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Full Length Research Paper

Semi-quantitative analysis of endometrial receptivity marker mRNA expression in the mid-secretory endometrium of patients with uterine fibromas

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In fertile women, expression of molecular marker of endometrial receptivity, HOXA11, leukemia inhibitory factor (LIF) and basic transcriptional element binding protein 1 (BTEB1), rises during the luteal phase with the peak occurring during the implantation window. We evaluated the transcript levels of HOXA-11, LIF and BTEB1 in the mid-secretory endometrium of infertile patients with uterine fibroid infertility (n = 8) and from normal fertile women (n = 8). Expression levels of HOXA11, LIF and BTEB1 mRNA were measured in endometrium during the mid-secretory phase using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Endometrial HOXA11, LIF and BTEB1 mRNA expression levels (normalized to ß-actin expression) were significantly decreased in endometrium of infertile patients with uterine fibroid as compared with healthy fertile controls at the time of implantation (P<0.05). The results suggest that the alteration in expression pattern of some genes could account for some aspects of infertility in patients with uterine fibroma.

Key words: Myoma, fibromas, implantation, HOXA11, leukemia inhibitory factor, basic transcriptional element binding protein 1.

INTRODUCTION

Uterine fibromas are the most frequent benign tumors in women during reproductive age and may affect fertility. Different types of fibroids may affect the reproductive outcome into a different extent being a cause of infertility. Fibroids (myomas) are present in approximately 5 to 10% of women with infertility and are the sole factor identified in 1 to 2.4% (Pritts, 2001). Depending on location in the uterus, myomas have been implicated in both recurrent pregnancy loss and infertility (Bajekal and Li, 2000). Fibroids that distort the endometrial cavity are associated with lower pregnancy, implantation and delivery rates in women undergoing IVF compared with infertile women without myomas (Pritts et al., 2009; Horne and Critchley, 2007). Reproductive outcomes improve after myomectomy for a submucosal myoma, and the difference is more pronounced if the myoma was the only identifiable etiology of infertility (Pritts, 2001; Goldenberg et al., 1995). It is plausible that myomas adversely affect the overlying endometrium and hence impair endometrial receptivity; however, little is known about the effect of myomas on known markers of endometrial receptivity. During the menstrual cycle, the ovarian steroid hormones, estrogen and progesterone control a dramatic transcriptional reprogramming of endometrial cells leading to a receptive state for blastocyst implantation and the establishment of pregnancy (Lynch et al., 2009). Optimal implantation conditions are preceded by proliferation and differentiation of elements including endometrial glands, stroma, blood vessels, smooth muscle cells and fibroblasts. Impaired endometrial growth and differentiation may be a significant factor contributing

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Abbreviations: LIF, Leukemia inhibitory factor; **BTEB1,** basic transcriptional element binding protein 1.

to infertility (Strowitzki et al., 2006). Several genes have been identified that are essential for preparing endometrium to receive embryo like HOXA11, leukemia inhibitory factor (LIF) and basic transcriptional element binding protein1 (BTEB1) (Haouzi et al., 2009). HOXA11 homebox genes are one of the best-known transcription factors participating in implantation. In the mid-secretory phase of a menstrual cycle which is coincide with the time of implantation, HOXA11 mRNA expression is up-regulated in both endometrial glandular and stromal cells in women (Wang et al., 2004; Haouzi et al., 2009). Findings regarding the importance of HOXA11 gene are contradictory. Some authors have reported a dramatic rise in the HOXA11 expression level in the mid-luteal phase (Wang et al., 2004; Skrzypczak et al., 2007), whereas other authors do not confirm this phenomenon (Haouzi et al., 2009). LIF expression is essential for embryo-endometrium interaction and for blastocyst implantation in mice and humans (Kimber, 2005). In humans, LIF mRNA and protein levels are low in the proliferative phase, and expression markedly increases in the secretory phase at the time of implantation (Rackow and Taylor, 2010). High LIF expression is an indicator of receptive endometrium in fertile women. However, in infertile individuals, the data on endometrial LIF expression and secretion are controversial (Aghajanova, 2010). BTEB1 is an endometrial transcription factor that may play a role in regulation of endometrial cell growth by modulating gene transcription (Du, 2006). This Kruppel-like family member gene directly interacts with the progesterone receptor to mediate progesterone-responsive gene expression in endometrial cells (Haouzi et al., 2009). Gene array studies have established that there are aberrantly expressed genes in endometrium of women with endometriosis compared to the women without endometriosis during the implantation window (Kao et al., 2003). But expression pattern of BTEB1 in fibromas has not been previously examined in luteal phase of human endometrium.

