FKBP4 is regulated by HOXA10 during decidualization and in endometriosis

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Abstract
FKBP4 (FKBP52) and FKBP5 (FKBP51) are progestin receptor (PR) co-chaperone proteins that enhance and inhibit, respectively, progestin-mediated transcription by PR. Here, we examined FKBP4 and FKBP5 expression in the eutopic endometrium of fertile women with endometriosis and effects of FKBP4 and FKBP5 on the decidualization of human endometrial stromal cells (HESCs), and assessed HOXA10 regulation of FKBP4. Expression of FKBP4 mRNA was increased in the late proliferative phase and remained elevated throughout the secretory phase. FKBP5 expression was low and remained constant throughout the menstrual cycle. Compared with controls, FKBP4 mRNA expression was decreased in the endometrium of women with endometriosis, whereas no significant endometriosis-related change was seen for FKBP5. Cultured HESCs were treated with either FKBP4 or FKBP5 siRNA and then decidualized by incubation with progesterone (P4) and 8-bromoadenosine cAMP. Treatment of HESCs with FKBP4 siRNA resulted in 60% lower IGFBP1 expression. In contrast, incubation with FKBP5 siRNA did not significantly decrease IGFBP1 expression during in vitro decidualization. HOXA10 and FKBP4 expression increased in parallel during in vitro decidualization. In HESCs, overexpressed HOXA10 enhanced FKBP4 mRNA and protein levels, whereas HOXA10 knockdown decreased FKBP4 mRNA and protein levels compared with controls. Similarly, during in vitro decidualization, FKBP4 expression was decreased in HOXA10-silenced cells. Enhanced HOXA10 expression in HESCs elicits a decidualization mediating increase in FKBP4 expression. The findings are consistent with the observation that women with endometriosis have diminished FKBP4 expression leading to impaired decidualization and infertility. The P4 resistance seen in endometriosis may be mediated through HOXA10-regulated FKBP4 expression.

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Introduction
Endometriosis is a benign gynecological disorder that affects ~10% of reproductive age and 30–50% of infertile women (Eskenazi & Warner 1997, Giudice & Kao 2004). Impaired decidualization of eutopic endometrium is a key contributor to infertility in women with endometriosis (Klemmt et al. 2006, Ulukus et al. 2006, Bulun 2009). Decidualization of human endometrial stromal cells (HESCs) is initiated in the secretory phase of the menstrual cycle and involves marked changes in morphological and biochemical differentiation that prepares the intrauterine environment for blastocyst implantation (Minici et al. 1997, Dey et al. 2004, Klemmt et al. 2006). In women with endometriosis, delayed transition from the proliferative to the secretory phase (Burney et al. 2007) is accompanied by reduced decidualization (Minici et al. 1997, Klemmt et al. 2006). Progesterone (P4) acts via cognate nuclear P4 receptor (PR) isoforms, PR-A and PR-B, to initiate decidualization in HESCs. Activation of PR alone elicits delayed decidualization in cultured HESCs, whereas initiation of more rapid decidualization requires both elevated cAMP levels and sustained activation of the protein kinase A pathway, which sensitizes the cells to P4 (Gellersen & Brosens 2003, Jones et al. 2006). Therefore, co-incubation of P4 with cAMP is frequently used to induce decidualization in vitro.

The developmentally regulated homeobox transcription factor, homeobox A10 (HOXA10: human/Hoxa10: mouse), is highly expressed in the endometrial glands and stroma of the developing human uterus (Taylor et al. 1997, Block et al. 2000). Expression of HOXA10 varies...
across the menstrual cycle with a marked increase in HOXA10 expression particularly evident in epithelial cells during the window of implantation of the secretory phase (Taylor et al. 1998, Gui et al. 1999, Sarno et al. 2005, Martin et al. 2007). In human endometrium, decidualizing stromal cells show elevated HOXA10 levels. However, endometrial HOXA10 expression is lower in women with endometriosis compared with controls (Taylor et al. 1999), and induced endometriosis in mice leads to diminished Hoxa10 (Lee et al. 2009). In mice, targeted deletion of Hoxa10 leads to severe decidualization defects (Benson et al. 1996). Similarly, blockade of Hoxa10 in the adult murine uterus results in impaired decidualization and diminished litter size (Bagot et al. 2000, Kim et al. 2003, Daftry & Taylor 2004). Taken together, these observations indicate that impaired decidualization induced by decreased HOXA10/Hoxa10 expression may contribute to endometriosis-associated infertility. The molecular mechanisms by which decidualization is impaired in Hoxa10−/− mice or in women with decreased HOXA10 expression is not fully characterized.

