Taiwanese Journal of Obstetrics & Gynecology 57 (2018) 507–516

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Endometrial L-selectin ligand is downregulated in the mid-secretory phase during the menstrual cycle in women with adenomyosis

Tsung-Hsuan Lai [a, b, c], Fung-Wei Chang [d, e], Jun-Jie Lin [a], Qing-Dong Ling [c, f, *, 1]

[a] Department of Obstetrics and Gynecology, Cathay General Hospital, Taipei, Taiwan
[b] School of Medicine, Fu Jen Catholic University, New Taipei City, Taiwan
[c] Institute of Systems Biology and Bioinformatics, National Central University, Taoyuan City, Taiwan
[d] Tri-Service General Hospital Penghu Branch, National Defense Medical Center, Taipei, Taiwan
[e] Department of Obstetrics and Gynecology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[f] Cathay Medical Research Institute, Cathay General Hospital, New Taipei City, Taiwan

ARTICLE INFO

Article history:
Accepted 26 April 2018

Keywords:
Adenomyosis
Endometrium
L-selectin ligand

ABSTRACT

Objective: Defects in L-selectin ligand (LSL) expression have been reported to cause implantation failure, but little is known about LSL expression in adenomyosis. This study evaluates LSL expression throughout the menstrual cycle in women with adenomyosis.

Materials and methods: Endometrial samples were obtained from reproductive-aged women with adenomyosis who underwent hysterectomy. A total of 42 endometrial biopsies were included. There were 12 women in proliferative phase, 10 in early-secretory phase, 9 in mid-secretory phase, and 11 in late-secretory phase. Immunohistochemistry, western blotting, and RT-PCR were performed to evaluate LSL expression. A non-parametric Kruskal–Wallis one-way analysis of variance with multiple comparisons was performed to examine differences among menstrual phases.

Results: Immunohistochemistry analysis with MECA-79 shows that LSL is expressed with weak intensity in the endometrium in all phases. In the luminal epithelium, MECA-79 reactivity increased from the proliferative to the late-secretory phase but decreased in the mid-secretory phase. There were significant differences in the mean histological scores (HSCOREs) among the proliferative, early-secretory, and late-secretory phases ($p < 0.05$). Five LSL genes were detected in the adenomyotic endometria: *PODXL*, *EMCN*, *CD300LG*, *GLYCAM1*, and *CD34*. The mRNA expression of LSL genes occurred differentially among phases. Moreover, *PODXL* differed significantly among phases ($p < 0.05$).

Conclusions: LSL expressions were downregulated in the luminal epithelium of adenomyotic endometria in the mid-secretory phase. The mRNA expressions of LSL genes also had differential expression patterns throughout the menstrual cycle, especially for *PODXL*. Our study showed that adenomyosis may cause abnormalities of LSL production in the mid-secretory phase, which may contribute to impaired endometrial receptivity and implantation failure.

Introduction

Adenomyosis is a common but benign uterine disease characterized by the presence of ectopic endometrial glands and stroma within the myometrium. In addition, the surrounding myometrium usually reveals hypertrophy [1]. The manifestation of the disease ranges from grossly visible nodules called adenomyoma to smaller forms that are only detectable by microscopy. Adenomyosis affects nearly 20% of the population of females of reproductive age. The most common symptoms are dysmenorrhea, abnormal uterine bleeding, hypermenorrhea, and infertility. Approximately 35% of women with adenomyosis are asymptomatic [2].

The relationship between adenomyosis and infertility is still unclear, but severe adenomyosis has a negative impact on fertility and could impair pregnancy rates when using artificial reproductive techniques [3,4]. Some reports show that women with

* Corresponding author. Institute of Systems Biology and Bioinformatics, National Central University, No.300, Jhongda Rd., Jhongli District, Taoyuan City 32001, Taiwan. Fax: +886 2 27092063.
E-mail addresses: tslai382@gmail.com (T.-H. Lai), qdling@hotmail.com (Q.-D. Ling).
[1] Cathay Medical Research Institute, Cathay General Hospital, No. 32, Ln. 160, Jiancheng Rd., Sijhih Dist., New Taipei City 22174, Taiwan.

https://doi.org/10.1016/j.tjog.2018.06.005

adenomyosis have poorer reproductive outcomes [5,6]. The pathophysiology of infertility associated with adenomyosis is related to gene dysregulation, altered uterine peristaltic activity, altered endometrial function, impaired implantation, altered decidualization, and abnormal concentrations of intrauterine free radicals [7]. Thus far, there has been little molecular data to explain the mechanism behind subfertility and infertility in adenomyosis. Furthermore, little is known about the effects of adenomyosis on known biomarkers of endometrial receptivity, such as L-selectin ligand (LSL).

LSLs are glycoproteins that bind to L-selectin (CD62P) on leukocytes in high endothelial venules (HEVs) of the lymph nodes [8]. Interestingly, evidence indicates that the initial attachment of an embryo to the endometrium depends on the binding of L-selectin expressed by the trophoblast and oligosaccharide-based ligands expressed by the endometrium [9,10]. More and more evidence is revealing that the expression of LSL might reflect endometrial receptivity [11]. In our previous study, LSL was expressed differentially between different phases of the natural cycle in the human endometrium. It was upregulated in the secretory phase and downregulated in the proliferative phase. The expression of LSL reached its highest level in the luminal epithelium in the mid-secretory phase, which coincides with the window of implantation [12]. Another study of our team revealed that oocyte donors had lower LSL expression than controls on cycle day 19 in the luminal epithelium and from cycle days 19–24 in the glandular epithelium. This time frame corresponds with the implantation window [13]. These results suggest that controlled ovarian hyperstimulation is associated with reduced LSL expression in the secretory phase, particularly during the implantation window.

Defects in LSL expression lead to implantation failure and early pregnancy loss [9]. Recently, a study showed that the absence of LSL during the implantation window in the secretory endometrium could lead to recurrent implantation failure (RIF) in patients who suffer from unexplained infertility [14]. A screening test for RIF's patients who lack LSL had a predictive value of 100% with 50% sensitivity and 100% specificity. The positive predictive value was 100%, and the negative predictive value was 87%. The study concluded that LSL plays a vital role in early human implantation. Screening for the absence of LSL might help many patients with RIF to avoid repeatedly undergoing failed treatment cycles [14].

To date, little is known about the patterns of LSL distribution in the different phases during the menstrual cycle in adenomyosis. In addition, little is known about whether adenomyosis impairs LSL expression in the human endometrium. Therefore, this study evaluates the expression pattern of LSL in different phases during the menstrual cycle in adenomyosis and explores the mRNA expressions of LSL genes in these phases. We hypothesized that adenomyosis could impair endometrial LSL expression in the mid-secretory phase (the period of embryo implantation).

Materials and methods

All procedures conformed to the Declaration of Helsinki for research involving human subjects. The Institutional Review Board of Cathay General Hospital approved the use of human specimens (*CGHIRB No.:* CT9681). Formal informed consent was obtained from all patients before sample collection. The medical records of all patients were reviewed retrospectively.

