

The effect of hysteroscopic polypectomy on the gene expression of endometrial receptivity markers: *HOXA10*, *HOXA11* and *LIF*

Research Article

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Summary

The aim of this study is to determine the effect of hysteroscopic polypectomy on the mRNA expression levels of the endometrial receptivity markers, namely, homeobox A10 (*HOXA10*), homeobox A11 (*HOXA11*) and leukemia inhibitory factor (*LIF*). Twenty-five reproductive-aged women with endometrial polyps underwent hysteroscopy. Samples were taken at the mid-secretory phase using hysteroscopic polypectomy and 4 months after polypectomy, and the change in mRNA expression levels of normalized *HOXA10*, *HOXA11* and *LIF* genes were determined using Reverse Transcription Quantitative Real Time–Polymerase Chain Reaction (RT-qPCR). The results show that mRNA levels of *HOXA10* and *HOXA11* taken prior to surgery and 4 months after the complete hysteroscopic removal of polyps were not significantly different ($P=0.79$ and $P=0.14$, respectively). Moreover, a marked difference could not be obtained between preoperative and postoperative endometrial *LIF* mRNA expression levels ($P=0.86$). As a conclusion, these results indicate that mRNA levels of *HOXA10*, *HOXA11* and *LIF* genes, three of molecular markers of endometrial receptivity, are not affected by hysteroscopic polypectomy.

I. Introduction:

Successful embryo implantation requires a well-coordinated reciprocal action between hormones, cytokines and growth factors (Carson *et al*, 2000; Dey *et al*, 2004; Tabibzadeh and Babaknia, 1995). Endometrial polyps, which are benign, hyperplastic-localized overgrowth of glands and stroma have been reported in 15-25% of infertile women and in about 1.4% of women undergoing *in vitro* fertilization (IVF) (de Sa Rosa e de Silva *et al*, 2005; Salm, 1972; Sanders, 2006; Shokeir *et al*, 2004). The mechanism by which endometrial polyp influences fertility is not clearly understood yet; however, it has been suggested that endometrial polyps may be related to the mechanical interference of sperm transport, embryo implantation or aberrant expression of endometrial markers consisting of *HOXA10* and *HOXA11*, insulin growth factor binding protein 1 (*IGFBP1*), tumor necrosis factor alpha (TNF alpha) (Elbeheri *et al*, 2011; Inagaki *et al*, 2003; Rackow *et al*, 2011). *HOXA10* is essential for both endometrial development during menstrual cycle and endometrial receptivity (Aghajanova *et al*, 2008; Rackow and Taylor, 2010; Taylor, 2000; Taylor *et al*, 1998; Taylor *et al*, 2003). An *in vitro* study has demonstrated that decreased *HOXA10* expression is associated with diminished implantation, which proposes that change in *HOXA10* levels modulates the degree of endometrial receptivity (Bagot *et al*, 2000). Similarly, an altered expression of *HOXA11* results in poor endometrial development and lower implantation rate (Gendron *et al*, 1997; Hsieh-Li *et al*, 1995).

Although evidence from previous reports has shown that *HOXA10* and *HOXA11* appeared to influence implantation, several other molecules are also needed in this highly coordinated process where hundreds of molecules are intertwining rather than only a single molecule (Altmae *et al*, 2014; Altmae *et al*, 2010; Giudice and Kao, 2004; Horcajadas *et al*, 2007; Koler *et al*, 2009).

We suggest that different mechanisms might be related with implantation failure. *LIF*, which is known to be a member of pleiotropic cytokine family, can exert proinflammatory and anti-inflammatory responses (Aghajanova *et al*, 2008; Giudice and Kao, 2004). In human, *LIF* is expressed in endometrial glands during menstrual cycles and its expression peaks in the middle-late secretory phase (Singh *et al*, 2011). It has also been reported that *LIF* might also be involved in embryonic implantation, and its expression level might decrease with unexplained infertility when compared to fertile controls during implantation window (Aghajanova, 2010; Aghajanova *et al*, 2009; Charnock-Jones *et al*, 1994). It has been reported that *LIF* gene activity was lower with women who had unexplained infertility rates, thus *LIF* signaling pathway could possibly offer an explanation for increased infertility rates (Aghajanova *et al*, 2009).