The analysis of assisted reproductive outcomes revealed that women with myomas and infertility who undergo *in vitro* fertilization and embryo transfer have pregnancy rates less than of women who undergo *in vitro* fertilization and embryo transfer who did not have fibroids (Check et al., 2002). Abnormalities in the endometrium resulting in failure of embryonic implantation are believed largely to account for the lower pregnancy rates in women with fibromas. However, the underlying mechanisms of fibroma-associated infertility are remained unclear. Many factors may be involved, and some studies suggest that endometrial molecular defects involved in implantation during the implantation window might be the cause of fibromas-associated infertility (Burney et al., 2007). Findings regarding the importance of HOXA11, LIF and BTEB1 genes in fibroma are contradictory. We investigated the effect of uterine fibromas on several markers of endometrial receptivity: HOXA11, BTEB1 and LIF.

MATERIALS AND METHODS

Patiants

The study included 16 women of reproductive age, 8 of whom had uterine fibroid with size less of 5 cm and were infertile. These 8 comprised the study group. The control group comprised 8 normal fertile women. All tissue samples were obtained with full and informed patient consent. Subjects had not used hormonal medications for at least 3 months before surgery and had regular menstrual periods. Subjects did not have any other condition previously demonstrated to affect endometrial receptivity such as endometriosis, PCOS or hydrosalpinges. Endometrial tissue biopsies were performed during 19 to 23 days of a menstrual phase using an endometrial suction catheter. The endometrial sample was then divided for pathology and laboratory evaluation. All samples underwent histological evaluation, and normal mid-secretory phase of endometrium was identified. Endometrium from subjects with fibroma and controls was evaluated for mRNA expression of HOXA11, BTEB1 and LIF.

RNA extraction

Endometrial tissue was obtained at the time of surgery, and was immediately placed in RNA extraction solution (RNX-Plus, Cinagene Company, Iran) and stored at – 80°C. To obtain total RNA, this solution is homogenized by shaking. The cellular lysate was incubated, chloroform 0.2 ml was added and the samples were centrifuged (17000 × g centrifugal force at 4°C for 15 min). The clear, aqueous phase was collected, and RNA was precipitated with isopropanol and washed with 75% ethanol. The RNA pellet was air-dried, then resuspended with RNase-free water (Alizadeh et al., 2011). From all obtained RNA samples, 2 µl was analyzed using the Epoch microplate spectrophotometer (BioTek, USA).

Reverse transcription

Single-stranded cDNA was synthesized using RivertAid™ First Strand cDNA Synthesis Kit (Frementas, Canada) using 4 µg of RNA according to the manufacturer's protocol. The transcription process included incubation of the reaction mixture at 42°C for 60 min, followed by 5 min at 70°C. The cDNA was stored at -80°C until further use for PCR.

Polymerase chain reaction

Primer pairs for the amplification of cDNA coding for HOXA11, BTEB1 and LIF were designed from the GenBank databases using the AlleleID 6 software and checked for minimum overlap. The sequences of primers used in the experiments are presented in Table 1. PCR were carried out using Mg^{+2} (3 mM), Taq-polymerase (2 unit), PCR buffer, dNTP (200 µ M) and a pair of specific primer (10 pmol/µl) in a final volume of 30 µl each tube. The PCR conditions were as follows: initial denaturation at 95°C for 2 min followed by 32 cycles for both LIF and BTEB1 and 29 cycles for HOXA11 and 26 cycles for β-actin (as control) of denaturation at 95°C for 30 s, annealing at 50°C for HOXA11, 58°C for LIF, 48°C for BTEB1 and 49°C for β-actin for 30 s, and extension at 72°C for 1 m and final extension at 72°C for 7 m. The expected length for PCR products is given in Table 1. PCR for each sample was duplicated.