Sample collection

Participants were included using the following criteria: 1) age ranging from 35 to 50 years; 2) regular menstrual cycle (28–35 days); 3) body mass index (BMI) less than 28; 4) no hormone therapy within at least 2 months before surgery; 5) no other gynecological diseases, such as pelvic inflammatory disease (PID), cancers, endometrial hyperplasia, submucosal myoma or endometrial polyps; 6) and no sexually transmitted diseases (STD). Patients were excluded from the study if they had 1) pregnancy, 2) coagulopathy, 3) psychological diseases, or 4) any other apparent reproductive tract pathology.

Endometrium tissue samples were collected from women with adenomyosis who underwent hysterectomy from August 2008 to July 2009. Samples with evidence of endometritis, endometrial polyps, endometrial hyperplasia, or other endometrial pathologies were excluded. There were 42 endometrial biopsies, which included 12 from the proliferative phase (days 7–14), 10 from the early-secretory phase (days 15–18), 9 from the mid-secretory phase (days 19–24), and 11 from the late-secretory phase (days $\geqq$ 25). In addition, 11 endometrial samples were obtained to compare the LSL mRNA expression from menopausal women with uterine prolapse who underwent vaginal hysterectomy. Tissue samples were frozen at −80 °C until immunohistochemistry analyses and RNA isolation.

Immunohistochemistry

One portion of the obtained tissues was fixed in buffered formalin and evaluated by a pathologist with experience in endometrial dating. Separate dating of the luminal and glandular epithelium was carried out according to the criteria reported by Noyes et al. [15]. A second portion of each biopsy sample was used to investigate the expression of LSL by immunohistochemistry. The expression of LSL was examined by immunolocalization with rat monoclonal antibody MECA-79 (BD Biosciences, San Jose, CA), which recognizes a high-affinity LSL carbohydrate epitope containing SO3 → 6GlcNAc [16].

The assay was performed as described previously [12,17]. Briefly, the samples were fixed in 10% buffered formaldehyde for 24 h and embedded in paraffin. Paraffin sections with thicknesses of 3–4 mm were prepared on positively charged slides prior to the immunohistochemical analysis. The sections were dewaxed with xylene, followed by descending grades of methanol solutions to distilled water. They were then pretreated at 90° for 20 min with Citra Buffer (Vector H3300, Vector Laboratories, Burlingame, CA) in a steamer (HA900; Black & Decker, Hampstead, MD).

The tissue sections were labeled with MECA-79 at a concentration of 3.3 μg/mL using a dilution of 1:30 in phosphate-buffered saline (PBS). Positive and negative (no antibody) controls were established using a section of tonsil and a section of endometrium tissue, respectively. For antigen retrieval, the slides were incubated in CC1 buffer (Ventana) for 1 h on plates heated at 100 °C with a Benchmark XT processor. The primary antibody incubation was performed for 32 min at a dilution of 1:30 and 37 °C. Positive binding of MECA-79 was detected by biotinylated rabbit anti-mouse secondary antibody (at a dilution of 1:800 with PBS), which cross-reacts with the rat primary antibody.

After the hybridization of primary and secondary antibodies, the HRP conjugated avidin-biotin peroxidase (ABC) complex was analyzed using a Ventana DAB Detection Kit (Ventana-Biotek Solutions Inc., Tucson, AZ). The slides were counterstained with hematoxylin, dehydrated, cleared, and mounted in DPX mountant. Finally, they were evaluated using an Eclipse 80i optical microscope (Nikon, Tokyo, Japan). The intensity of staining of the antibody was then analyzed in both the glandular and luminal epithelium by a semi-quantitative histological scores (HSCOREs) system [18]. HSCOREs for the tissue section were derived as the sum of the component HSCOREs weighted by the fraction of each component observed in the tissue section. The HSCOREs were calculated using

the equation HSCOREs = Σ P*i* (*i* + 1), where *i* is the intensity of the stained epithelium (1 = weak, 2 = moderate, and 3 = strong), and P*i* is the percentage of stained epithelial cells for each intensity and varies from 0 to 100%. This semi-quantitative analysis has been shown to have low intra-observer and inter-observer error [19].

Western blot analysis

Western blot analysis was performed according to a previous report [17]. Endometrial samples were homogenized in 10 mM potassium homogenization buffer containing 250 mM of sucrose, 1 mM of EDTA, and 0.1 mM of phenylmethylsulfonyl fluoride, which was adjusted to pH 7.6. Homogenates were centrifuged at 3000 × g for 5 min and 9000 × g for 15 min. The endometrial homogenates (50 μg) were separated by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane in a transfer buffer (50 mM Tris, 95 mM glycine, and 20% ethanol) at 100 V at 4 °C for 1 h.

The membranes were blocked overnight with 5% bovine serum albumin (BSA) diluted in 0.1% Tween-20-Tris buffered saline (TTBS, blocking buffer) for MECA-79 detection. Membranes were subsequently incubated for 1 h at 48 °C with rat anti-MECA-79 monoclonal antibody diluted to 1/1000 in 5% BSA-TTBS. After washing five times with TTBS, blots were incubated for 1 h at room temperature with the appropriated secondary antibody coupled to horseradish peroxidase. Between the various incubation steps, the membranes were washed several times with TTBS.

The samples were processed in parallel using rabbit anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotech) and the appropriated secondary antibody in order to normalize the protein load in each well. The membranes were washed with TTBS and then developed using enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL). The relative intensity of the bands was determined using UnScanit software (Silk Scientific, Inc., Orem, UT). The background was subtracted from each band volume using local background subtraction. The band intensities acquired from each protein extract were normalized against corresponding values for bands of the house-keeping protein GAPDH.

Reverse-transcription polymerase chain reaction (RT-PCR)

The mRNA expressions of LSL genes were analyzed using one-step quantitative RT-PCR. There are 5 genes of LSL-peptide components found in HEVs of the lymph node in mouse: *podocalyxin (PODXL)*, *endomucin (EMCN)*, *nepmucin (CD300LG)*, *glycosylation-dependent cell adhesion molecule 1 (GLYCAM1)*, and *CD34*. Two main HEV-expressed sulfotransferases are involved in the generation of LSL epitopes and LSL activity: carbohydrate sulfotransferase 2 (*CHST2*) and carbohydrate sulfotransferase 4 (*CHST4*) [8,20]. One-step quantitative RT-PCR was also performed to analyze the gene expression of *CHST2* and *CHST4*.

Total RNA was isolated using 3-*Zol* reagent (MDBio, Taipei, Taiwan). The RNA samples were treated with Dnase I (Promega, Madison, WI) to remove traces of genomic DNA. The buffer conditions in the RNA solution were adjusted to assure optimal Dnase I activity. RNA absorbance at 260 nm was measured using a spectrophotometer to obtain a yield in micrograms per microliter (μg/μL). Quantitative RT-PCR was performed with 1 μg of total RNA from the endometrial specimens. The cDNA was synthesized and amplified using a Titanium One-Step RT-PCR Kit (BD Biosciences Clonetech, Palo Alto, CA). The cDNA levels of the genes of interest were measured using specific primer pairs, which are described in detail in Table 1. *GAPDH* was used as an internal control.

