3-Phosphoglycerate dehydrogenase expression is regulated by HOXA10 in murine endometrium and human endometrial cells

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Abstract

3-Phosphoglycerate dehydrogenase (PHGDH, 3-PGDH) is an enzyme necessary for *de novo* L-serine biosynthesis. HOXA10 expression is required for endometrial receptivity; however, few target genes of HOXA10 regulation are known. Using a microarray we identified *Phgdh* as a target of HOXA10 regulation in murine endometrium and confirmed this regulatory relationship in human endometrial cells. PHGDH was downregulated 2.0-fold by HOXA10 and upregulated 4.4-fold by HOXA10 antisense *in vivo*. In human endometrial cells, real-time PCR results show that pcDNA3.1/HOXA10 transfection decreased *PHGDH* mRNA expression to 40% of pretreatment level ($P < 0.05$), while *PHGDH* mRNA expression was increased 2.1-fold ($P < 0.05$) by *HOXA10* siRNA. Western blot results confirmed the regulatory relationship in both primary human endometrial stromal and epithelial cells, as well as in human endometrial stromal cells and Ishikawa cells. In human cycling endometrial tissue, immunohistochemical results showed that PHGDH expression is relatively high in the proliferative phase in glandular cells and lower in the secretory phase. Here we report novel expression and regulation of PHGDH in murine and human endometrium. PHGDH is expressed in both endometrial epithelial and stromal cells. HOXA10 represses endometrial PHGDH expression. PHGDH is necessary for serine biosynthesis, which serves as a substrate for protein synthesis. One mechanism by which HOXA10 regulates cellular differentiation may involve limiting protein synthesis in the secretory phase.

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Introduction

The limiting step of implantation is the ability of the blastocyst to implant into a receptive endometrium. Transient, predictable molecular events within the endometrial glands and stroma render a normally hostile environment receptive to implantation. Thus, the cascade of signaling events that occur in both fetal and maternal tissues at the time of implantation establishes an appropriate milieu critical to the development and survival of the fetus. Defects in formation of this network and the inability to sustain this crosstalk are believed to result in various implantation-associated issues that may manifest as implantation failures or as pregnancy complications throughout gestation (Aplin 2000, Red-Horse *et al.* 2004).

The homeobox (Hox) genes encode transcription factors that guide embryologic development as well as regulate differential gene expression within the endometrium with each menstrual cycle (Taylor *et al.* 1997). In particular, a member of this family, *HOXA10*, encodes a transcription factor required for both endometrial receptivity and blastocyst implantation. The *HOXA10* gene product is expressed by endometrial glands and

stroma throughout the menstrual cycle (Taylor *et al.* 1998, 1999) with peak expression coincident with the implantation window of the midsecretory phase (Taylor *et al.* 1997, Sarno *et al.* 2005).

Selective alteration of endometrial *Hoxa10* expression in mice through the use of liposome-mediated gene transfection dramatically alters implantation (Bagot *et al.* 2000). While similar studies have not yet been performed in higher animal models or humans, transfection of a human endometrial adenocarcinoma cell line (Ishikawa cells) with a *HOXA10* antisense oligodeoxynucleotide also resulted in decreased *HOXA10* expression (Bagot *et al.* 2000). Furthermore, efficient transfection and expression of an *Escherichia coli* lacZ reporter gene has been accomplished in intact human uteri *ex vivo* (Daftary & Taylor 2003, 2004). Thus, differential maternal expression of endometrial HOXA10 is essential for implantation as this transcription factor regulates the molecular switches that promote the appropriate endometrial receptivity.

To identify candidate genes that may be regulated by differential HOXA10 expression, we employed our mouse model that allows for transient expression of plasmid-originated select gene products during the

peri-implantation period (Bagot *et al.* 2000). Employing a microarray screen, we selected for candidate gene products that were regulated differentially when HOXA10 was transiently overexpressed in endometrium in comparison to vehicle only control tissues (Vitiello *et al.* 2008). Many of these genes demonstrating altered expression represent commonly occurring gene products within the cell rather than unique regulatory products. The cellular ontogenies of differentially expressed genes included cell adhesion molecules, signal transduction factors as well as metabolic regulators.

