Endometrial expression of telomerase, progesterone, and estrogen receptors during the implantation window in patients with recurrent implantation failure

N. Long[1,2], N. Liu[1], X.L. Liu[1], J. Li[2], B.Y. Cai[3] and X. Cai[1]

[1]Reproductive Center of the First Affiliated Hospital of Xinjiang Medical University, Urumqi, China
[2]Department of Reproductive Health of Jinan University School of Medicine affiliated with the Shenzhen Baoan Maternal and Child Health Hospital, Shenzhen, China
[3]Department of Pathology of Jinan University School of Medicine affiliated with the Shenzhen Baoan Maternal and Child Health Hospital, Shenzhen, China

Corresponding author: X. Cai
E-mail: xiacai_cc@sina.com

ABSTRACT. Telomerase plays a critical role in cell proliferation and senescence, but the exact involvement of endometrial telomerase in recurrent implantation failure (RIF) is unknown. We collected endometrial biopsies from RIF patients (N = 30) and fertile women (N = 30). Real-time PCR was performed for detecting changes in telomerase reverse transcriptase (*Tert*), *ER alpha*, and *PR* expression at the transcript level, and the correlation between the variable expressions of these genes was tested using regression analysis. Then, western blot and immunohistochemistry were used to analyze the expression profiles of TERT and ER alpha at the protein level. Compared to the

control, *Tert* expression was substantially increased, whereas *ER alpha* expression significantly decreased in the endometrium with RIF. No change was observed in *PR* expression. *Tert* expression was inversely associated with *ER alpha* expression. TERT protein expression in RIF patients was also clearly elevated, and was localized to both the endometrial epithelium and stromal cells. However, the signals for ER alpha in the stromal cells were weaker than those in the control. Expression of endometrial telomerase was substantially enhanced as ER alpha decreased in RIF patients during the implantation window.

Key words: Telomerase; ER alpha; RIF; Implantation window

INTRODUCTION

Embryo implantation is a prerequisite for a successful pregnancy (Carson et al., 2000), and implantation failure is the primary cause of poor natural conception rate in humans (Norwitz et al., 2001; Cha et al., 2012). Preparing the endometrium for implantation is essential for the establishment of the implantation window, which is a critical period both for natural and in vitro fertilization (IVF)-assisted pregnancies (Carson et al., 2000; Cha et al., 2012). In humans, the putative implantation window occurs at the mid secretory phase, and is precisely controlled by synergistic signals of progesterone and estrogen, which bind to the progesterone receptor (PR) and estrogen receptor (ER), respectively (Carson et al., 2000; Groothuis et al., 2007; Large and DeMayo, 2012). In the endometrium with recurrent implantation failure (RIF), approximately 8% of the downregulated genes are associated with compromised estrogen signals, due to decreased expression of ER alpha (Carranza-Lira et al., 2000; Koler et al., 2009). However, the molecular mechanisms responsible for recurrent implantation failure remain largely unclear.

Telomerase is a critical ribonucleoprotein polymerase that maintains telomeres at chromosome ends by adding the telomeric repeat TTAGGG, and stabilizes chromosome ends by preventing their degradation, fusion, rearrangements or sequence loss during cell division (Greider and Blackburn, 1989; Yu et al., 1990). Telomerase has been well studied in the context of cell oncogenesis, senescence, and immortalization (Stewart et al., 2012; Ye et al., 2014). Recently, several studies have also revealed that telomerase is closely involved in female reproductive health (Kim et al., 2007; Hapangama et al., 2008a; Hapangama et al., 2008b; Chen et al., 2011; Mafra et al., 2014). In the human endometrium, the mRNA of telomerase reverse transcriptase (*Tert*), which encodes the catalytic subunit of telomerase, is highly expressed in epithelial cells (Tanaka et al., 1998; Hapangama et al., 2008b). Telomerase expression and activity are high during the proliferation phase but wane during the secretory phase, indicating an inverse correlation with serum estrogen and progesterone levels (Tanaka et al., 1998; Williams et al., 2001; Hapangama et al., 2008b). Additionally, ER alpha activity is widely considered to affect telomerase activity and TERT expression (Cha et al., 2008; Calado et al., 2009; Li et al., 2010). However, the role of endometrial TERT during the human implantation window is still ambiguous.

This study aims to investigate the relationship between telomerase and steroid receptor expression in recurrent implantation failure during IVF. By analyzing the changes in the expression of endometrial TERT and steroid receptors during the implantation window, we

demonstrate that TERT expression increases while ER alpha decreases in the RIF endometrium. Therefore, we concluded that the induction of TERT is significantly relevant to the compromised expression of ER alpha, which in turn is directly responsible for embryo implantation failure.