Table 1. Primer sequenses used in PCR.

Gene	Product Size (bp)	Primer sequence
HOXA11	412	Sense: 5´-TTCCAGCACCACACTCAG-3´ Anti-sense: 5´-AGCATTTCCCTAACTCTTTCC-3´
LIF	710	Sense: 5-TTGAAGTGTGCTGTGAAC-3´ Anti-sense: 5´-GGAAGAGAACGAAGAACC-3´
BTEB1	638	Sense: 5´-TGGTCTCCTTCCTGTGTTC-3´ Anti-sense: 5´-TAGTGATGGCTGTTGTATTGG-3´
β-actin	360	Sense: 5´-CGTACCACTGGCATCGTGAT-3´ Anti-sense: 5´-GTGTTGGCGTACAGGTCTTTG-3´

Optimizing PCR conditions for semi-quantitative analysis

For semi-quantitative analysis, the PCR must be in its exponential range providing doubling of the products amount with each cycle (Rey et al., 2000; Marone, 2001). To define the exponential range, several independent PCRs were performed where 3.5 µl of the reaction mix was taken out every determined cycle. The PCR products were loaded on a 1% agarose gel and evaluated for the relative increase in the PCR product obtained with each cycle. The optimal numbers of PCR cycles determined were 29, 32, 32 and 26 for HOXA11, LIF, BTEB1 and β-actin, respectively.

Gel electrophoresis

The PCR products of each interested gene and β-actin were loaded onto the same ethidium bromide-stained agarose gels (1%). Stained gels were recorded and the band intensity was evaluated using the TotalLab TL 120 2009 software. Band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be determined and β-actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization to β-actin (Marone, 2001; Berljak et al., 2005).

RESULTS AND DISCUSSION

Compared with normal uteri, endometrial HOXA11 mRNA expression (normalized to ß-actin expression) was significantly decreased in biopsies from uterine with myomas (17.71 ± 0.68 and 48.95 ± 4.23, respectively; $P<0.05$; Figure 1). Endometrial BTEB1 mRNA expression (normalized to ß-actin expression) was significantly lower in uteri with myomas than normal uteri (22.29499 ± 2.471 and 50.57 ± 1.93, respectively; $P<0.05$; Figure 1). Analysis of endometrial LIF mRNA expression demonstrated a similar trend between uteri with myoma and normal uteri (21.51 ± 1.18 and 55.35 ± 0.27, respectively; $P<0.05$; Figure 1).

Uterine fibroma are associated with poor reproductive outcome (Pritts et al., 2009). Several theories have been proposed to explain the effects of fibroids on fertility. The effect of cavity distortion from fibroids on implantation is understandable, however the mechanism by which fibroids in an infertile patient with a normal uterine cavity might interfere with implantation is unclear. The interference of uterine fibroids with gamete transport, normal uterine contractility and in particular completion of the process of implantation has been a point of concern. Alterations in uterine artery blood flow, alterations in gene expression, implantation site and local cytokine release may also play a role (Horne and Critchley, 2007). Although, defective implantation is likely due to an endometrial defect, no specific endometrial deficiency has been identified that would explain these clinical findings. Histological characterization of endometrium from uteri with myomas revealed no consistent endometrial abnormality (Rackow and Taylor, 2010). Therefore, histology alone cannot effectively assess endometrial receptivity; molecular evaluation of the endometrium is a potential means of identifying defects in receptivity. Alterations in endometrial expression of some genes have been identified in several clinical conditions associated with impaired endometrial receptivity: endometriosis, idiopathic infertility and hydrosalpinges (Alizadeh et al., 2011; Szczepańska et al., 2011; Bildirici et al, 2001). In this study, the effect of myomas on the endometrium was evaluated using several established molecular markers of endometrial receptivity: HOXA11, LIF as well as BTEB1.Homeobox (Hox/HOX) genes encode transcription factors that mediate embryonic development. In human, HOXA11 is expressed in endometrial glands and stroma throughout the menstrual cycle (Szczepańska et al., 2010). HOXA11 have been demonstrated to be necessary for implantation in mice. Targeted disruption of this gene results in sterility. The knock-out mice ovulate, but their embryos do not implant in their uterus. Wild-type embryos also do not implant in the knock-out mice; however, knock-out mouse embryos implant in a wild-type uterus. This targeted disruption results in an endometrial defect, not an embryo defect in which implantation is severely altered (Gendron et al.,