3-Phosphoglycerate dehydrogenase (PHGDH, 3-PGDH), a key enzyme necessary for *de novo* L-serine biosynthesis (Ichiara & Greenberg 1957), demonstrates clear differential expression patterns in the peri-implantation mouse uterus. The human PHGDH gene is regulated at the transcriptional level in a tissues and it is dependent on cellular proliferation status (Cho *et al.* 2000). It is the first committed enzyme for the *de novo* phosphorylated pathway of serine synthesis catalyzing the transition of 3-phosphoglycerate into 3-phosphohydroxypyruvate using NAD⁺ as a cofactor and resulting in an increase in the serine pool. Serine itself, a nonessential amino acid, plays a central role in cellular proliferation serving as an indispensable precursor for the synthesis of biomolecules, such as proteins, membrane lipids, other amino acids, and nucleotides (Snell & Weber 1986, Narkevicz *et al.* 1996, Xue *et al.* 1999). It is expressed at high levels in diverse tissue including: prostate, testis, ovary, brain, liver, kidney and pancreas (Snell 1984). The activity of PHGDH is elevated in some cancers, such as rat hepatomas, rat sarcoma and human colon carcinoma (Snell & Weber 1986, Snell *et al.* 1987, 1988).

Hox genes were first recognized as an evolutionarily conserved family of transcription factors critical to the control of early embryonic development. Their differential regulation in human endometrium leads to tissue specialization (Taylor *et al.* 1998). Estrogen and progesterone regulate HOXA10 expression in both the embryonic and the adult reproductive tracts (Taylor *et al.* 1998, Block *et al.* 2000). HOXA10 expression is required for endometrial receptivity, however few target genes of HOXA10 regulation are known. Using microarray we identified *Phgdh* as a target of HOXA10 in murine endometrium and confirmed this regulatory relationship in human endometrial cells.

Results

Phgdh is a target of HOXA10 regulation in murine endometrium

To identify putative gene regulated by HOXA10, we performed an initial screen of these targets employing a microarray. Thirty-nine thousand unique mouse transcripts were probed for differences in expression. The *Phgdh*

gene product was noted to be reciprocally regulated according to HOXA10 expression. When HOXA10 was overexpressed, the microarray results demonstrated an almost 2.0-fold decrease in *Phgdh* mRNA expression. Conversely, transfection of an enzyme resistant *Hoxa10* antisense oligonucleotide demonstrated a 4.4-fold increase in *Phgdh* mRNA expression (Fig. 1).

PHGDH protein expression in human cycling endometrial tissue

To determine the expression pattern of PHGDH through the menstrual cycle, we used immunohistochemical analysis on human endometrial samples which were collected from subjects in secretory phase and proliferative phases of the menstrual cycle. PHGDH protein was found in the cytoplasm of both stromal and glandular cells in human endometrium. Immunohistochemical results show PHGDH expression is relatively high in the proliferative phase (P) and lower in the secretory phase (S) in glandular epithelial cells of human cycling endometrium. Immunohistochemical results also show that PHGDH expression is relatively lower in endometrial stromal cells than in glandular epithelial cells in the proliferative phase. PHGDH expression is also lower in the secretory phase than the proliferative phase in stromal cells of human cycling endometrium (Fig. 2 and Table 1).

HOXA10 regulated PHGDH mRNA expression in human endometrial cell lines and primary human endometrial cells

To confirm the regulatory relationship identified in the murine microarray, we first used the human endometrial stromal cell line (HESC) and the human endometrial epithelial cell line Ishikawa. We have previously described HOXA10 gene expression in HESC and Ishikawa cells (Taylor *et al.* 1998). HOXA10 mRNA

Figure 1 Identification of *Phgdh* as a target of HOXA10 in murine endometrium as assessed by microarray analysis. Murine uterine endometrium was transfected with a *Hoxa10* expression vector or enzyme-resistant *Hoxa10* antisense oligonucleotides *in vivo* and then microarray analysis was performed on RNA extracted from those tissues. *Phgdh* was downregulated by HOXA10 2.0-fold and upregulated by *Hoxa10* antisense 4.4-fold in the murine microarray.