Although endometrial polyps have been identified as a possible factor for infertility, their implication on endometrial molecular markers involved in implantation has not been studied extensively. It was postulated that the presence of endometrial polyps in uterine cavity may alter endometrial signaling pathways leading to impaired endometrial receptivity; therefore, removal of polyps will have beneficial impact on endometrial receptivity (Rackow *et al*, 2011). Based on evidence from aforementioned studies, we attempted to evaluate the impact of hysteroscopic endometrial polypectomy on established endometrial receptivity markers including *HOXA10*, *HOXA11* and *LIF*.

II. Materials and Methods:

A. Biopsy

This prospective interventional study was carried out at Inonu University, Turgut Ozal Medical Centre. From unselected population of non-pregnant, reproductive aged women, 25 consecutive subjects (Six of patients did not have any children and one of these women lost a baby by abortion) who were diagnosed of endometrial polyp during transvaginal ultrasound (TV-USG) were invited to be involved in this study. The study protocol was approved by Institutional Ethical Committee for Research on Human Subjects. Informed written consent form was obtained from all participants.

All participants meets following inclusion criteria: 1) ages ranging from 22 to 49 years; 2) regular menstrual cycles; 3) presence of endometrial polyp on TV-USG that was also confirmed on saline infusion sonohysterogram; 4) acceptance of second examination at follow-up screening; 5) not taking any hormonal medications or treatments at least 3 months before surgery. The exclusion criteria were determined as the following: 1) having any confounding medical conditions known to affect endometrial receptivity such as endometriosis, polycystic ovarian syndrome (PCOS) or hydrosalpinges; 2) presence of any other endometrial pathology including Asherman Syndrome, submucosal fibroids, endometrial cancer or endometrial hyperplasia; 3) diagnosis of pelvic inflammatory disease at the time of the study; 4) previous history of endometrial surgery; 5) history of habitual abortion.

All women had two-dimensional transvaginal ultrasound scan (Toshiba Xario, Japan) using high frequency transducers of 5-7.5 MHz, followed by saline infusion hysterosonography for the confirmation of endometrial polyps (Lee *et al*, 2006).

Subsequently, all the women recruited for the study had endometrial polyps removed through pipelle in the secretory phase of menstrual cycle during the implantation window. The endometrial specimens were divided into two groups for both pathological evaluation and biochemical-molecular analysis. Endometrial polyps were excised under hysteroscopic guidance. The secretory phase was calculated as 7 to 9 days after the ultrasonographic confirmation of ovulation. Subsequently, endometrial samples were obtained 4 months

after polypectomy. Biopsy samples were evaluated by an independent pathologist experienced in gynecological pathology and only patients who are having polyps were included in the study. All endometrial biopsy samples were taken 7-9 days after ovulation and also maturation of endometrium was evaluated by Noyes criteria. Age of the patients, time of the surgery, obstetric and gynecological history, medical conditions, last menstrual period, preoperative and postoperative diagnoses data were recorded for all patients. Follow-up hysteroscopy was performed after four months at mid-luteal phase of menstrual cycle, and endometrial samples were collected from all participants.

B. RT-qPCR

All samples were stored in 1 ml of RNA-later solution at -80 °C until they were analyzed for the expression levels of *HOXA10*, *HOXA11*, *LIF* and β -*actin* mRNA. Endometrial samples and polyps that were obtained prior to and after polypectomy were subjected to manufacturer's total RNA isolation protocol (RNeasy Mini Kit, QIAGEN, Hilden, Germany). Total extracted RNA was run on 1% agarose gel and integrity of the mRNA was confirmed by visualization of ribosomal bands with ethidium bromide staining over a UV transilluminator (data not shown). The concentration of the total purified RNA was determined by NanoDrop® Spectrophotometer. SuperScript® III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) was used for reverse transcription (RT) reactions with minor changes. Equal amounts of total RNA were added into each RT reaction and oligo dT-18 primer was used to extend all mRNAs in the first strand cDNA synthesis. Primers were designed by BLAST primer designing software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) using human gene as a template (Table 4). Quantitative PCR was carried out by using cDNA as template with 96 well qPCR platform (Roche LC480). Briefly, PCR amplification mixture (20 μ l) contained 1 μ l of RT reaction mixture (cDNA), 1 μ l of forward (10 pmol/ μ l), 1 μ l of reverse primers (10 pmol/ μ l) and 10 μ l of 2x SYBR Green I Master Mix (LC480 SYBR Green I