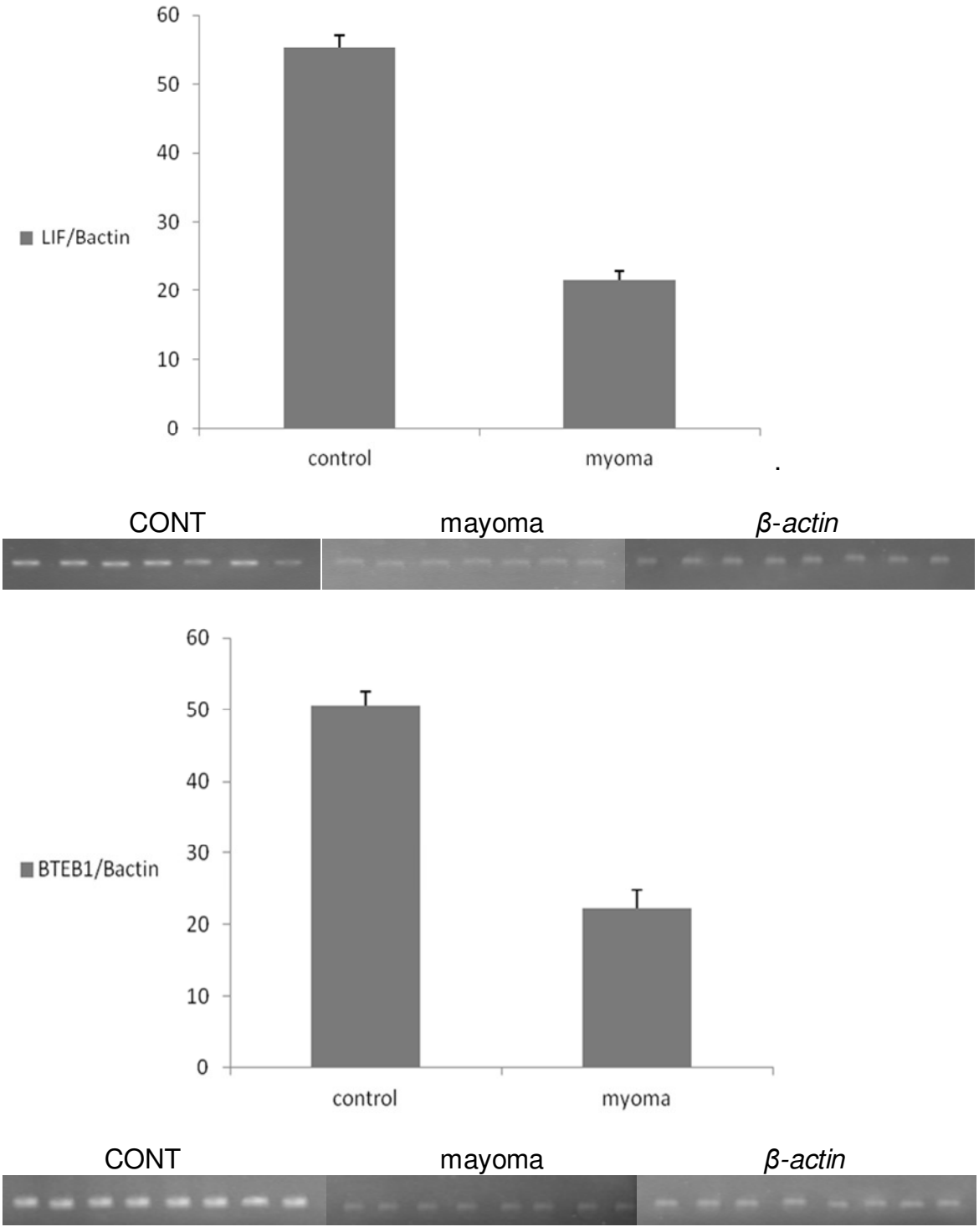

Figure 1. Mean endometrial HOXA11, BTEB1 and LIF mRNA expression (normalized to β-actin). Endometrium was sampled from uteri with mayoma and compared with control groups (CONT). Details of the original gels are shown on the bottom of each blot.

1997). Similarly, a defect in HOX expression in patients with endometriosis and idiopathic