Figure 2 PHGDH protein expression in human cycling endometrium tissue. PHGDH protein was expressed in the cytoplasm of both stromal and glandular cells in human endometrium. Immunohistochemical results show PHGDH expression is relatively high in the proliferative phase and lower in the secretory phase. The left column shows the first half of each phase, the center column shows the mid portion of each phase (including the window of implantation). The right column shows the luminal epithelial surface at each phase. Arrows point to the luminal epithelial surface (S, stroma; G, glands). The insert shows a negative control. Photomicrographs taken at $\times 600$ magnification.

was not endogenously expressed in the ovarian cancer cell line A2780, and therefore this cell line was used as a control (Fig. 3A). Quantitative real-time RT-PCR (qRT-PCR) results demonstrated that *HOXA10* gene expression increased over 1000-fold after transfection with the pcDNA–*HOXA10* vector in HESC and Ishikawa cell lines. *HOXA10* mRNA expression was decreased 40–50% of pretreatment level after transfection of *HOXA10* siRNA in Ishikawa cell lines, and 60–70% of pretreatment level after transfection of *HOXA10* siRNA in HESC cells (Fig. 4A). As a control A2780 cells were transfected with the *HOXA10* expression vector or siRNA (Fig. 3). *HOXA10* was not endogenously

Table 1 *H*-score analysis (mean $\pm$ S.E.M.).

	Stroma	Glands	<i>P</i> values
Proliferative phase (<i>n</i> =10)	2.63 $\pm$ 0.253	3.20 $\pm$ 0.228	<i>P</i> <0.05
Secretory phase (<i>n</i> =10)	1.78 $\pm$ 0.032	2.165 $\pm$ 0.225	<i>P</i> <0.05
	<i>P</i> <0.05	<i>P</i> <0.05	

expressed by these cells, and could be induced by transfection with pcDNA3.1/*HOXA10*. *PHGDH* expression was not changed by transfection with either the *HOXA10* expression vector or *HOXA10* siRNA in the A2780 cell line. However, pcDNA3.1/*HOXA10* transfection did result in decreased *PHGDH* mRNA expression to 40% of pretreatment level (*P*<0.05) in HESC cells, a small increase in *PHGDH* mRNA expression was seen after *HOXA10* siRNA transfection in these cells (Fig. 4B). *PHGDH* mRNA expression was increased 2.1-fold (*P*<0.05) by *HOXA10* siRNA transfection in Ishikawa cells (Fig. 4B). There was no significant change in *PHGDH* mRNA expression after pcDNA3.1/*HOXA10* transfection in Ishikawa cells (Fig. 4B).

We also used the primary human endometrial stromal and epithelial cells to confirm the regulatory relationship identified in the cell lines. qRT-PCR results demonstrated that *HOXA10* gene expression increased over 1000-fold after transfection with the pcDNA–*HOXA10* vector in primary human endometrial cells. *HOXA10* mRNA expression was decreased around 70% of pretreatment level after transfection of *HOXA10* siRNA in both primary human endometrial stromal and epithelial cells (Fig. 5A). pcDNA3.1/*HOXA10* transfection result in decreased *PHGDH* mRNA expression to 70% of pretreatment level (*P*<0.05) in primary HESC (Fig. 5B). *PHGDH* mRNA expression was increased 1.7-fold (*P*<0.05) by *HOXA10* siRNA transfection in primary human endometrial epithelial cells (Fig. 5B).