Hoxa10−/− mice exhibit decreased FKBP4 (FKBP52) expression, suggesting that this molecule may be involved in the decidualization defect (Daikoku et al. 2005). The large immunophilin FKBP (FK506)-binding proteins, FKBP4 and FKBP5 (FKBP51), contain a tetracopeptide repeat (TPR) domain that binds to the C terminus of heat-shock protein 90 (HSP90). This binding enables both the immunophilins to act as chaperones with HSP90 and interact with the ligand-binding domain of the PR (Lydon et al. 1995, Cheung-Flynn et al. 2005, Tranguch et al. 2005, Yang et al. 2006). Consequently, FKBP4 enhances and FKBP5 inhibits progesterin-initiated transcription. This role, as a co-chaperone of HSP90, enables FKBP4 to govern normal PR function in the mouse uterus (Reynolds et al. 1999, Riggs et al. 2003, Davies et al. 2005, Davies & Sanchez 2005). Fkbp4−/− mice have reduced P4 binding to PR, diminished PR transcriptional activity, and down-regulation of several P4-regulated genes in the uterus (Tranguch et al. 2005). Women with endometriosis show diminished levels of FKBP4 (Hirota et al. 2008). P4 resistance is an established risk factor for endometriosis, suggesting that insufficient FKBP4 expression elicits PR dysfunction and that the resulting P4 resistance leads to endometriosis. Gene knockout models help to elucidate the physiological importance of FKBP4 expression. Female mice lacking PR and PR-A expression are infertile due to ovulation failure, embryo implantation, and decidualization (Yang et al. 2006, Tranguch et al. 2007). However, only implantation and decidualization are impaired in Fkbp4 knockout mice, indicating the selectivity and specificity of FKBP4 in mediating these progestin effects (Tranguch et al. 2005). Eutopic and ectopic endometria of women with endometriosis show significantly lower FKBP4 levels in both glands and stromal cells compared with women without endometriosis (Hirota et al. 2008). Moreover, lower FKBP4 levels are reported in the endometria of baboons with experimentally induced endometriosis (Jackson et al. 2007). There are no studies evaluating the involvement of FKBP5 in either decidualization or endometriosis.

The current study sought to assess the mechanisms underlying the regulation of FKBP4 and FKBP5 expression during in vitro decidualization of HESCs. This question was approached initially by confirming a report that FKBP4 mRNA levels are significantly lower in menstrual cycle-matched specimens of eutopic endometrium of women with endometriosis compared with endometriosis-free women (Hirota et al. 2008) and extending these observations to include FKBP5. Subsequently, in vitro experiments evaluated the HOXA10, FKBP4, and FKBP5 mRNA and protein expressions...
during decidualization of HESCs. Our observation that siRNA-mediated silencing of FKBP4, but not FKBP5, blocked enhanced expression of IGFBP1, a well-characterized decidualization marker, prompted further determination as to whether HOXA10 regulates FKBP4 in the presence or absence of decidualization stimulation.

Results

The potential involvement of FKBP5 and FKBP4 in endometriosis was investigated by measuring FKBP5 and FKBP4 mRNA levels in eutopic endometrium of subjects with endometriosis compared with menstrual cycle phase-matched controls. As shown in Fig. 1, FKBP5 mRNA levels were low and did not vary significantly across the menstrual cycle. FKBP4 mRNA levels were increased in the late proliferative phase and remained elevated in the secretory phase. Secretory-phase levels of FKBP4 mRNA did not vary significantly and were similar to the late proliferative phase; hence, the secretory-phase endometrial samples are presented together, while the proliferative phase was analyzed as two separate components (Fig. 1). FKBP4 mRNA levels were lower in endometriotic vs control endometrial specimens at all menstrual cycle stages studied with reductions by 11 and 18% of control levels in late proliferative and secretory phases (P<0.01). FKBP4 mRNA expression was similar in women with or without endometriosis. These results suggest that FKBP4 gene expression is repressed in endometriosis.