infertility may lead to a decrease in implantation without an appreciable pathology noted on histological examination (Alizadeh et al., 2011; Szczepańska et al., 2011). We found statistically significant lower HOXA11 transcript levels in infertile women with uterine fibromas compared to the fertile healthy women.

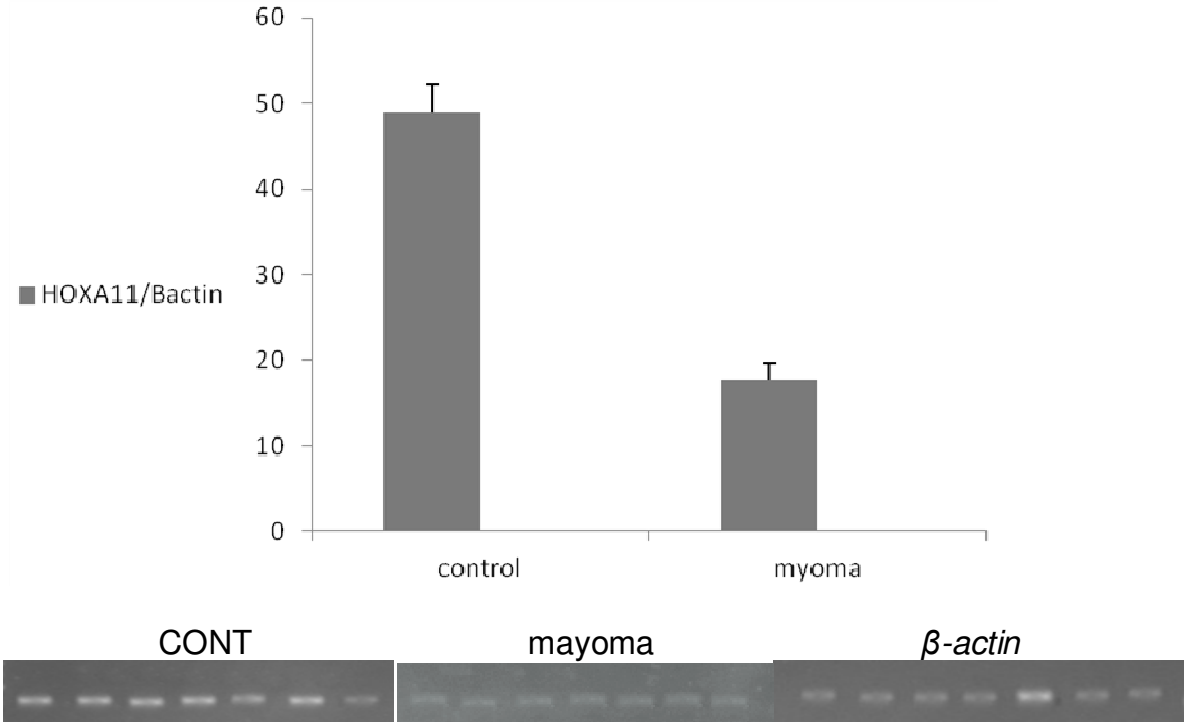

Figure 1. Contd.