MATERIAL AND METHODS

Patients and endometrial samples

Endometrial biopsies for this study were obtained from women with their informed consent, according to the Ethical Committee of Baoan Maternal and Child Health Hospital, Shenzhen, China. All women exhibited regular menstrual cycles of between 27 and 35 days, and had not used any hormonal treatment for at least 3 months before the biopsy. Daily measurements of LH in urine samples, starting from Day 9 of the menstrual cycle were used to identify the LH surge (designated LH + 0). The LH surge was determined using a commercially available urine LH kit (Kunming Yunda Bio-Technology Co., Ltd., GB/ T 18990.2-2003). Putative implantation window in the mid secretory phase was determined as Day 8-9 after the LH surge (LH + 8/+ 9). RIF was defined as the failure to detect positive serum hCG after transfer of good quality embryos during IVF, at least three times. None of RIF patients (N = 30) had ever had a successful full term pregnancy. Fertile women (N = 30) in the control group were recruited within 5 years of the last successful pregnancy. None of the women included in the study had a history of conditions that can lead to pregnancy failure, including thrombophilia, antiphospholipid syndrome, morphological uterine anomalies, ovarian diseases such as polycystic ovarian syndrome, hormone abnormalities, and other endocrinopathies. On the day of endometrial collection, 5 mL venous blood was collected from each woman to determine serum estradiol and progesterone by immunofluorometric assay using commercial kits (Beckman Coulter, Inc. Estradiol-E$_2$, 9Z3HV7SGT2VYBTN956; Progesterone, X843H1NNMTKJCPSX39). Age and body mass index of the participants as well as endometrial thickness were also recorded, as seen in Table 1.

Table 1. Characteristics of women participating in the study.

	Control group	RIF group	P value
Number of patients	30	30	-
Age (year)	31.8 (28-37)	32.1(28-38)	0.892
Body mass index	23.4 (20.9-28.1)	24.2 (21.6-28.8)	0.677
Plasma progesterone (ng/mL)	29.2 (18-45)	27.6 (18-48)	0.624
Plasma estrogen (pg/mL)	72 (59-107)	79(47-128)	0.585
Endometrial thickness (mm)	10.3 (9-11)	9.8 (8-11)	0.522

Real-time polymerase chain reaction (Real-time PCR)

Total RNA was extracted from human endometrium using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), then digested with RQ1 deoxyribonuclease I (Promega, Fitchburg, WI, USA), and reverse transcribed into cDNA using the PrimeScript reverse transcriptase reagent kit (TaKaRa, Dalian, China). Before real-time PCR, cDNA was amplified using a SYBR Premix Ex Taq kit (TaKaRa) on the Rotor-Gene 3000A system (Corbett Research, Mortlake, Victoria, Australia). The real-time PCR protocol had the following conditions: 95°C for 10 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 34 s. All reactions were conducted in

triplicate. Data from real-time PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method. The specific primer sequences for each gene are given in Table 2.

Table 2. Primers for Real-time PCR used in this study.

Gene name	Primer sequences	Accession number	Size (bp)
Tert	F: 5' TATGCCGTGGTCCAGAAG 3' R: 5' CCACGAACTGTCGCATGTAC 3'	NM_198253	103
ER alpha	F: 5' CTCAACAGCGTGTCTCCG 3' R: 5' GGCTCGTTCTCCAGGTAG 3'	NM_000125	110
PR	F: 5' TACCAGTGTTCCCGTCTT 3' R: 5' TCCAGCATAAGCATTCACAG 3'	NM_001202474	98
Gapdh	F: 5' GAAGGTGAAGGTCGGAGT 3' R: 5' GATGGCAACAATATCCACTT 3'	NM_001256799	94

Western blot

Whole endometrial tissue lysate was prepared and homogenized in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.25% sodium deoxycholate). Protein concentration was measured and adjusted by using the BCA Reagent kit (Applygen, Beijing, China). Samples were run on a 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking in 5% nonfat dry milk in TBST (0.1% Tween 20 in TBS) for 1 h, membranes were incubated overnight at 4°C with the following primary antibodies: Rabbit anti-human telomerase (ab68781, Abcam, Cambridge, UK), ER alpha (sc-7207, Santa Cruz, CA, USA) or anti-human Gapdh (sc-25778, Santa Cruz). After three washes in TBST, membranes were incubated with secondary antibodies conjugated with horseradish perioxidase for 1 h at about 25°C. The signals were developed with the ECL Chemiluminescent kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Films were scanned using a flat-bed scanner. The intensities of the bands representing ER alpha and TERT were evaluated using the Image J analysis software (Rawak Software, Inc., Germany).