The role of FKBP5 and FKBP4 in decidualization was assessed in vitro using siRNA to downregulate FKBP5 or FKBP4 expression and then examine the induction of IGFBP1 expression as a measure of decidualization. Figure 2 demonstrates the FKBP5 and FKBP4 dependence of decidualization in HESCs. FKBP5 and FKBP4 mRNA (A) and protein (B) expressions are decreased significantly after siRNA treatment, indicating that expression was successfully repressed. We subsequently exposed the cells to P4/cAMP to induce decidualization. Cells pretreated with the FKBP4 siRNA showed less cuboidal morphology compared with cells treated with negative control siRNA, suggesting a reduced level of decidualization (results not shown). Furthermore, IGFBP1, a marker of decidualization, was expressed at significantly lower levels in HESCs pretreated with the FKBP4 siRNA. In cells treated with FKBP5 siRNA, decidual cell morphology was similar to controls with no loss of IGFBP1 expression. Taken together, our results
indicate that downregulation of FKBP4, but not FKBP5, substantially reduces the degree of decidualization of HESCs. The current results confirm previous studies demonstrating that HOXA10 is required for decidualization. Specifically, treatment with HOXA10 siRNA decreased the degree of decidualization of HESCs as assessed by IGFBP1 production (Fig. 2D).

Previous studies demonstrated that expression of FKBP4 is decreased in Hoxa10 knockout mice and that HOXA10 is also a key regulator of human decidualization (Lim et al. 1999). We thus hypothesized that HOXA10 may affect FKBP2 and/or FKBP5 expression during the process of decidualization. Therefore, expressions of HOXA10, FKBP5, and FKBP4 were measured during in vitro decidualization of HESCs. As shown in Fig. 3, FKBP5 and FKBP4 mRNA were expressed at low levels before decidualization. FKBP5 mRNA levels increased rapidly on day 1 after exposure to P4/cAMP, reached a peak on day 4 (10.3 ± 1.3), and remained elevated. Similarly, HOXA10 mRNA levels were also elevated in P4/cAMP-treated HESCs. Levels of HOXA10 mRNA reached a maximum on day 4 (27.0 ± 4.0) and were maintained at high levels over the remaining days of the incubation period. Unlike FKBP4, FKBP5 expression did not significantly increase during the observed time course. These results demonstrated a parallel pattern of HOXA10 and FKBP4 expression during the process of induced decidualization.

To determine whether HOXA10 is involved in regulating FKBP4 expression in endometrial cells, the expression of FKBP4 was assessed in HESCs in response to either increasing or silencing HOXA10 expression (Fig. 4). The effectiveness of constitutive HOXA10 expression and HOXA10 knockdown was confirmed by real-time PCR (Fig. 4A and B) and western blotting (Fig. 4E and F). Interestingly, constitutive expression of HOXA10 led to a 3.7-fold increase in the expression level of FKBP4 mRNA (P < 0.05), whereas knockdown of HOXA10 reduced FKBP4 mRNA levels by 36% of control cells treated with vector alone. The effect of HOXA10 on FKBP4 expression was further confirmed at the protein level by western blotting (Fig. 4E and F). These results suggest that HOXA10 is involved in regulating the expression of FKBP4 in HESCs.

Similarly, we examined the effect of HOXA10 on FKBP4 specifically in decidualizing cells. We demonstrated that both HOXA10 and FKBP4 increased simultaneously in P4/cAMP-induced decidualization. Here, we determined whether the increased expression of FKBP4 depended on the expression of adequate level of HOXA10. As shown in Fig. 5, knockdown of HOXA10 blocked the upregulation of FKBP4 during in vitro decidualization. PR expression was not decreased (data not shown). In HESCs treated with P4/cAMP, mRNA levels of FKBP4 increased by fourfold (negative siRNA control), whereas this increase was not observed in HOXA10-silenced HESCs.