For quantitative analysis, it is necessary to determine the linear correlation window of PCR cycles for each sample. Control experiments were conducted to determine the range of PCR cycles where the amplification efficiency remained constant. The gene products were amplified using 30 s at 94 °C for denaturation, 30 s at 65 °C for annealing, and 1 min of extension at 68 °C for 18 to 32 cycles, depending on the transcript abundance and template complexity, followed by a final extension step at 68 °C for 2 min (Table 1).

After an indicated number of PCR cycles (between 18 and 32), 3-μL aliquots of PCR reaction mixtures were collected. PCR products were separated by electrophoresis in 2% agarose gels and ethidium bromide staining. Images of the electrophoresis gels were obtained using a Typhoon 9410 multiple image scanner (GE, Little Chalfont, Buckinghamshire, United Kingdom) with a 610-nm band-pass emission filter. The densities of target bands in the electrophoresis gel were measured and quantified using ImageQuant software (Amersham Pharmacia Biotech).

Statistical analysis

A non-parametric Kruskal–Wallis one-way analysis of variance was performed to compare the mean HSCOREs. When a significant difference was identified in the main effect, a Dunn post-hoc test

Table 1
Sequences of primers used for RT-PCR in this study.

Gene Symbol	Accession No.[a]	Sequence (5′ to 3′)[b]	Product (bp)[c]	Cycle	Annealing temp (°C)
PODXL	NM_001018111	F_AAAGGCCAAAAGCTCAGACA R_GGACGTTCCCACAACAGTCT	154	29	56
EMCN	NM_001159694	F_GAGCGTGAAGCTTCTTACCG R_GGTATGGAAGTCGGGATTGA	193	26	56
GLYCAM1	NR_003039	F_CTGCTACAGCTCCACCATGA R_GTTCTGCAGCTTTCCACCTC	215	37	56
CD34	NM_001025109	F_GCAAGCCACCAGAGCTATTC R_TGCATGTGCAGACTCCTTTC	181	29	56
CHST2	NM_004267	F_TCCAAGCCTTTCGTGGTATC R_GGGAACCCCTTTTAGAGACG	209	29	56
CHST4	NM_001166395	F_TGGCCATCTTGGCTCTATTC R_CTGCTTGAAGGTCATCCACA	203	29	56
CD300LG	NM_001168322	F_GCCTGACCTAGAAGCGTTTG R_CCTCAAACTCAAGTGCGACA	487	29	60
GAPDH	NM_001256799	F_ACCACAGTCCATGCCATCAC R_TCCACCACCCTGTTGCTGTA	480	18	56

[a] Accession No. refers to the registered number of each respected mRNA.
[b] F: Forward primer; R: Reverse primer.
[c] Amplicon size in base pairs.

Table 2
Demographic data of the subjects at different phases of the menstrual cycle.

Phases	Proliferative (day 7–14)	Early-secretory (day 15–18)	Mid-secretory (day 19–24)	Late-secretory (day ≧ 25)	Menopause	*P* value[a]
No. of subjects	12	10	9	11	11	
Mean ± (SE) age (y)	42.3 ± 3.7	46.8 ± 2.6	46.4 ± 2.8	41.0 ± 8.1	60 ± 7.6	0.07
Mean ± (SE) BMI (kg/m^2)	23.1 ± 3.5	23.8 ± 3.8	23.8 ± 2.9	23.2 ± 1.8	23.9 ± 5.8	0.79

BMI: body mass index; No.: Number; SE: standard error.
[a] *p* value was K–W test between four phases in adenomyosis.

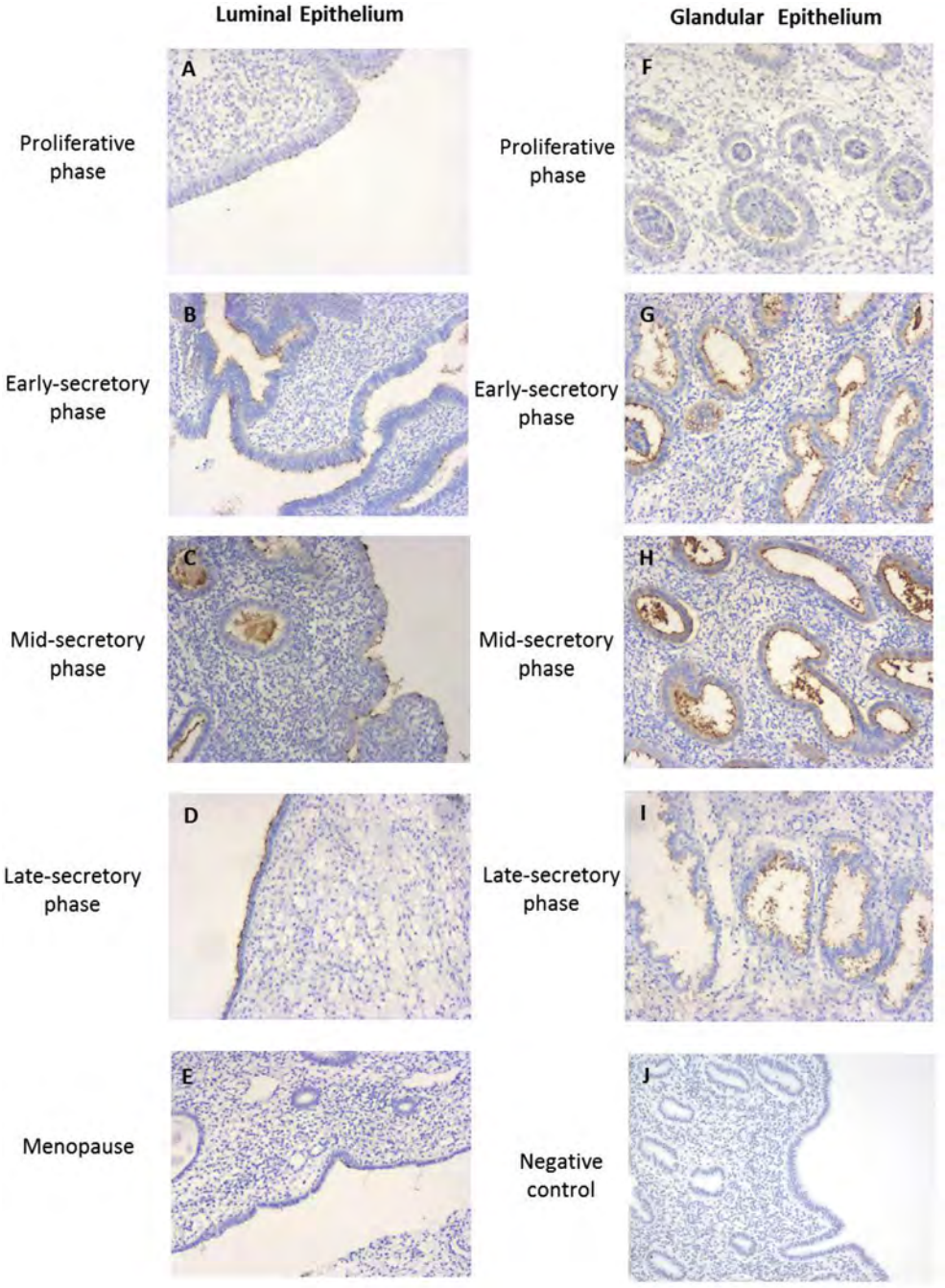

Fig. 1. Immunohistochemical staining with MECA-79 for LSL expression in adenomyotic endometria. LSLs were weakly expressed in the luminal and glandular epithelia of the endometrium in all the phases. Luminal epithelium at (A) proliferative (cycle days 7–14), (B) early-secretory (cycle days 15–18), (C) mid-secretory (cycle days 19–24), (D) late-secretory (cycle days ≧ 25) phases, and (E) menopause. Glandular epithelium at (F) proliferative, (G) early-secretory, (H) mid-secretory, (I) late-secretory phases, and (J) negative control. Original magnification 40×. LSL: L-selectin ligand.

was performed to identify the differences between the phases. $P < 0.05$ was considered as statistically significant.