***HOXA10* regulated *PHGDH* protein expression in human endometrial cell lines and primary human endometrial cells**

Western blot results demonstrated that *HOXA10* gene expression increased after transfection of the pcDNA–*HOXA10* vector and decreased after transfection with *HOXA10* siRNA in the HESC, Ishikawa cells and primary human endometrial cells (Fig. 6a). Figure 6b shows western blot results after pcDNA/*HOXA10* transfection of HESC and Ishikawa cells; *PHGDH* protein expression was decreased in these cells. Densitometry revealed that *HOXA10* transfection significantly decreased *PHGDH* protein expression by 62 ± 5.2 and $30 \pm 3.2\%$ in HESC and Ishikawa cells, respectively. After siRNA transfection, *PHGDH* protein expression was increased in these cells. siRNA treatment increased *PHGDH* protein by 70 ± 12.4 and $45 \pm 5.6\%$, respectively, in these cells. Similarly, *PHGDH* protein expression was increased by *HOXA10* siRNA transfection in primary human endometrial cells. Densitometry revealed a 25 ± 2.6 and $40 \pm 1.7\%$ increase in *PHGDH* expression in primary stromal and epithelial cells respectively. There was a similar decrease in *PHGDH* protein expression in primary human endometrial cells after pcDNA3.1/*HOXA10* transfection, indicating submaximal repression at

Figure 3 *HOXA10* and *PHGDH* mRNA expression in the control A2780 ovarian cancer cell line. (A) RT-PCR results show absent *HOXA10* mRNA expression in A2780 cells after transfection with empty pcDNA vector. *HOXA10* mRNA expression was seen only after transfection with the *HOXA10* vector. (B) *PHGDH* mRNA expression was not regulated in the A2780 cell line after transfection with the pcDNA-*HOXA10* vector. (C) *PHGDH* mRNA expression was not regulated in the A2780 cell line after transfection with the *HOXA10* siRNA. Shown are mean $\pm$ s.d.

endogenous levels of *HOXA10* expression. Densitometry revealed a 33 ± 6.1 and $23 \pm 3.1\%$ decrease in *PHGDH* expression in primary stromal and epithelial cells respectively. Taken together, these data confirmed the regulatory relationship determined in both HESC and Ishikawa cells in mRNA expression. As a control, A2780 cells were transfected with the *HOXA10* expression vector. *HOXA10* protein could be induced by transfection with pcDNA3.1/*HOXA10*. *PHGDH* protein expression was not changed by transfection with either the *HOXA10* expression vector or *HOXA10* siRNA in the A2780 cell line.

Discussion

Uterine endometrium undergoes dramatic proliferation and growth, regenerating completely each menstrual/estrus cycle. The molecular signaling pathways that regulate this growth and subsequent differentiation are still incompletely characterized. Few genes are known to be essential for this process. It is intuitive that genes regulating metabolism and protein synthesis would be required for these functions; however, few have been identified and the regulation of their expression has not been described. The *PHGDH* gene encodes PHGDH, which catalyzes the first step in the phosphorylated pathway of serine biosynthesis.

In the adult, the expression of this gene has been associated with cellular proliferative status. In monocytic cells, for example, experimentally-induced cell differentiation results in growth arrest and abrupt

downregulation of *PHGDH* (Cho *et al.* 2000). Regulation of enzymes that are necessary for protein synthesis is linked to proliferative potential. Our data suggest that a similar mechanism exists in the endometrium, where *PHGDH* is downregulated in the secretory phase. It is necessary for a cell to have the ability to regulate enzymes essential for distinct cellular functions

Figure 4 (A) *HOXA10* mRNA expression in human endometrial cell lines. (a) Real-time PCR results show that pcDNA3.1/*HOXA10* transfection increased *HOXA10* mRNA expression over 1000-fold ($P < 0.05$) in HESC cells. (b) *HOXA10* mRNA expression decreased to 60% ($P < 0.05$) of pretreatment level after transfection of *HOXA10* siRNA in HESC cells. (c) Similarly, real-time PCR results show that *HOXA10* mRNA expression was increased over 1000-fold ($P < 0.05$) by pcDNA3.1/*HOXA10* transfection in Ishikawa cells. (d) *HOXA10* mRNA expression decreased to $\sim 40\%$ ($P < 0.05$) of pretreatment level after transfection with *HOXA10* siRNA in Ishikawa cells. Shown are mean $\pm$ s.d. *Indicates $P < 0.05$. (B) *HOXA10* regulated *PHGDH* mRNA expression in human endometrial cell lines. (a) Real-time PCR results show that pcDNA3.1/*HOXA10* transfection decreased *PHGDH* mRNA expression to 40% of pretreatment level ($P < 0.05$) in HESC cells. (b) *PHGDH* mRNA expression was not significantly increased after transfection of *HOXA10* siRNA in HESC cells. (c) *PHGDH* mRNA expression was not significantly changed after transfection with pcDNA3.1/*HOXA10* in Ishikawa cells. (d) Real-time PCR results show that *PHGDH* mRNA expression was increased 2.1-fold ($P < 0.05$) by *HOXA10* siRNA transfection in Ishikawa cells. Shown are mean $\pm$ s.d. *Indicates $P < 0.05$.