**Figure 3** HOXA10 and FKBP4 mRNA levels increased during in vitro decidualization. Human endometrial stromal cell cultures were treated with DMSO vehicle, 0.5 mM 8-bromoadenosine cAMP and 0.1 μM progesterone (P4), or only DMSO (d0). Media were changed every 48 h. Cells were collected at day 0 (d0), day 1 (d1), day 2 (d2), day 4 (d4), day 6 (d6), day 8 (d8), day 10 (d10), and day 12 (d12). Real-time PCR were used to analyze HOXA10, FKBP5, and FKBP4 mRNA expression. Each point represents the mean of at least three experiments, and the error bars enclose ± 1 S.E.M. Significant differences were found in expression of HOXA10 and FKBP4 between cultures treated with DMSO (d0) and P4/cAMP on days 1, 2, 4, 6 and 8, and 10. FKBP1 expression remained low throughout this time course. The appearance of a small increase at d2 did not reach statistical significance. HOXA10 expression was increased following treatment and is shown superimposed on FKBP4 expression to illustrate the similarities in the pattern of expression between these two genes.

**Discussion**

In humans, decidualization is initiated by P4 acting on estradiol-primed stromal cells during the secretory phase of the menstrual cycle. In the event of pregnancy, decidualized stromal cells play a critical role in promoting receptivity of the endometrium for the implanting blastocyst, whereas defective decidualization elicits infertility (Lockwood et al. 2009, Das 2010). Although transgenic mice that rendered null for Fkbp4 gene expression are infertile due to impaired decidualization and embryo implantation (Yang et al. 2006, Tranguch et al. 2007), the underlying mechanism(s) by which FKBP4 affects these events has not been fully characterized at the molecular level. Unlike decidualization in humans, which is initiated by hormonal stimulation during each potentially fertile cycle, initiation of decidualization in mice requires not only proper hormonal priming of the endometrium but also the presence of a blastocyst or a surrogate stimulus. Moreover, decidualization in mice is constrained near the site of embryo implantation, whereas decidualization spreads throughout the late secretory-phase human endometrium.

The established importance of local FKBP4 expression in regulating decidualization taken together with the unique features exhibited by human decidualization as
well as the marked decrease in FKBP4 expression in eutopic and ectopic endometrium of women with endometriosis (Hirota et al. 2008) prompted assessment of the regulation of FKBP4 expression in a well-characterized model of human decidualization. Thus, the current study demonstrates that during co-incubation with P4 and cAMP, knockdown of FKBP4 significantly decreased the degree of decidualization in HESCs. While FKBP4 is documented to promote PR transcription, in the current report, parallel studies with knockdown of FKBP5, which inhibits PR transcription, did not affect HESC decidualization. The decreased degree of decidualization in FKBP4-silenced cells and diminished P4 response is consistent with impaired decidualization and failed embryonic implantation in Fkbp4 knockout female mice.

In preparation for hormone binding, the PR assembles sequentially with various chaperones. In the final construction stage, HSP90 associates with the PR ligand-binding domain to stabilize PR ligand binding (Smith 1993). Hormone binding interrupts the steroid receptor-chaperone assembly cycle resulting in the activation of PR as a transcription factor. FKBP4 potentiates and FKBP5 attenuates hormone responses of the PR. Thus, the PR activity reflects the net effect of FKBP4 vs FKBP5. In this regard, the current results reveal the overwhelming dominance of FKBP4 in mediating decidualization. Thus, in comparison with the prolonged augmentation of FKBP4 expression observed during in vitro decidualization of HESCs, only a relatively slight transient increase in FKBP5 expression was detected. Moreover, unlike the marked inhibition in the expression of a well-documented decidualization marker by FKBP4 siRNA, FKBP5 siRNA did not affect IGFBP1 expression during in vitro decidualization of HESCs.