Some of the key players in the receptivity of the endometrium are cytokines belonging to IL-6 superfamily, namely LIF and IL-11 (Mikolajczyk et al., 2006). An important role for LIF in implantation was shown on LIF knockout mice when embryo implantation did not occur. In endometrium of healthy women, LIF and LIF mRNA are expressed throughout the menstrual cycle (Kimber, 2005). Also, it has been shown that infertile women exhibited low levels of LIF in endometrial flushing and immunohistochemical staining (Mikolajczyk et al., 2006). In contrast, other studies report no change in endometrial LIF mRNA or secretion by endometrial biopsies from infertile compared to fertile women (Sherwin et al., 2002). In the present study, endometrial LIF mRNA expression was significantly decreased in biopsies from endometrium of uterine fibroid in mid secretory as compared with controls. Our results are consistent with Sinclair et al. (2011). They evaluated the effect of leiomyoma on endometrial gene expression essential for implantation and hemostasis both *in vivo* and in primary endometrial stromal cells (ESC). ESCs were treated with recombinant human (rh) BMP-2 or rhTGF-β3. Expression of LIF and some other genes was assessed by quantitative RT-PCR. LIF expression increased after rhBMP-2 treatment of normal but not leiomyoma-associated ESCs.

BTEB1 is an endometrial transcription factor that may play a role in regulation of endometrial cell growth by modulating gene transcription (Du et al., 2006). Targeted mutation in BTEB1 has been shown to result in subfertility and uterine hypoplasia (Simmen et al., 2004). We examined the expression of this gene in human uterine fibroma and detected a significant decrease in endometrial BTEB1 expression of mRNA in infertile patients in comparison with normal fertile controls.

Rackow and Taylor (2010) studied expression level of HOXA11, BTEB1 and LIF in infertile women with submucousal myoma. They showed that HOXA11 and BTEB1 mRNA was significantly lower in endometrium from women with submucousal myoma compared to the control. Their endometrial sampling was performed during the proliferative phase and our sampling was done in secretory phase. These results show that expression of these two genes were significantly lower during both proliferative and secretory phases. In Rackow and Taylor's (2010) study, endometrial LIF mRNA expression did not differ in the myomas or control groups. This marker of endometrial receptivity is minimally expressed in the proliferative phase and has higher level of expression in the secretory phase. Because their study sampled endometrium in the proliferative phase, the timing may have impaired the evaluation of LIF mRNA expression.

In this study, endometrial sampling was performed during the secretory phase. Our results show that LIF mRNA expression significantly decrease during the mid-luteal phase at the time of implantation. The present study indicates the possibility that endometrium of some infertile women with fibroma has abnormalities in expression of LIF which may contribute to altered uterine receptivity, resulting in infertility. Moreover, it has shown

that endometrial mRNA expression was globally affected in the presence of a submucosal myoma, rather than focally changed in the endometrium over the myoma (Rackow and Taylor, 2010). In our study, endometrial sampling were done out of site of myoma and our results were the same as theirs. Uterine receptivity and implantation are complex processes requiring the co-coordinated expression of molecules by the embryo and uterus during implantation. HOXA11, LIF and BTEB1 are expressed in different kinds of endometrial cells throughout the menstrual cycle and show a dramatic increase during the mid-luteal phase at the time of implantation in response to estrogen and progesterone. This study has identified lower expression of HOXA11, LIF and BTEB1 in women with myoma that may result in inadequate preparation of a receptive endometrium. However, HOXA11, LIF and BTEB1 mRNA are not the only molecules responsible for successful implantation. Many different molecules could be involved in implantation failure and different underlying molecular defects might be involved in the molecular mechanisms of fibromas-associated infertility.

Further studies are needed to investigate other endometrial molecular defects during the window of implantation in infertile patients with myomas.

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