorrhea group was higher than that in the minimal dysmenorrhea group, and highest in the severe dysmenorrhea group in EU and EC (all $P < 0.05$). In contrast, ARHGAP26 protein expression was lower

in the moderate dysmenorrhea group than that in the minimal dysmenorrhea group and was lowest in the severe dysmenorrhea group in EU and EC (all $P < 0.05$) (Figure 3c, d).

Associations between ROCK1, ROCK2, RhoA, and ARHGAP26 expression and the severity of menorrhagia

The patients were further divided into two subgroups according to the severity of menorrhagia. ROCK1, ROCK2, and RhoA gene and protein expression in the menorrhagia group was significantly higher than that in the normal group in EU and EC (all $P < 0.01$). In contrast, ARHGAP26 gene and protein expression in the menorrhagia group was significantly lower than that in the normal group in EU and EC (all $P < 0.01$) (Figure 4).

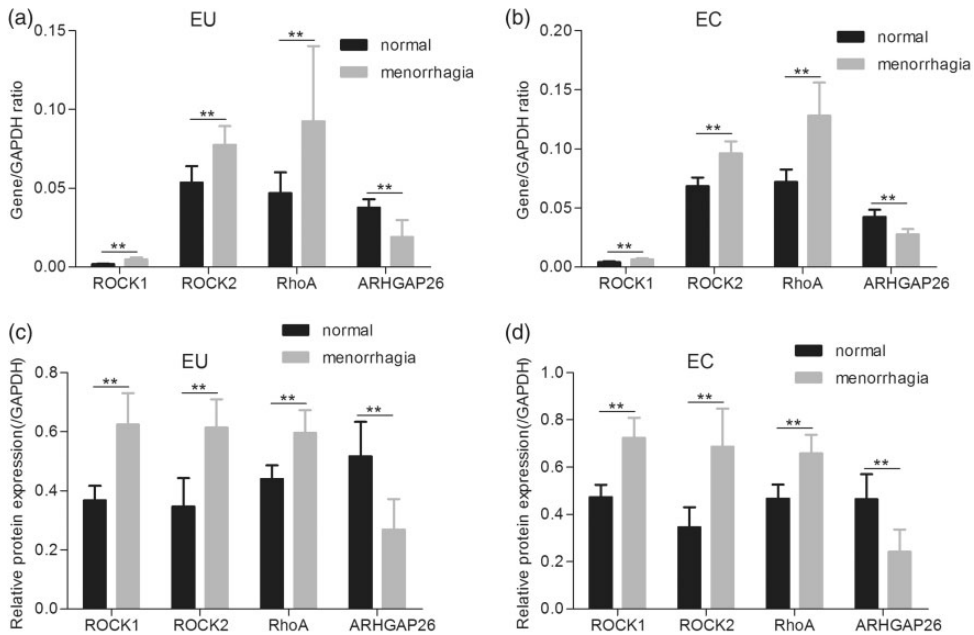


Figure 4. Associations between ROCK1, ROCK2, RhoA, and ARHGAP26 expression and the severity of menorrhagia

ROCK: RhoA-associated coiled-coil containing protein kinase; ARHGAP26: Rho GTPase-activating protein 26; CE: control endometrium without adenomyosis; EU: eutopic endometrium; EC: ectopic endometrium; NS: nonsignificant ($P > 0.05$). * $P < 0.05$; ** $P < 0.01$

Discussion

As a special form of endometriosis, the adenomyotic uterus is characterized by ectopic, nonneoplastic endometrial glands and stromal cells surrounded by a hypertrophic and hyperplastic myometrium at the light microscopic level.¹⁴ Adenomyosis is a common and intractable gynecological disease mostly affecting women of reproductive age. Dysmenorrhea and menorrhagia are the two most important reasons for patients to obtain medical treatment.¹⁵ The Rho family GTPase proteins play a role in molecular switching in a series of intracellular activities in reorganizing the cytoskeleton and regulating cellular shape, adhesion, and migration via activation of effector proteins, such as ROCK.¹⁰ Numerous studies have shown that enhanced myometrial contractility is

mediated in part through increased Rho/ROCK in late gestation, suggesting that the RhoA/ROCK system plays a vital role in uterine smooth muscle contraction.⁹ Emerging evidence suggests that RhoA/ROCK expression is also significantly increased in some tumors, particularly during their progression to more invasive and metastatic phenotypes.¹¹ Although adenomyosis is a type of benign gynecologic disease, it has malignant biological behavior such as infiltration, metastasis, and recurrence.¹⁶ The RhoA/ROCK signaling pathway also plays important regulatory roles in a series of biological behaviors of the ectopic endometrium, and is believed to be related to the pathophysiological processes in adenomyosis.^{9,11}