For the calculation of LSL mRNA expression, we assumed that the LSL mRNA expression in menopausal endometria was as basic expression dosages without sex hormone stimulation. The relative fold changes of LSL mRNA expression in reproductive endometria were divided by those in menopausal endometria, which can represent the expression patterns of LSL mRNAs in different phases during the menstrual cycle induced by cyclic sex hormone stimulation. The values obtained from each sample were normalized to *GAPDH* expression, and the data are presented as the means ± the standard error of the mean.

A nonparametric Kruskal–Wallis (K–W) one-way analysis of variance with multiple comparisons was performed to examine differences between menstrual phases. Findings with a two-sided P value < 0.05 were considered to indicate statistically significant differences between phases. All data analyses were performed using SPSS version 13.0 (Chicago, IL, USA).

Results

Table 2 summarizes the demographic data of the subjects. The mean ages of cases in the proliferative, early-secretory, mid-secretory, and late-secretory phases were 42.3 ± 3.7, 46.8 ± 2.6, 46.4 ± 2.8, and 41.0 ± 8.1 years, respectively. The average BMIs were 23.1 ± 3.5 kg/m^2 for the proliferative phase, 23.8 ± 3.8 kg/m^2 for the early-secretory phase, 23.8 ± 2.9 kg/m^2 for the mid-secretory phase, and 23.2 ± 1.8 kg/m^2 for the late-secretory phase. The mean age and BMI of the menopausal group were 60.0 ± 7.6 years and 23.9 ± 5.8 kg/m^2, respectively. The mean age and BMI of the patients were not significantly different between phases ($p > 0.05$).

Immunohistochemistry analysis with MECA-79 showed that LSL was weakly expressed in the luminal and glandular epithelium of the endometrium in all phases (Fig. 1). Interestingly, weak immunostaining was also found in the luminal epithelium of the menopausal endometria (Fig. 1E). In the luminal epithelium, MECA-79 reactivity was relatively weak in the proliferative phase (Fig. 1A). The reactivity increased by the early-secretory phase (Fig. 1B) but fell during the mid-secretory phase (Fig. 1C) and then rose to its peak level during the late-secretory phase (Fig. 1D).

The mean HSCOREs among the proliferative, early-secretory, and late-secretory phases were significantly different (P < 0.05) (Fig. 2A). In the glandular epithelium, LSLs increased gradually to reach their peak level in the mid-secretory phase and then fell by the late-secretory phase (Fig. 1F–I). No significant differences were observed in the mean HSCOREs of the glandular epithelium among phases (Fig. 2B). No immunoreactivity was detected when monoclonal antibody MiTF was used as the primary antibody in the negative control staining (Fig. 1J).

LSL expression was confirmed via MECA-79 antibody detection using western blot in all four phases of the menstrual cycle (Fig. 3A). Five LSL genes were detected in adenomyotic and menopausal endometria: *PODXL*, *EMCN*, *CD300LG*, *GLYCAM1*, and *CD34* (Fig. 3B). The mRNA expressions of the LSL genes occurred differentially among the phases, but only *PODXL* differed significantly. A post-hoc test revealed *PODXL* differed significantly between the proliferative, early secretory, and mid-secretory phase. *PODXL* also had statistically significant differences between the late-, early-, and mid-secretory phases ($p < 0.05$) (Fig. 4A).

Comparison of the mRNA expression in the proliferative and mid-secretory phases indicated that the expression of all LSL genes were higher in the mid-secretory phase than in the proliferative phase. Moreover, *PODXL* expression was significantly different from that of *EMCN* and *CD34* ($p < 0.05$) (Fig. 4B). The mRNA expressions of the *CHST2* and *CHST4* genes were expressed without significant differences among phases (Fig. 5).

Discussion

Immunohistochemistry analysis with MECA-79 revealed that LSL was expressed in the luminal and glandular epithelium of the adenomyotic endometrium throughout the menstrual cycle (Fig. 1).

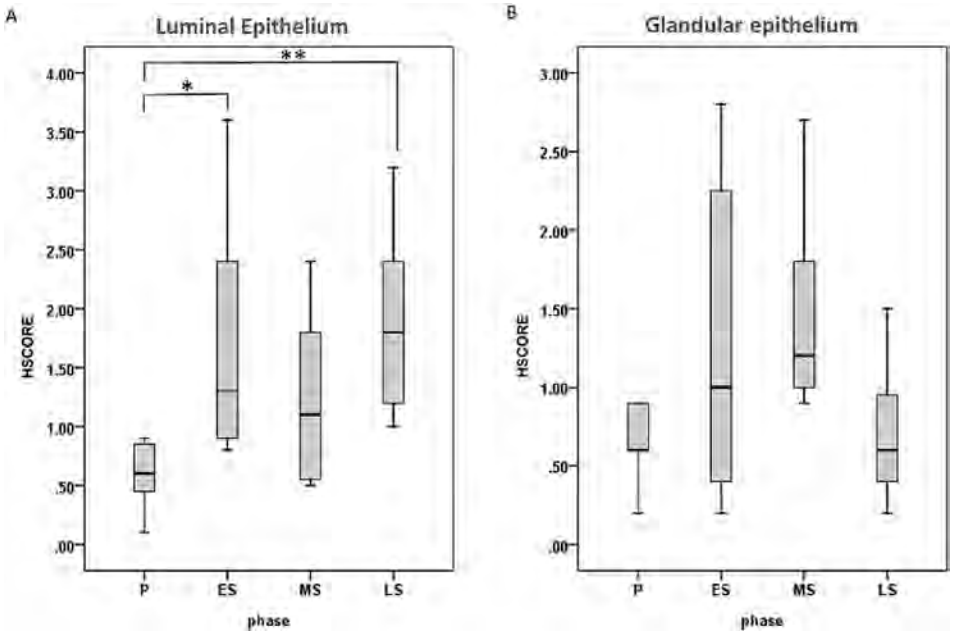

Fig. 2. Mean (±SEM) HSCOREs of MECA-79 for LSL in adenomyotic endometria throughout menstrual cycle. (A) Decreased expression of LSL was found in the mid-secretory phase in the luminal epithelium. Significant differences were found between the proliferative and early-secretory phases and between the proliferative and late-secretory phases. *$p < 0.05$. (B) LSLs increased gradually from the proliferative to mid-secretory phases and then fell by the late-secretory phase in the glandular epithelium. No significant difference was observed among the phases. P: proliferative phase; ES: early-secretory phase; MS: mid-secretory phase; LS: late-secretory phase; LSL: L-selectin ligand.