Master Mix, Roche 04707516001). Amplification was performed at 95°C for 10 min, followed by 45 cycles of 95°C for 20 sec, 60°C for 20 sec, and 70°C for 30 sec heating and cooling cycles. Melting analysis was also performed at the end of PCR. Ct values were determined by automatic setting of the software. All qPCR were performed in triplicates in the same plate with β -actin housekeeping gene. For each gene, relative mRNA expression levels were calculated according to housekeeping genes, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

C. Statistical Analysis

The data were analyzed using the Statistical Package for Social Sciences software 19.0 for Windows package software (SPSS, Inc., Chicago, IL). Power analysis revealed that 16 patients are required for *HOXA11* gene with the following conditions: *HOXA11* average: 0.045; standard deviation: 0.052; type I error (alpha): 0.05 and type II error (beta: power): 0.10.

The distribution normality of data was evaluated with the Kolmogorov–Smirnov test and gene expression levels was found to be non-normally distributed ($P < 0.05$). The results were presented as median, minimum and maximum. Comparison of *HOXA10*, *HOXA11* and *LIF* concentrations in endometrial samples prior to and after polypectomy was performed using Wilcoxon Signed-Rank Sum Test. Correlation of variables was assessed using Spearman's correlation test. For all comparisons, probability of < 0.05 was considered to be significant.

III. Results

The basal characteristics and serum hormone levels of patients are presented in **Table 1**. All endometrial tissue samples and hysteroscopically excised endometrial polyps underwent histological evaluation and endometrial polyps were identified as polyps and biopsy samples revealed normal secretory endometrium. The mean time interval between two endometrial samplings was 105.4 ± 53.6 days.

To compare *HOXA10*, *HOXA11* and *LIF* mRNA levels prior and after polypectomy, total RNA was isolated from all samples and cDNA was transcribed from all mRNA. mRNA levels of *HOXA10*, *HOXA11*, *LIF* and β -actin mRNA levels were measured by qPCR method and Ct values were calculated from SYBR Green fluorescence amplification graphs (**Figure 1.A**). Melting analysis of PCR products revealed a single peak (**Figure 1.B**) which proves that SYBR Green fluorescence signal belongs to only one kind of PCR product. Since melting analysis does not give information about the PCR product size, PCR products were also run in DNA agarose gels and correct sized PCR products were obtained for *HOXA10*, *HOXA11* and *LIF* genes as well as for the control β -actin housekeeping gene, 110 bp, 128 bp, 113 bp and 112 bp, respectively (**Figure 2, Table 2**).

Expression levels *HOXA10*, *HOXA11* and *LIF* mRNA were normalized according to β -actin housekeeping gene and they were presented in Table 3. The expression levels of *HOXA10*, *HOXA11* and *LIF* mRNA of endometrial samples taken prior to polypectomy were not significantly different than endometrium samples obtained 4 months after removal of whole endometrial polyp ($P=0.79$, $P=0.14$ and $P=0.86$, respectively). The size of polyps and expression levels of *HOXA10*, *HOXA11* and *LIF* ($r=0.252$, $P=0.29$; $r=0.391$, $P=0.88$; $r=0.152$, $P=0.52$, respectively) were compared but a significant correlation was not observed. The comparison of patient age with expression levels of *HOXA10*, *HOXA11* and *LIF* also did not correlate significantly ($r=-0.307$, $P=0.19$; $r=0.157$, $P=0.51$; $r=-0.197$, $P=0.41$, respectively).