RhoA and ROCK1 expression in EC is higher than that in EU of endometriosis. Furthermore, blockage with small-interfering

RNA decreases RhoA and ROCK1 expression and migration of EU.¹¹ These findings suggest that the RhoA/ROCK pathway participates in regulation of endometrial cell migration in endometriosis. In our study, ARHGAP26 gene and protein expression in EC and EU was significantly lower than that in CE. Because a reduced level of ARHGAP26 was consistent with elevated RhoA activity, we examined ROCK1, ROCK2, and RhoA expression in EC, EU, and CE. We found that ROCK1, ROCK2, and RhoA gene expression in EU was significantly higher than that in CE, and was highest in EC. Similarly, we found the same outcome at the protein level, which is consistent with the gene expression. Our findings indicate that the RhoA/ROCK signaling pathway might be involved in formation of the pathological state of adenomyosis.

The RhoA/ROCK signaling pathway regulates the actin cytoskeleton and promotes actin globulin contraction, and subsequently regulates cell function.⁷ Uterine contractions are dependent on the individual contractile activity of the cellular element, uterine myocytes. The RhoA/ROCK signaling pathway plays a major role in regulating the contractility of various types of smooth muscle cells. Wang et al.⁹ found that estrogen may affect uterine junctional zone contraction of adenomyosis by enhancing RhoA/ROCK1 signaling. Wang et al.¹² found that RhoA and ROCK1 expression in the junctional zone in the adenomyosis group was higher than that in the control group, and positively correlated with the severity of dysmenorrhea in the adenomyosis group. In our study, patients with adenomyosis were divided into several subgroups according to their clinical characteristics. With regard to the degree of dysmenorrhea, the higher the gene and protein expression of ROCK1, ROCK2, and RhoA, the lower the gene and protein expression of

ARHGAP26, and the more serious dysmenorrhea was. These data are generally consistent with previously published studies.¹² With regard to menorrhagia, of ROCK1, ROCK2, and RhoA gene and protein expression was positively associated with the severity of menorrhagia, but this association was negative for ARHGAP26. The associations between expression of these parameters and disease severity suggest that the degree of dysmenorrhea and menstrual capacity are in parallel with increased expression of ROCK1, ROCK2, and RhoA, and decreased expression of ARHGAP26. Therefore, we conclude that high expression of the RhoA/ROCK-mediated signaling pathway might be involved in abnormal contraction of the uterine myometrium and positively associated with dysmenorrhea and menstrual capacity in adenomyosis. However, ARHGAP26 appears to be negatively associated with dysmenorrhea and menstrual capacity in adenomyosis.

Despite our important findings, there are some limitations in this study. First, we did not detect peritoneal fluid and serum expression levels of ROCK1, ROCK2, RhoA, and ARHGAP26. Additionally, the menstrual data that were used in this study were obtained only by review of the medical records without histological confirmation. Therefore, further studies with larger sample sizes are required to avoid these limitations and verify the findings of the present study.

In summary, our study shows that RhoA, ROCK1, and ROCK2 expression is upregulated, and that of ARHGAP26 is downregulated in patients with adenomyosis. Furthermore, high expression of the RhoA/ROCK-mediated signaling pathway is positively associated with dysmenorrhea and menstrual capacity in adenomyosis. These findings may help gain insight into the role of the RhoA/ROCK-mediated signaling pathway in development of

adenomyosis and its association with the clinical characteristics of dysmenorrhea and menstrual capacity in patients with adenomyosis. Future studies in cell or animal models are required to further clarify the role of the RhoA/ROCK signaling pathway in adenomyosis at the molecular level.

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Author Contributions

All authors contributed to management of the patients and writing the report. W.G., R.C., and H.K. conceived and designed the experiments; C.J. performed the experiments and wrote the main manuscript; X.Q. and W.Y. contributed to statistical analysis and revision of the manuscript; W.G. participated in sample collection; and Z.C. supervised the study. All authors reviewed the manuscript. Written consent to publish was obtained.

Data availability statement

All data are available within the manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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Note

All of the images/graphs were created by C.J.

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