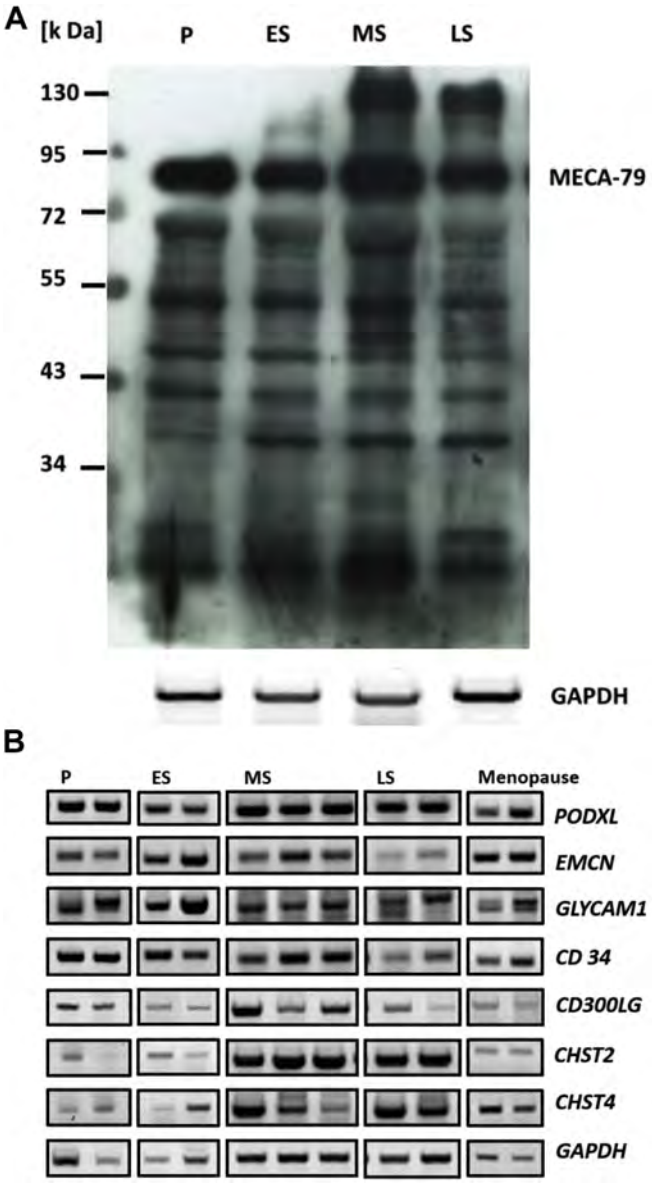

Fig. 3. A. Western blot to confirm LSL expression via MECA-79 antibody detection in different phases during the menstrual cycle in adenomyotic endometria. Strong band density at 79k Da was observed in the four phases. The band density was normalized to GAPDH. P: proliferative phase; ES: early-secretory phase; MS: mid-secretory phase; LS: late-secretory phase; LSL: L-selectin ligand; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. B. Examples of RT-PCR gel electrophoresis for the mRNA expressions of LSL genes in different phases during the menstrual cycle in adenomyotic endometria. Cropped gels of RT-PCR products in selected samples showed that the LSL genes *PODXL*, *EMCN*, *GLYCAM1*, *CD34*, and *CD300LG* were differentially expressed in the four phases of the menstrual cycle and menopausal endometria. The mRNA expressions of *CHST2* and *CHST4* were also found in the four phases of the menstrual cycle and menopausal endometria. The mRNA levels were normalized to *GAPDH* mRNA levels. P: proliferative phase; ES: early-secretory phase; MS: mid-secretory phase; LS: late-secretory phase; LSL: L-selectin ligand.

The expression pattern and immunostaining intensity were different from those found in the normal endometrium of the natural cycle in our previous study [12]. In that study, LSL had differential but strong expression in the endometrium among the phases of the natural cycle in women with myoma. However, the immunostaining intensity of LSL was relatively weak in the luminal epithelium of the adenomyotic endometrium compared to a normal endometrium using HSCOREs (Fig. 2A). Moreover, the LSL expression decreased in the mid-secretory phase (the period of embryo implantation).

Statistically significant differences were found between the proliferative, early-secretory, and late-secretory phases (Fig. 2A). These results suggested that weak LSL expression in the luminal epithelium throughout the menstrual cycle, especially decreased LSL expression in the mid-secretory phase, might be related to impaired endometrial receptivity and cause implantation failure in women with adenomyosis. In addition, we also found that LSL expression was very weak in the luminal epithelium of the menopausal endometrium (Fig. 1F). In summary, these results indicate that LSL may usually be present in the endometrium, regardless of whether a woman is at reproductive or menopausal age as well as with adenomyosis. This implies that LSL might serve as a basic adhesion molecule in the human endometrium, which is not only largely expressed during the window of embryo implantation but also in normal, adenomyotic, and menopausal endometria.

We first reported the mRNA expression of the LSL genes (*PODXL*, *EMCN*, *CD300LG*, *GLYCAM1*, and *CD34*) in human endometrium throughout the menstrual cycles in women with myoma and in menopausal women. Among the LSL genes, *EMCN* is the only one that is significantly different between the proliferative and early-secretory phases [21]. Interestingly, these LSL genes are the same as those found in the HEVs of mouse lymph nodes in previous studies [8,22–24]. LSLs may serve as common adhesion molecule in the immune and reproductive systems in mammals [24].

As far as we know, this is the first study to examine the mRNA expression patterns of LSL genes in adenomyotic endometria throughout the menstrual cycle. The discoveries in our serial studies suggest that LSL genes that translate the peptide components of LSL molecules are relevant in the production and modification of LSL molecules. Recent studies on endometriosis or adenomyosis show that endometrial receptivity is affected by cell adhesion molecules and their ligands, apoptosis, homeobox genes, and the direct action of gonadotrophin-releasing hormone analogues [25]. Moreover, impaired expressions of adhesion molecules during the implantation window were observed in women with adenomyosis and endometriosis [19,26,27]. Abnormal expression of adhesion molecules, such as integrin and LSL in the endometrium of adenomyosis, may contribute to subfertility and infertility.

The mRNA expressions of the LSL genes revealed differential patterns among phases. *PODXL* showed statistically significant differences between the proliferative, early-secretory, and mid-secretory phases, as well as between the late-, early-, and mid-secretory phases ($p < 0.05$) (Fig. 4A). Moreover, *PODXL* had statistically significant differences from *EMCN* and *CD34* ($p < 0.05$) (Fig. 4B). These results suggest that *PODXL* plays a relatively vital role in the production of LSL in adenomyosis.