Consistent with a previous report, the current study revealed decreased expression of FKBP4 mRNA in the endometrium of women with endometriosis (Hirota et al. 2008). Defective decidualization accompanied by infertility is common in women with endometriosis (Ulukus et al. 2006). The current results suggest that impaired decidualization as a consequence of decreased expression of FKBP4, but not FKBP5, may significantly contribute to such infertility. Endometriosis is resistant to P4 treatment due to PR dysfunction (Conneely et al. 2001, Daikoku et al. 2005). As decidualization is P4/PR dependent and FKBP4 is a known regulator of PR activity (Fang et al. 2004, Tranguch et al. 2007), defective decidualization in endometriosis may thus reflect low FKBP4 expression resulting in inadequate P4/PR function.

Previously, our laboratory demonstrated that HOXA10 is downregulated in eutopic endometrium of women with endometriosis (Taylor et al. 1999). The current study confirmed this observation and extended it to include parallel downregulated expression of FKBP4. Methylation by HOXA10 has been shown to initiate decidualization and regulate the expression of multiple genes crucial for implantation. The current study demonstrates that both HOXA10 and FKBP4 are upregulated during P4/cAMP-induced decidualization of primary HESCs. Moreover, Hoxa10 knockout mice show significantly lower FKBP4 levels (Daikoku et al. 2005), suggesting that a regulatory interrelationship exists between HOXA10 and FKBP4. To address this supposition, HOXA10 expression and knockdown experiments were designed to further investigate whether HOXA10
modulates FKBP4 expression. The current study found that FKBP4 expression is positively regulated by HOXA10 in HESCs. However, this regulation proved to be independent of P4/cAMP-induced decidualization. Taken together with previous reports, the current results suggest that decreased endometrial HOXA10 expression contributes significantly to decreased FKBP4 expression in endometriosis. Further experiments are needed to determine whether HOXA10 regulates FKBP4 directly or whether this regulation is mediated by another transcription factor.

In summary, results presented in the current study suggest that FKBP4, but not FKBP5, is involved in the induction of decidualization. These PR co-chaperones regulate PR activity in response to ligand in the endometrium. The current observations also identify HOXA10 as a regulator of FKBP4 during human decidualization. Our results provide new insight into the regulation of decidualization and imply that downregulation of FKBP4 may significantly contribute to infertility in some patients with endometriosis.

Materials and Methods

Tissue collection

Endometrial tissue samples were collected by Pipelle biopsy from 15 women (age, 28–40 years) with surgically and histologically confirmed endometriosis. Endometriobiopsy specimens were also obtained from 15 healthy female controls who underwent tubal sterilization and were confirmed laparoscopically to be free of endometriosis. All the subjects were cycling regularly and had not received hormonal therapy for at least 3 months before surgery. The endometrial tissue samples were snap frozen on dry ice or used immediately for cell culture. Informed consent was obtained from each patient before surgery. Samples from ten subjects were included for each phase of the menstrual cycle examined, five obtained from controls and five from women with endometriosis. All protocols were approved by the Yale University School of Medicine Human Investigations Committee.

Cell culture

Tissues were minced and digested in Hanks’ balanced salt solution containing 1% penicillin, 1% streptomycin, 5% collagenase, and 0.5% deoxyribonuclease at 37 °C for 30 min in a shaking water bath. Dispersed endometrial cells were separated by filtration through a wire sieve (73 mm diameter pore, Sigma). Endometrial glands (largely undispersed) were retained by the sieve, whereas the dispersed stromal cells passed through the sieve into the filtrate. HESCs were seeded onto 12-well plates in DMEM/F12 phenol red medium (Gibco Invitrogen) containing 10% fetal bovine serum (Gibco), maintained at 37 °C in a humidified atmosphere (5% CO2 in air), and grown to confluence. Cells obtained after the first passage were characterized as described previously (26). To study decidualization of HESCs, cell cultures were treated with either DMSO vehicle (mock) or 0.1 μM P4 (Sigma–Aldrich) and 0.5 mM 8-bromoadeinosenine cAMP (Sigma–Aldrich) (P4/cAMP). Media were exchanged every 48 h for fresh media with P4 and cAMP, and the cultures were evaluated for decidualization-related morphological changes via light microscopy. Real-time PCR evaluated insulin-like growth factor binding protein 1 (IGFBP1) mRNA expression as a marker of decidualization. All experiments were performed in triplicate and repeated three times.

