FKBP4 is regulated by HOXA10 during decidualization and in endometriosis

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Abstract

FKBP4 (FKBP52) and FKBP5 (FKBP51) are progesterin receptor (PR) co-chaperone proteins that enhance and inhibit, respectively, progesterin-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, Daftary & 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 tetracoordinate repeat (TPR) domain that binds to the C terminus of heat-shock protein 90 (HSP90). This binding enables both the immunophilins to act as co-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 progesterin 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

Figure 1 *FKBP5* and *FKBP4* mRNA levels in the endometrium of women with and without endometriosis. *FKBP5* and *FKBP4* mRNA levels were measured by real-time qPCR using biopsies obtained from 15 fertile women with endometriosis and 15 cycle-matched controls confirmed to be free of endometriosis. Mean values $\pm$ S.E.M. are shown. *FKBP5* was expressed at low levels throughout the menstrual cycle, and mRNA levels were similar in women with or without endometriosis. *FKBP4* mRNA expression was increased in the later half of the proliferative phase and remained at that level throughout the secretory phase. As the secretory-phase mRNA levels did not change, this phase is presented here as a single grouping. Expression of *FKBP4* mRNA was significantly lower in the endometrium obtained from women with endometriosis compared with controls in the late proliferative and secretory phases of the menstrual cycle. *Significant difference between controls and subjects with endometriosis ($P < 0.01$).

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 ($P < 0.01$). *FKBP5* 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 P_4 /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

Figure 2 Knockdown of *FKBP5* or *FKBP4* mRNA in cultured human endometrial stromal cells (HESCs) and its effect on the expression of *IGFBP1* during *in vitro* decidualization. (A) *FKBP5* and *FKBP4* expressions were decreased by 11 and 32%, respectively, of pretreatment level after transfection with *FKBP4* siRNA in HESCs ($*P < 0.01$). (B) Western blot demonstrated that *FKBP4* siRNA transfection decreased *FKBP4* protein level. N, mock transfection without siRNA; C, Control: transfection with control siRNA; S, siRNA transfection with *FKBP5* (left) or *FKBP4* (right). (C) HESCs were transfected with control siRNA, *FKBP5* siRNA, or *FKBP4* siRNA for 3 days and then cultured in the presence of P_4 /cAMP for 3 days. Cells were collected and total RNA was extracted. Real-time PCR results indicated that *IGFBP1* mRNA level in *FKBP4* siRNA/ P_4 /cAMP-treated cells (siFKBP4) was decreased significantly compared with control siRNA/ P_4 /cAMP cells. Blockade of *FKBP5* expression had no effect on *IGFBP1* expression ($*P < 0.05$). (D) We have previously shown that inhibition of *HOXA10* expression with siRNA also results in decreased *IGFBP1* expression; here, we confirmed those results in this model system ($*P < 0.01$).

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 P_4 /cAMP, reached a peak on day 4 (10.3 ± 1.3), and remained elevated. Similarly, *HOXA10* mRNA levels were also elevated in P_4 /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.

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 μ M progesterone (P_4), 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 P_4 /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.

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 P_4 /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 P_4 /cAMP, mRNA levels of *FKBP4* increased by fourfold (negative siRNA control), whereas this increase was not observed in HOXA10-silenced HESCs.

Discussion

In humans, decidualization is initiated by P_4 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

Figure 4 HOXA10 regulated *FKBP4* expression in HESCs. (A) *HOXA10* expression was increased more than 300-fold ($*P < 0.05$) after transfection with the pcDNA3.1/*HOXA10* vector in HESCs compared with vehicle-transfected cells or those transfected with the empty pcDNA3.1 vector. (B) *HOXA10* expression was decreased by 48% ($*P < 0.05$) of pretreatment level after transfection with *HOXA10* siRNA in HESCs. (C) Real-time PCR results showed that *FKBP4* expression increased more than three-fold after pcDNA3.1/*HOXA10* transfection ($*P < 0.05$). (D) Conversely, *HOXA10* siRNA transfection decreased *FKBP4* mRNA expression level by 36% of control ($*P < 0.05$). (E) Western blot results demonstrated that pcDNA/*HOXA10* transfection increased both *HOXA10* and *FKBP4* protein levels. (F) Western blot demonstrated that *HOXA10* siRNA transfection decreased both *HOXA10* and *FKBP4* protein levels.

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 P_4 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 P_4 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 P_4 treatment due to PR dysfunction (Conneely *et al.* 2001, Daikoku *et al.* 2005). As decidualization is P_4 /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 P_4 /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*. Mediation 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 P_4 /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*

Figure 5 HOXA10 regulated *FKBP4* expression in HESCs during *in vitro* decidualization. HESCs were transfected with *HOXA10* siRNA for 3 days and then exposed to P_4 /cAMP (labeled P). (A) Real-time PCR results showed that *FKBP4* mRNA level increased in the cells exposed to P_4 /cAMP (P) and control siRNA compared with vehicle and siRNA control (Ctl). However, *HOXA10* siRNA transfection significantly attenuated this effect despite treatment with P_4 /cAMP. $*P < 0.01$ compared with either control or siRNA treatment. (B) Representative western blot demonstrating the effect of P and *HOXA10* siRNA on *FKBP4* protein expression. The effect of each treatment results in changes in *FKBP4* protein that are similar to the mRNA changes demonstrated earlier.

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 P_4 /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 down-regulation 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. Endometrial biopsy 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 P_4 (Sigma–Aldrich) and 0.5 mM 8-bromoadenosine cAMP (Sigma–Aldrich) (P_4 /cAMP). Media were exchanged every 48 h for fresh media with P_4 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 purchased from Qiagen (Germantown, MD, USA). HESCs, grown to 60–70% confluence, were transfected using TransIT-LT1 (Mirus Bio LLC, Madison, WI, USA) with either pcDNA3.1 (+)/*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.). GAPDH 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: FKBP5: 5'-AAAAGGCCAAGGAGCACAAC-3' (sense), 5'-TTGAGGAGGGGCCGAGTTC-3' (antisense); FKBP4: 5'-AGATGACAGCCGAGGAGATG-3' (sense), 5'-AATTGTCTTGC-GATCCAG-3' (antisense); HOXA10: 5'-AGGTGGACGCTGCGGCTAATCTCTA-3' (sense), 5'-GCCCTTCCGAGAGCAGCAAAG-3' (antisense); GAPDH: 5'-ATGACCCCTTC-ATTGACC-3' (sense), 5'-GAA GAT GGT GAT GGG ATT TC-3' (antisense); IGFBP1: 5'-CTATGATGGCTCGAAGGCTC-3' (sense), 5'-TTCTTGTTCAGTTTGGCAG-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 FKBP4 (1:500, ab54991; Abcam, Inc., Cambridge, MA, USA), a goat polyclonal antibody to HOXA10 (1:200, SC-17158; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a mouse monoclonal antihuman GAPDH (1:5000; Abcam, Inc.) with gentle shaking followed by incubation with HRP-conjugated secondary antibodies. The reactions were detected by an enhanced chemiluminescence assay. GAPDH 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 $\pm$ 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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