PODXL is a transmembrane sialomucin that is similar in structure to *CD34* and belongs to the *CD34* family of sialomucins. It has previously been shown to be present in the foot processes of podocytes in the kidney glomerulus, as well as the vascular endothelium in other organs. It acts as an adhesion molecule and may be important for LSL function [28]. *PODXL* plays an essential role in the regulation of endothelial permeability by influencing the mechanisms involved in restoring the integrity of the endothelial barrier after injury [29]. In addition, Chen et al. reported that *PODXL* that is likely derived from maternal endothelial cells is present in pregnant serum and is significantly increased in early onset pre-eclampsia [30]. Recently, several studies reported that *PODXL* and *CD34* are markers of stem cells and are involved in differentiation and migration in embryonic, cancer, and induced pluripotent stem cells [31–36]. To date, the role of *PODXL* in endometrial receptivity is unclear. Further studies should be conducted to examine the

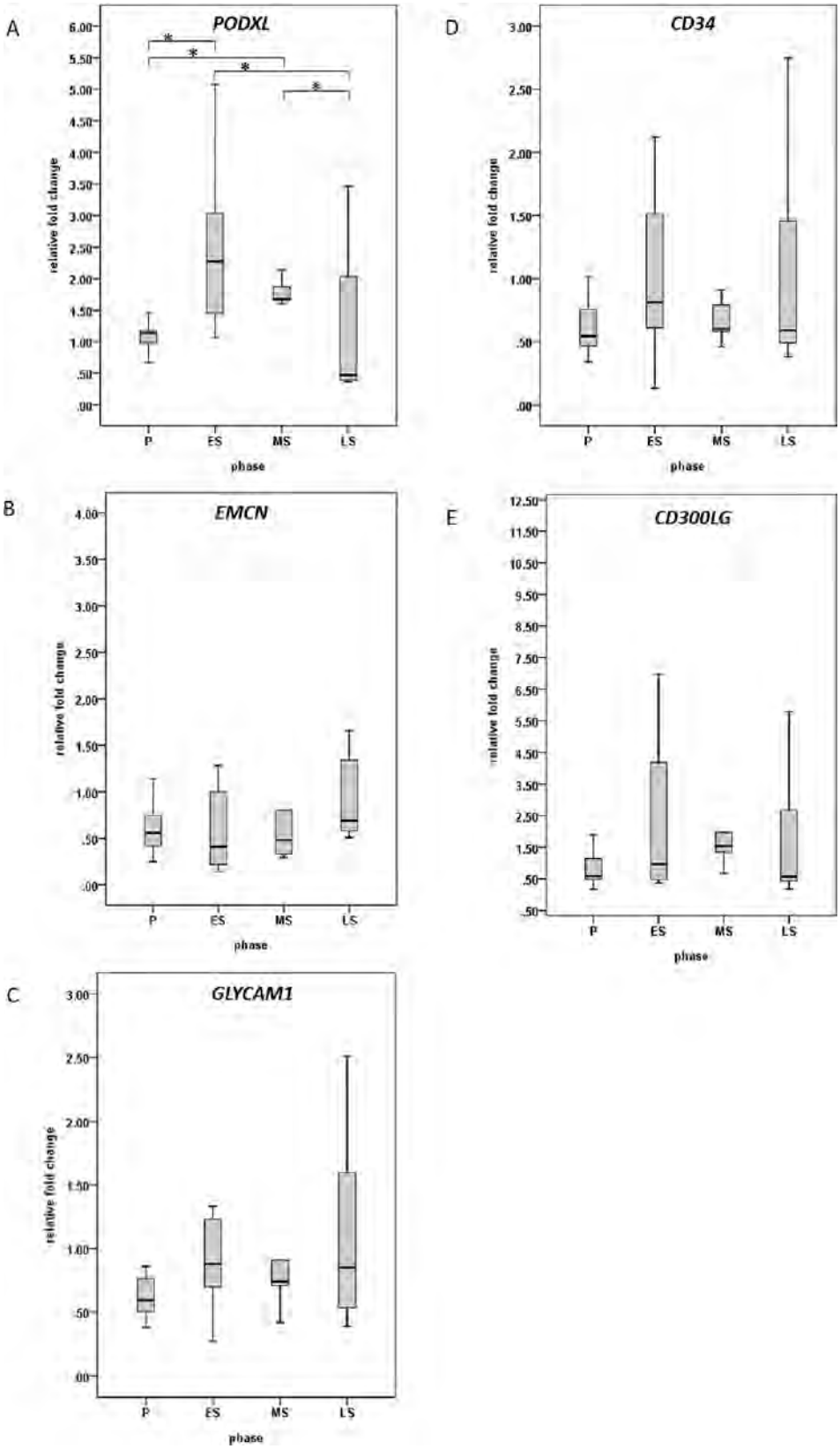

Fig. 4A. The mRNA expression patterns of LSL genes in the four phases during the menstrual cycle in adenomyotic endometria. The mRNA expressions of LSL genes including *PODXL*, *EMCN*, *GLYCAM1*, *CD34* and *CD300LG* were different between phases. *PODXL* differed significantly between the proliferative, early-secretory, and mid-secretory phases. *PODXL* also had significant differences between the late-secretory, early-secretory, and mid-secretory phases. $^{*}p < 0.05$. Results are expressed as mean ± SE. P: proliferative phase; ES: early-secretory phase; MS: mid-secretory phase; LS: late-secretory phase. LSL: L-selectin ligand.

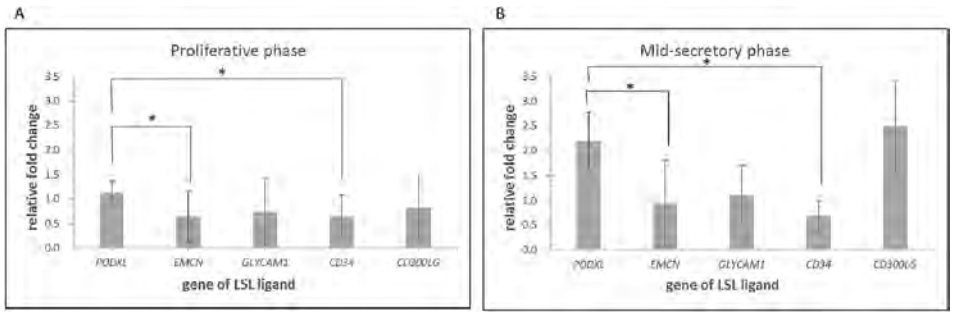

Fig. 4B. The mRNA expression patterns of LSL genes between the proliferative and mid-secretory phases in adenomyotic endometria. The mRNA expressions of LSL genes were all higher in the mid-secretory phase than in the proliferative phase. *PODXL* was significantly different from *EMCN* and *CD34* in the proliferative and mid-secretory phases ($p < 0.05$), (A) and (B). LSL: L-selectin ligand.

mechanism of local regulation on the expression of *PODXL* in relation to endometrial receptivity in adenomyosis.