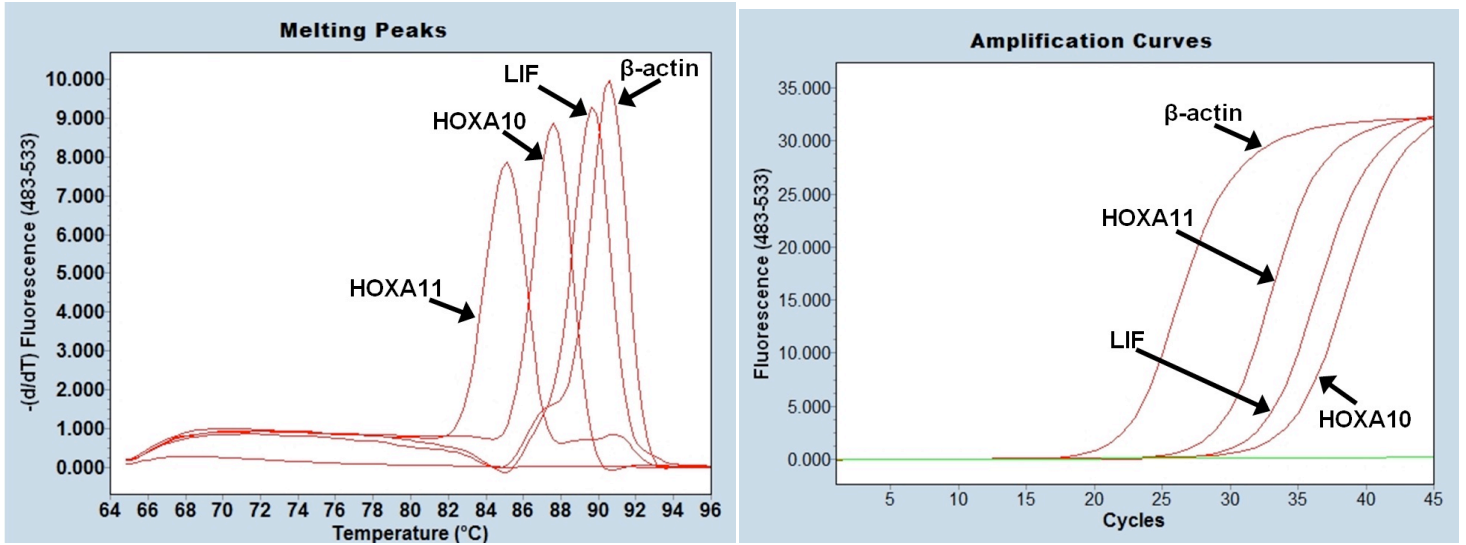


Figure 1: RT-qPCR amplification and melting analysis of HOXA10, HOXA11, LIF and β-actin mRNA. HOXA10, HOXA11, LIF and β-actin mRNA samples were subjected to reverse transcription and SYBR Green based Real Time PCR analysis (A). After RT-qPCR, products were subjected to melting analysis (B).

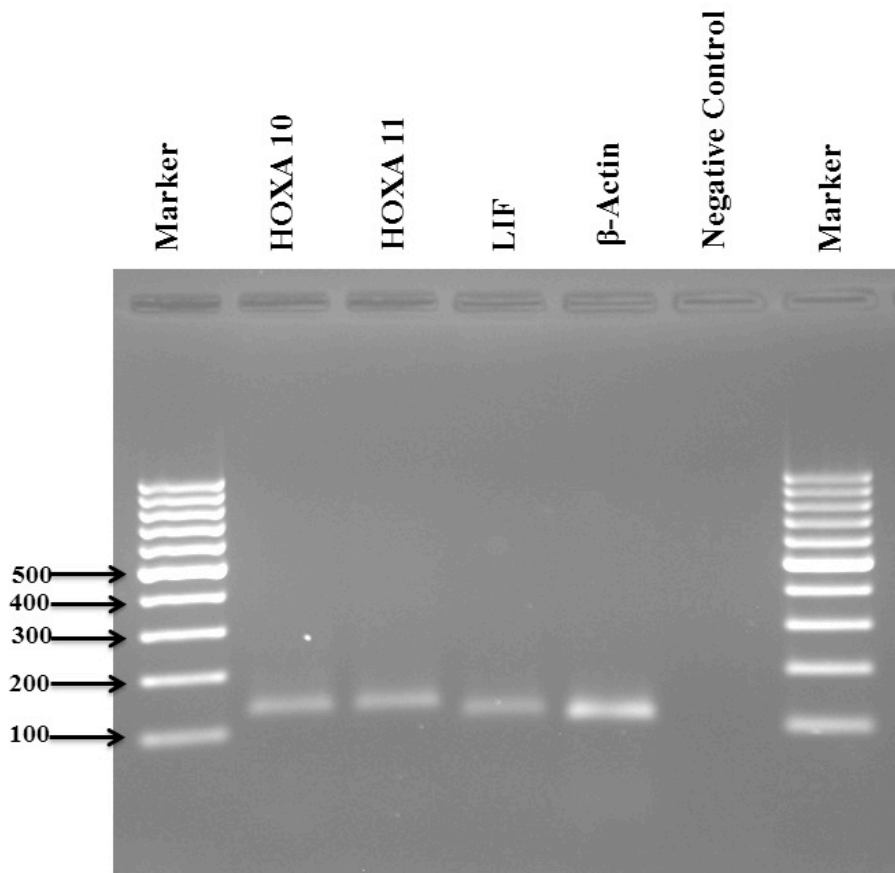


Figure 2: DNA gel electrophoresis of RT-qPCR of HOXA10, HOXA11, LIF and β-actin mRNA. PCR products were loaded on 1.5% DNA agarose gel and the size of each mRNA was determined by using of DNA marker (100 bp, Fermentas). The expected sizes of the PCR products were given in **Table 4**.