Immunohistochemistry

Each endometrial sample was fixed in paraformaldehyde, embedded in paraffin, and 7 μm sections were prepared. IHC was performed according to the procedure described below. Briefly, sections were rehydrated and retrieval repaired using a microwave for 10 min in 10 mM sodium citrate buffer, pH 6.0. Endogenous horseradish peroxidase (HRP) activity was inhibited with 3% H_2O_2. After blocking with 10% normal horse serum in PBS, sections were incubated overnight with rabbit anti-ER alpha (sc-7207, Santa Cruz) or anti-human telomerase (ab27573; Abcam) at 1:100 dilution at 4°C. Normal rabbit anti-GFP (sc-8334, Santa Cruz) served as the negative control. On the next day, slides were stained with biotinylated secondary antibodies, and then incubated with diaminobenzidine. Signal color was developed using the DAB-HRP reaction system, and brown staining indicated a positive signal. The sections were lightly counterstained with haematoxylin, and then mounted.

Ethical approval

This study was approved by the Ethical Committee of Reproductive Center of the First Affiliated Hospital of Xinjiang Medical University and Ethical Committee of Baoan Maternal and Child Health Hospital, Shenzhen, China.

Statistical analysis

Values are expressed as the mean ± SEM. Each experiment was repeated at least twice independently. Equal variance was tested by the F-test. If variance was not equal, *t*-test assuming unequal variances was performed, else equal variance *t*-test was used. Linear regression analysis and Spearman factor (SPSS; Chicago, IL, USA) were used to assess the relationship between ER alpha and telomerase expression in each endometrial sample with RIF. In all the cases, $P < 0.05$ was considered significant.

RESULTS

Changes in *Tert*, *ER alpha* and *PR* mRNA expression during implantation window in RIF patients

Since telomerase is closely related with uterine health (Hapangama et al., 2008a; Mafra et al., 2014), we initially speculated that endometrial telomerase expression might change in RIF patients during the implantation window. Using a specific primer for human *Tert*, real-time PCR was performed for investigating the mRNA level of *Tert*. Additionally, changes in *ER alpha* and *PR* expression were also analyzed as they are indispensible in embryo implantation (Carranza-Lira et al., 2000; Koler et al., 2009; Cha et al., 2012). Compared to the control, the RIF group showed a substantial increase in *Tert* expression and a significant reduction in *ER alpha* expression, whereas no evident change was observed in *PR* expression (Figure 1A).
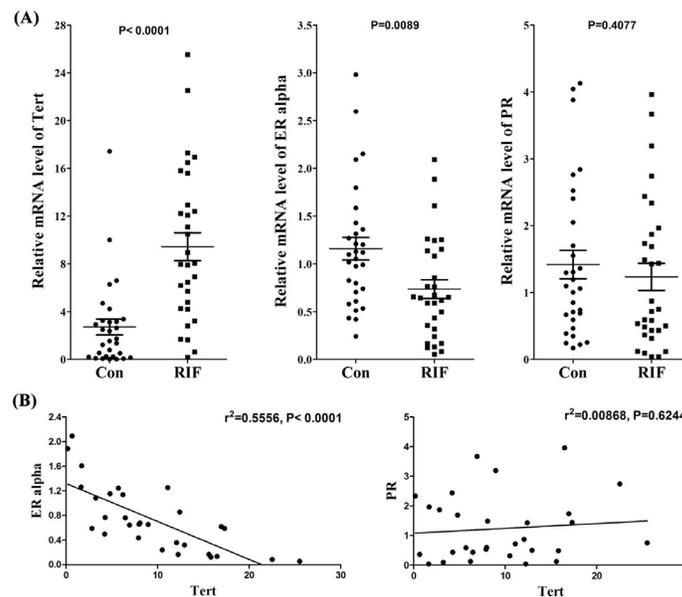
Figure 1. Changes of endometrial steroid receptors and *Tert* mRNA expression in RIF patients. **A.** Real-time PCR analysis shows an increase on *Tert* expression, a reduction on *ER alpha* and not significant changes in *PR* expression; **B.** Regression analysis using Spearmen factor in the different expression of *Tert* and the steroids receptors in RIF patient during implantation window.

In order to evaluate the correlation of the expression of *Tert* and steroid receptor mRNA, we employed the Spearmen correlation factor and performed a regression analysis without the constant term. A significant correlation was found between *ER alpha* and *Tert* expression, whereas *Tert* expression was not correlated with *PR* expression (Figure 1B).