In addition, we compared the expression patterns of LSL genes at different phases of the menstrual cycles between myotic and adenomyotic endometria with the data presented in our previous study [21]. We assumed that the LSL mRNA expression in menopausal endometria was as basic expression dosages without sex hormone stimulation. The relative fold changes of LSL mRNA expression in myotic and adenomyotic endometria were divided by those in menopausal endometria, which can represent the expression patterns of LSL mRNAs in different phases during the menstrual cycle induced by cyclic sex hormone stimulation. The relative fold changes of *EMCN* in women with myoma are significantly different between the proliferative and early-secretory phases ($p < 0.05$). However, in women with adenomyosis, *PODXL* showed statistically significant differences between the proliferative, early-secretory, and mid-secretory phases, as well as between the late-, early-, and mid-secretory phases ($p < 0.05$). These findings revealed that there are different mechanisms regulating the gene expressions of LSL subtypes between myoma and adenomyosis in human endometrium throughout the menstrual cycle.

Our data showed that all LSL genes showed higher expression in the mid-secretory phase than in the proliferative phase (Fig. 4B). However, in the mid-secretory phase, there was no significant decrease in the mRNA expressions of two sulfotransferase genes, *CHST2* and *CSHT4*, which are involved in the generation of LSL epitopes and LSL activity (Fig. 5). These results suggest that the decrease of LSL expression in the luminal epithelium in the mid-secretory phase may be a key event in the impairment of endometrial receptivity in adenomyosis.

It is reasonable to infer that decreased LSL expression in the luminal epithelium may result from the impact of adenomyosis. A series of metabolic and molecular abnormalities that increase angiogenesis and proliferation and decrease apoptosis allow for the local production of estrogens, create progesterone resistance, and impair cytokine expression in the eutopic and ectopic endometria of women with adenomyosis [3,37]. We speculate that abnormal cytokines, chemokines, inflammatory factors, free radicals and paracrines produced by adenomyotic lesions may affect LSL production in the luminal and glandular epithelium of the endometrium. Further studies *in vitro* and *in vivo* are needed to elucidate the possible mechanisms.

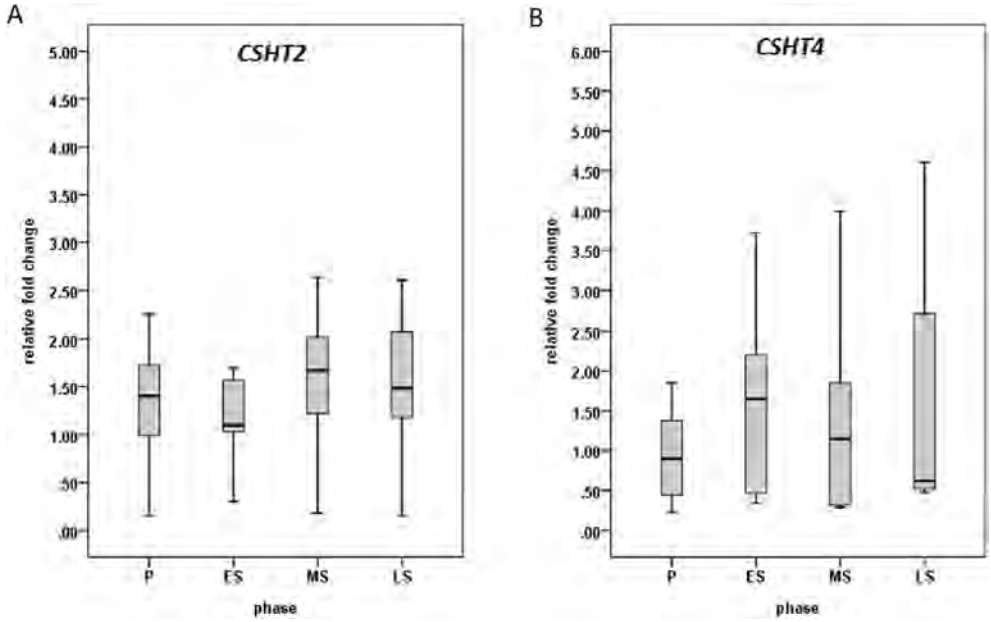

Fig. 5. The mRNA expression patterns of *CHST2* and *CHST4* in the phases during the menstrual cycle in adenomyotic endometria. The mRNA expression of *CHST2* and *CHST4* were different between phases. However, there was no significant difference between the phases (A and B). Results are expressed as mean ± SE. *CHST2*: the gene of carbohydrate sulfotransferase 2; *CHST4*: the gene of carbohydrate sulfotransferase 4. P: proliferative phase; ES: early-secretory phase; MS: mid-secretory phase; LS: late-secretory phase. LSL: L-selectin ligand.

In humans, during a natural cycle, the embryo enters the uterine cavity about 4–5 days after ovulation. The endometrium becomes receptive to blastocyst implantation 6–8 days after ovulation and remains receptive for 4 days (cycle days 20–24) [38,39]. The duration of receptive endometrium for implantation coincides with the mid-secretory phase. Several reports had mentioned that defects in LSL expression during mid-secretory phase could lead to implantation failure and early pregnancy loss [9,14,17,40]. In this study, we found that LSL expression decreases in the luminal epithelium of adenomyotic endometria in the mid-secretory phase. Our findings showed that adenomyosis may cause abnormalities in the production of LSL during the mid-secretory phase, which may contribute to impaired endometrial receptivity and implantation failure.

There are certain limitations in this study that need to be addressed. The small number of samples could potentially limit the power of the study. Furthermore, the heterogeneity of the samples in each phase of the menstrual cycle could impact the results. Finally, there are no normal endometrial samples available in each phase as control to compare the differences with samples from adenomyosis. It is very difficult to obtain endometrial samples from healthy women. Moreover, endometrial biopsies from healthy women may raise ethical concerns. Therefore, we used endometrial samples collected from menopausal women who underwent hysterectomy as controls to compare the mRNA expressions of LSL genes. This is the most feasible approach to obtaining human endometrial tissue and has also been used in previous studies.

In conclusion, LSL expressions were downregulated in the luminal epithelium of adenomyotic endometria in the mid-secretory phase. The mRNA expressions of LSL genes also had differential expression patterns throughout the menstrual cycle, especially for *PODXL*. Our study showed that adenomyosis may cause abnormalities of LSL production in the mid-secretory phase, which may contribute to impaired endometrial receptivity and implantation failure. Further studies *in vitro* and *in vivo* are required to determine the mechanisms related to the decrease in LSL expression in adenomyosis.

Conflicts of interest

The authors declare that they have no conflicts of interest that could be perceived as prejudicing the impartiality of this research.

Acknowledgements

This research was supported by a research grant (CGH-MR-9705) from Cathay General Hospital, Taipei City, Taiwan. The authors would like to thank Professor Chien-An Sun for his invaluable suggestions for the statistical analysis. The authors also thank Ms. Sin-Bay Wu for her excellent technical assistance.