Table 1: The basal characteristic of patients and serum level of FSH, LH and E2.

Age mean±SD	PS (mm) mean±SD	NP median (min-max)	G median (min-max)	P median(min-max)	FSH (mU/ml) median (min-max)	LH(mU/ml) mean±SD	E2 (pg/ml) median(min-ma)
36.6±7.7	18.75±8.44	1 (1-5)	2.5 (0-8)	2 (0-5)	7 (1.7-64)	4.9±2.3	112 (51-690)

PS; Polyp size, NP; Number of polyps, G; Gravida, P; Para, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, E2; Estradiol

Table 2: Primer sequence and expected PCR product size for endometrial receptivity markers and internal standard β -actin mRNA.

Genes ^{a, b}	Primer sequences	PCR product size (bp)
β -Actin-F	5'-CGAGCACAGAGCCTCGCCTT-3'	112
β -Actin-R	5'-TGCACATGCCGGAGCCGTTG-3'	
HOXA10-F	5'-GTGCTCTTTCGCGCAGAACATCAAA-3'	110
HOXA10-R	5'-GCGGGAAGGGAGCCAGTTCG-3'	
HOXA11-F	5'-GGCTCCAGTGGCCAACGCAC-3'	128
HOXA11-R	5'-ATGCGGGACAGTTGCAGGCG-3'	
LIF-F	5'-CTCAACGCCACCGCCGACAT-3'	113
LIF-R	5'-CCCGAGGTGTCAGGGCCGTA-3'	

^a β -actin: Beta-actin gene, HOXA10: Homeobox A10 gene, HOXA11: Homeobox A11 gene, LIF: Leukemia Inhibitory Factor gene.

^b F: forward primer, R: reverse primer

Primer sequences were designed by BLAST primer designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) using human genes as template.

Table 3: Comparison of expression of endometrial receptivity markers including HOXA10, HOXA11 and LIF between pre- and post-polypectomy in the whole group. Values are presented as “median (min-max)”. Comparison between groups was performed using “Wilcoxon Signed-Rank Sum Test.

Variables	Preoperative (n=25)	Postoperative (n=25)	P values
	Median (min-max)	Median (min-max)	
HOXA10	0.00051 (0.00013-0.015)	0.00055 (0.00003-0.025)	0.79
HOXA11	0.027 (0.04-0.20)	0.039 (0.07-0.18)	0.14
LIF	0.0043 (0.00006-0.020)	0.0029 (0.00028-0.023)	0.86

IV. Discussion

In this study, the expression levels of HOXA10, HOXA11 and LIF genes were investigated before and after endometrial polyp removal and it was found that endometrial polyp removal does not change the expression levels of HOXA10, HOXA11 and LIF genes. Any significant correlation was not also noted with the polyp size and age of the patients. As a conclusion, we suggest that several other genes may also be involved in the regulation of endometrial polyp removal. In literature, several observational studies stated that the removal of endometrial polyp with infertility resulted in a significant improvement in pregnancy rate in subfertile or infertile women who underwent hysteroscopic endometrial polyp removal (Delage *et al*, 1995; Shokeir *et al*, 2004; Spiewankiewicz *et al*, 2003; Varasteh *et al*, 1999). The mechanism of endometrial polyp infertility has not been determined yet, but several hypotheses were proposed including mechanical inhibition of sperm and embryo transport, altered endometrial receptivity or impairment of endometrial vascularity adversely affecting embryo

implantation (Rackow *et al*, 2011). The result of one randomized controlled trial concerning the effect of endometrial polypectomy on subfertility has demonstrated that pregnancy rate considerably increased in women who underwent endometrial polypectomy (Perez-Medina *et al*, 2005).