Changes in TERT and ER alpha expression during implantation window in RIF patients

To determine whether the same expression pattern of TERT and ER alpha also existed at the protein level, we extracted endometrial protein and performed a western blot. Compared to the control group, TERT was notably promoted, while ER alpha was suppressed in RIF patients (Figure 2A). To evaluate the changes, band intensity was determined by densitometry (Figure 2B). Further, a regression analysis showed that TERT expression was significantly correlated with ER alpha expression in RIF patients (Figure 2C).
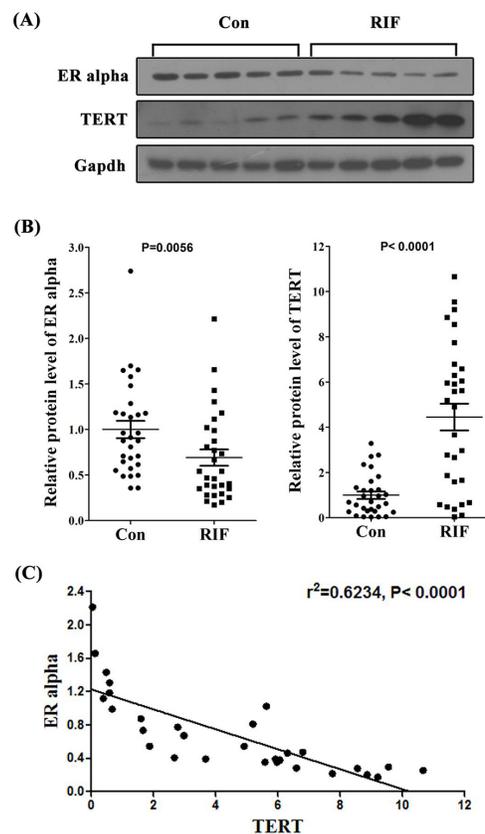
Figure 2. Changes of ER alpha and TERT expression in protein level in RIF patients. **A.** The endometrium in RIF patients present induced TERT expression and decreased ER alpha expression by performing Western blot; **B.** Changes of TERT and ER alpha expression was quantitatively evaluated by analyzing the blotting bands intensity; **C.** Regression analysis using spearmen factor in the different expression of TERT and ER alpha in the endometrium with RIF during implantation window.

Localization of endometrial TERT and ER alpha in RIF patients

   To further elucidate the expression profiles of telomerase and ER alpha in the human endometrium, we carried out immunohistochemistry to determine TERT and ER localization. As shown in Figure 3, consistent with our earlier western blot data, TERT signals were low in the control, but evidently enhanced in RIF samples. Concurrently, ER alpha expression in RIF patients was weaker compared to that in the control. Moreover, TERT was primarily located in the epithelial cells and secondarily in the stromal cells, showing an expression pattern similar to that of ER alpha. Collectively, these data suggested that compromised ER alpha expression might result in TERT induction in the RIF endometrium during the implantation window.
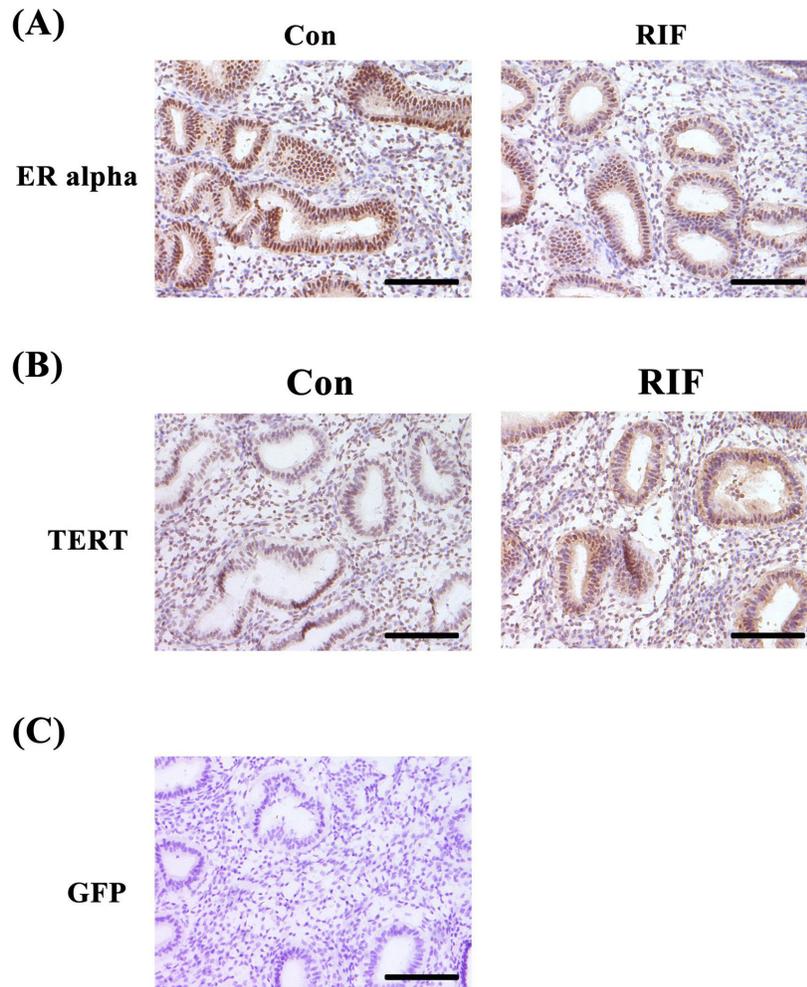
Figure 3. Localization of ER alpha and telomerase expression in endometrium during implantation window. **A.** Immunohistochemistry data shows the signals of ER alpha both in control and RIF group; **B.** Immunostaining signals of TERT in the endometrium during implantation window; **C.** No signal is observed for GFP, which is served as negative control. Bar = 100µm.