References

[1] Dueholm M. Uterine adenomyosis and infertility, review of reproductive outcome after in vitro fertilization and surgery. Acta Obstet Gynecol Scand 2017;96:715–26.
[2] Fischer CP, Kayisili U, Taylor HS. HOXA10 expression is decreased in endometrium of women with adenomyosis. Fertil Steril 2011;95:1133–6.
[3] Wang PH, Su WH, Sheu BC, Liu WM. Adenomyosis and its variance: adenomyoma and female fertility. Taiwan J Obstet Gynecol 2009;48:232–8.
[4] Soave I, Wenger JM, Pluchino N, Marci R. Treatment options and reproductive outcome for adenomyosis-associated infertility. Curr Med Res Opin 2018;34:839–49.
[5] Harada T, Khine YM, Kaponis A, Nikellis T, Decavalas G, Taniguchi F. The impact of adenomyosis on Women's fertility. Obstet Gynecol Surv 2016;71:557–68.
[6] Maheshwari A, Gurunath S, Fatima F, Bhattacharya S. Adenomyosis and subfertility: a systematic review of prevalence, diagnosis, treatment and fertility outcomes. Hum Reprod Update 2012;18:374–92.
[7] Campo S, Campo V, Benagiano G. Adenomyosis and infertility. Reprod Biomed Online 2012;24:35–46.
[8] Zak I, Lewandowska E, Gnyp W. Selectin glycoprotein ligands. Acta Biochim Pol 2000;47:393–412.
[9] Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Ilic D, Singer MS, et al. Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. Science 2003;299:405–8.
[10] Fazleabas AT, Kim JJ. Development. What makes an embryo stick? Science 2003;299:355–6.
[11] Feng Y, Ma X, Deng L, Yao B, Xiong Y, Wu Y, et al. Role of selectins and their ligands in human implantation stage. Glycobiology 2017;27:385–91.
[12] Lai TH, Shih Ie M, Vlahos N, Ho CL, Wallach E, Zhao Y. Differential expression of L-selectin ligand in the endometrium during the menstrual cycle. Fertil Steril 2005;83(Suppl 1):1297–302.
[13] Lai TH, Zhao Y, Shih Ie M, Ho CL, Bankowski B, Vlahos N. Expression of L-selectin ligands in human endometrium during the implantation window after controlled ovarian stimulation for oocyte donation. Fertil Steril 2006;85:761–3.
[14] Foulk RA, Zdravkovic T, Genbacev O, Prakobphol A. Expression of L-selectin ligand MECA-79 as a predictive marker of human uterine receptivity. J Assist Reprod Genet 2007;24:316–21.
[15] Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Fertil Steril 1950;1:3–25.
[16] Hemmerich S, Butcher EC, Rosen SD. Sulfation-dependent recognition of high endothelial venules (HEV)-ligands by L-selectin and MECA 79, and adhesion-blocking monoclonal antibody. J Exp Med 1994;180:2219–26.
[17] Margarit L, Gonzalez D, Lewis PD, Hopkins L, Davies C, Conlan RS, et al. L-selectin ligands in human endometrium: comparison of fertile and infertile subjects. Hum Reprod 2009;24:2767–77.
[18] Budwit-Novotny DA, McCarty KS, Cox EB, Soper JT, Mutch DG, Creasman WT, et al. Immunohistochemical analyses of estrogen receptor in endometrial adenocarcinoma using a monoclonal antibody. Cancer Res 1986;46:5419–25.
[19] Lessey BA, Castelbaum AJ, Wolf L, Greene W, Paulson M, Meyer WR, et al. Use of integrins to date the endometrium. Fertil Steril 2000;73:779–87.
[20] Uchimura K, Rosen SD. Sulfated L-selectin ligands as a therapeutic target in chronic inflammation. Trends Immunol 2006;27:559–65.
[21] Lai TH, Chang FW, Lin JJ, Ling QD. Gene expression of human endometrial L-selectin ligand in relation to the phases of the natural menstrual cycle. Sci Rep 2018;8:1443.
[22] Shimizu Y, Newman W, Tanaka Y, Shaw S. Lymphocyte interactions with endothelial cells. Immunol Today 1992;13:106–12.
[23] Picker LJ, Butcher EC. Physiological and molecular mechanisms of lymphocyte homing. Annu Rev Immunol 1992;10:561–91.
[24] Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. Annu Rev Immunol 2004;22:129–56.
[25] Selam B, Arici A. Implantation defect in endometriosis: endometrium or peritoneal fluid. J Reprod Fertil Suppl 2000;55:121–8.
[26] Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W, et al. Aberrant integrin expression in the endometrium of women with endometriosis. J Clin Endocrinol Metab 1994;79:643–9.
[27] Xiao Y, Li T, Xia E, Yang X, Sun X, Zhou Y. Expression of integrin beta3 and osteopontin in the eutopic endometrium of adenomyosis during the implantation window. Eur J Obstet Gynecol Reprod Biol 2013;170:419–22.
[28] Sassetti C, Tangemann K, Singer MS, Kershaw DB, Rosen SD. Identification of podocalyxin-like protein as a high endothelial venule ligand for L-selectin: parallels to CD34. J Exp Med 1998;187:1965–75.
[29] Horrillo A, Porras G, Ayuso MS, Gonzalez-Manchon C. Loss of endothelial barrier integrity in mice with conditional ablation of podocalyxin (Podxl) in endothelial cells. Eur J Cell Biol 2016;95:265–76.
[30] Chen Q, Wang Y, Li Y, Zhao M, Nie G. Serum podocalyxin is significantly increased in early-onset preeclampsia and may represent a novel marker of maternal endothelial cell dysfunction. J Hypertens 2017;35:2287–94.
[31] Zhang H, Nieves JL, Fraser ST, Isern J, Douvaras P, Papatsenko D, et al. Expression of podocalyxin separates the hematopoietic and vascular potentials of mouse embryonic stem cell-derived mesoderm. Stem Cell 2014;32:191–203.
[32] Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. Concise review: evidence for CD34 as a common marker for diverse progenitors. Stem Cell 2014;32:1380–9.
[33] Tateno H, Matsushima A, Hiemori K, Onuma Y, Ito Y, Hasehira K, et al. Podocalyxin is a glycoprotein ligand of the human pluripotent stem cell-specific probe rBC2LCN. Stem Cells Transl Med 2013;2:265–73.
[34] Thankamony SP, Sackstein R. Enforced hematopoietic cell E- and L-selectin ligand (HCELL) expression primes transendothelial migration of human mesenchymal stem cells. Proc Natl Acad Sci U S A 2011;108:2258–63.
[35] Schopperle WM, Lee JM, Dewolf WC. The human cancer and stem cell marker podocalyxin interacts with the glucose-3-transporter in malignant pluripotent stem cells. Biochem Biophys Res Commun 2010;398:372–6.
[36] Schopperle WM, DeWolf WC. The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. Stem Cell 2007;25:723–30.
[37] Benagiano G, Brosens I. The endometrium in adenomyosis. Womens Health (Lond Engl) 2012;8:301–12.

[38] Bergh PA, Navot D. The impact of embryonic development and endometrial maturity on the timing of implantation. Fertil Steril 1992;58:537–42.
[39] Diedrich K, Fauser BC, Devroey P, Griesinger G. The role of the endometrium and embryo in human implantation. Hum Reprod Update 2007;13:365–77.
[40] Zhong Y, Li J, Wu H, Ying Y, Liu Y, Zhou C, et al. Effect of surgical intervention on the expression of leukemia inhibitory factor and L-selectin ligand in the endometrium of hydrosalpinx patients during the implantation window. Exp Ther Med 2012;4:1027–31.