Another prospective study comparing concentrations of endometrial markers in consecutive endometrial flushing fluid prior to post hysteroscopic polypectomy has shown a significant increase in *IGFBP1*, TNF alpha and osteopontin concentrations after hysteroscopic polypectomy (Ben-Nagi *et al*, 2009).

Further, a case-controlled study has reported significantly lower mRNA expression of *HOXA10* and *HOXA11* in endometrium from women with polyps than controls in the proliferative phase with infertile subjects (Rackow *et al*, 2011). The endometrial polyp is type-a benign tumor and it is the result of monoclonal neoplastic proliferation of stromal cells with a non-neoplastic glandular component (Christopher P. Crum, 2011).

The endometrial polyps are characterized with the following features: thick-walled vessels, fibrous or collagenous stroma or irregular gland structure. The possible explanation of this discrepancy between the previous reports with our result may be the paucity of pathological discrimination of endometrial polyps. It has been demonstrated that endometrial polyps can be divided into two patterns including proliferative/hyperplastic, atrophic or functional, and these patterns frequently overlap (Robert J. Kurman, 2011). The other explanation for this difference may be the variation of methodology that other studies did not re-evaluate these endometrial receptivity markers postoperatively.

Despite an obvious improvement in fertility rate after removal of endometrial polyps reported previously, the significance of the location and the size of polyp still remain controversial. Evidence from retrospective data suggests that removal of endometrial polyp improves the pregnancy rate in infertile women, irrespective of the number or size of present polyps (Livak and Schmittgen, 2001; Preuthipan and Herabutya, 2005; Rackow and Taylor, 2010). A randomized controlled study on the effect of size of endometrial polyp reported that significant differences were not observed in the pregnancy rate after polypectomy between the groups with different size of polyps; polyp diameter ranged <5, 5-10, 11-20 and >20 mm (Perez-Medina et al, 2005). In a case-controlled study, Rackow et al. (Rackow et al, 2011) demonstrated that expression levels of endometrial receptivity markers (*HOXA10* and *HOXA11*) did not change significantly between a single polyp and multiple polyps. The same study also suggests that the size of polyps was not associated with the endometrial receptivity markers; *HOXA10* and *HOXA11* (Rackow et al, 2011). *LIF* gene, also considered as endometrial receptivity marker, was also investigated in another study and it was suggested that the expression levels of *LIF* mRNA did not significantly differ between

submucosal myoma carrying women and control subjects (Rackow and Taylor, 2010). On the contrary, a recent report presented lower *LIF* expression in patients with an endometrial polyp than the controls at the midsecretory phase (Hasegawa et al, 2012). The development and characteristics of endometrial polyps and fibroids varies from the onset of disease. Moreover, the finding of this study demonstrated that *LIF* expression did not alter after removal endometrial polyp. The plausible reason for this controversial result may be due to the small number of cases (n=5), which was analyzed in the previous study (Hasegawa et al, 2012). Since *LIF* expression was also assessed in the endometrium of different individuals, rather than the same patients, the discrepancy between the results of our study with Hasegawa's study might be explained with grouping bias.

In summary, the present findings suggest that endometrial polyps may not alter the expression of established implantation factors during midsecretory implantation window. Earlier studies reported that removal of endometrial polyps improves fertility (Perez-Medina et al, 2005; Shokeir et al, 2004; Spiewankiewicz et al, 2003; Varasteh et al, 1999). Based on our study, it is not possible to comment on the implication of endometrial polypectomy on the fertility rate.

The other reason for this discrepancy may be explained with the short period when the expressions of implantation genes were up-regulated to normal levels after the removal of endometrial polyp; however, evidence from previous study has shown that the *HOXA10* expression improved to normal levels 4 months after salpingectomy (Daftary et al, 2007). This study is the first report demonstrated that the endometrial polyp does not adversely affect the expressions of three implantation genes, *HOXA10*, *HOXA11* and *LIF* genes. It is also possible that several other implementation genes other than *HOXA10*, *HOXA11* and *LIF* genes may be regulated by the removal of

endometrial polyp. In addition to RT-PCR analysis of gene expression, microarray technology can also be used to scan several genes at the same time and endometrial receptivity array (ERA) was developed to determine transcriptomic signature of potential endometrial receptivity biomarkers cluster (Diaz-Gimeno *et al*, 2011) and to treat patients with repeated implantation failure (Ruiz-Alonso *et al*, 2013).

Declaration of interest: The authors declare that they have no conflict of interest.

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