DISCUSSION

A healthy and receptive endometrium is required for proper embryo implantation. The receptors for estrogen and progesterone play an essential role during the implantation window (Cha et al., 2012). After examining the expression profile of TERT and steroid receptors in the RIF endometrium during the mid secretory phase, we discovered that increased telomerase expression is clearly related with reduction in ER alpha, both at the mRNA and protein level. This indicates that telomerase is closely associated with estrogen signals and may be needed for successful implantation.

However, our observation that there is an inverse correlation between ER alpha and TERT expression may be controversial, as some studies have demonstrated that ER alpha induces telomerase in pluripotent and tumor cells (Cha et al., 2008; Calado et al., 2009; Li et al., 2010). Additionally, progesterone signals can also affect *Tert* transcription in breast and endometrial cancer cells (Wang et al., 2000), whereas there is no obvious relationship between TERT and PR protein expression as per our observations. To the best of our knowledge, inhibition of telomerase by ER alpha has not been reported in literature. However, telomerase expression and activity show an inverse correlation with the level of serum estrogen and progesterone during human menstruation (Williams et al., 2001). Thus, we presume that the signaling molecular factors mediating the effects of ER alpha on telomerase expression in endometrial cells are quite different from those in pluripotent or cancer cells.

Interestingly, although telomerase activity in the human endometrium is ameliorated by anti-estrogen drugs *in vivo*, estrogen or progesterone treatment exhibits little effect on telomerase activity in cultured human endometrial cells (Tanaka et al., 1998). In our study, RIF patients show an insignificant difference in serum estrogen and progesterone levels (Table 1), but show increased TERT expression and decreased ER alpha expression both in the endometrial epithelium and stroma. Thus, we speculate that ER alpha might influence endometrial telomerase expression independent of the serum steroid concentration.

Several studies have demonstrated that both telomerase activity and expression are significantly upregulated in the endometrium with endometriosis, which also shows subfertility and low implantation rate (Kim et al., 2007; Hapangama et al., 2008b; Mafra et al., 2014). Considering our data, we concluded that telomerase may contribute to a dysfunctional endometrium during the implantation window in RIF patients. During the mid secretory phase, the proliferation of endometrial epithelial and stromal cells is decreased, not only making space for the invading embryo as epithelial cells undergo apoptosis, but also allowing the transformation of stromal cells into decidual cells to support embryo implantation (Ma et al., 2003; Hapangama et al., 2008a; Cha et al., 2012; Large and DeMayo, 2012). It is known that telomerase activity is closely associated with endometrial proliferation during human menstruation (Kyo et al., 1997; Tanaka et al., 1998). In RIF patients, we found that telomerase is increased both in the epithelial and stromal cells. In agreement with our findings, Hapangama et al. (2008a) also reported elevated telomerase activity in several RIF patients. Collectively, these data suggest that the process of apoptosis and decidualization might be disrupted due to over expression of telomerase in the RIF endometrium, and low telomerase expression might be necessary to restrict cell proliferation and enable embryo implantation.

The etiology of RIF is extremely complicated and varied (Timeva et al., 2014). By describing the relationship between ER alpha and telomerase expression, our study sheds light on the involvement of telomerase, regulated by estrogen signals, during the implantation window. Moreover, these results might aid future clinical studies aimed at developing therapies for RIF.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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