Transient gene transfection in HESCs

HOXA10 cDNA was cloned into the EcoRI site of pcDNA3.1 (+) (Invitrogen). pcDNA3.1 (+) without the HOXA10 insert was used as a control (Invitrogen). SiGenome duplex HOXA10 siRNA (catalog no. D-006336–01) and control nontargeting siRNA (catalog no. D-001210–02) were purchased from Dharmacon (Chicago, IL, USA). FKBP5 siRNA, FKBP4 siRNA (S10278407), and negative control siRNA (S103650318) were both purchased from Qiagen (Germantown, MD, USA). HESCs, grown to 60–70% confluence, were transfected with TransIT-LT1 (Mirus Bio LLC, Madison, WI, USA) with either pcDNA3.1 (+) or HOXA10 (4.0 μg for a 6-well plate and 12 μg for a 10 cm dish) or HOXA10 siRNA (20 μM for a 6-well plate and 60 μM for a 10 cm dish) using empty pcDNA3.1 (+) or nonspecific siRNA as respective controls. HESCs were transfected using Lipofectamine 2000 (Invitrogen) with FKBP5 siRNA, FKBP4 siRNA (33 μM for a 6-well plate and 60 μM for a 10 cm dish), or nonspecific siRNA as a control. After 8 h, the media were exchanged for fresh...
media, and cells were incubated for an additional 20 h in OPTI-MEM 1 Reduced Serum Medium (Invitrogen), without serum and antibiotics. Twenty-four hours post-transfection, total RNA was isolated, and 72 h post-transfection, total protein was extracted. All transfections were performed in triplicate and repeated three times.

**Real-time PCR analysis**

Total RNA was extracted using Trizol Reagent (Invitrogen) and reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc.). RT-PCR was performed before quantitative real-time PCR. The mRNA levels were determined by real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.). GADPH was used as an internal control to normalize for differences in the amount of total RNA in each sample. The primer sequences were as follows: FKB5: 5’-AAAAAGCCAAAGGACACAAC-3’ (sense), 5’-TTGAGGA-GGCGCCGAGTTG-3’ (antisense); FKB4: 5’-AGATGAC-AAGGAGGAGATG-G-3’ (sense), 5’-ATATGGCTCTCGGATCCAG-3’ (antisense); HOXA10: 5’-AGGTGACGC- TGGGGCTAATCTCTA-3’ (sense), 5’-GCCCTTCGAGAGAGCA-AAAAGGCCAAGGAGCACAAC-3’ (antisense); FKBP4: 5’-ATGACCCCTCTCATTGACC-3’ (sense), 5’-GAGA TAT GGT GAT GGG ATT TC-3’ (antisense); IGFBP1: 5’-CTATGATGCTGCTCAAGGGCTC-3’ (sense), 5’-TTCTGTTCAGATTTGCCAG-3’ (antisense).

**Western blotting**

Cellular protein was extracted and its concentration was measured by the BCA method. Samples were electrophoresed on SDS–PAGE gels and then transferred to nitrocellulose membranes and immunoblotted overnight at 4°C with a primary mouse MAB to FKB5 (1:500; ab54991; Abcam, Inc., Cambridge, MA, USA), a goat polyclonal antibody to HOXA10 (1:200, SC-17158; Santa Cruz Biotechology, Santa Cruz, CA, USA), or a mouse monoclonal antihuman GADPH (1:5000; Abcam, Inc.) with gentle shaking followed by incubation with HRP-conjugated secondary antibodies. The reactions were detected by an enhanced chemiluminescence assay. GADPH was used as an endogenous control for normalization. All western blots were generated at least three times from independent experiments and representative results are shown.

**Statistical analysis**

The results are presented as mean ± s.d. Statistical analyses were performed using Kruskal–Wallis test on data derived from human samples and one-way ANOVA on data derived from in vitro experiments using SPSS software 15.0 for Windows with P<0.05 being considered significant.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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