Figure 5 (A) *HOXA10* mRNA expression in primary human endometrial cells. (a) Real-time PCR results show that pcDNA3.1/*HOXA10* transfection increased *HOXA10* mRNA expression over 1000-fold ($P < 0.05$) in primary human endometrial cells. (b) *HOXA10* mRNA expression decreased around 70% ($P < 0.05$) of pretreatment level after transfection with *HOXA10* siRNA in primary human endometrial cells. (c) Similarly, real-time PCR results show that *HOXA10* mRNA expression increased over 1000-fold ($P < 0.05$) after pcDNA3.1/*HOXA10* transfection in primary endometrial epithelial cells. (d) *HOXA10* mRNA expression decreased to 70% ($P < 0.05$) of pretreatment level after *HOXA10* siRNA transfection in primary human endometrial epithelial cells. Shown are mean $\pm$ s.d. *Indicates $P < 0.05$. (B) *HOXA10* regulated *PHGDH* mRNA expression in primary human endometrial cells. (a) Real-time PCR results show that pcDNA3.1/*HOXA10* transfection decreased *PHGDH* mRNA expression around 70% of pretreatment level ($P < 0.05$) in primary human endometrial cells. (b) *PHGDH* mRNA expression showed no significant increase after transfection of *HOXA10* siRNA in primary human endometrial cells. (c) *PHGDH* mRNA expression was not significantly changed after transfection with pcDNA3.1/*HOXA10* in primary human endometrial cells. (d) Real-time PCR results show that *PHGDH* mRNA expression was increased 1.7-fold ($P < 0.05$) by *HOXA10* siRNA transfection in Ishikawa cells. Shown are mean $\pm$ s.d. *Indicates $P < 0.05$.

throughout the course of the reproductive cycle; however, the molecular mechanisms responsible for this regulation are largely unknown.

HOXA10 is a transcription factor necessary for endometrial receptivity demonstrating heightened endometrial expression during the implantation window. Previously, we have reported that *HOXA10* regulates the

expression of other transcription factors and molecules that are important for embryo attachment at the epithelial cell surface (Daftary *et al.* 2002, Troy *et al.* 2003). Targeted disruption of *Hoxa10* in mice results in loss of endometrial receptivity (Satokata *et al.* 1995) while diminished expression in mice or humans similarly leads diminished embryo implantation (Bagot *et al.* 2000, Daftary *et al.* 2002, Cermik *et al.* 2003).

Here we demonstrate that *HOXA10* also has a regulatory effect on genes necessary for regulation of protein synthesis. We speculate that *HOXA10* is involved in modulating epithelial cell metabolism. When proliferation and demand for protein synthesis is at its peak, there is minimal glandular *HOXA10* gene-product expression; and consequently, there is no repression of enzymes (such as *PHGDH*) needed for protein synthesis, thus meeting the increasing metabolic demands. However, in the secretory phase, the glands' cellular contents have been expelled into the uterine lumen, glandular proliferation ceases, and the demand for new protein synthesis is minimal. As the concentrations of *HOXA10* gene product increases, the production of *PHGDH* decreases.

Not surprisingly, the *in vitro* results reflect the underlying physiology where *HOXA10* represses *PHGDH* expression. In Ishikawa cells, *HOXA10* siRNA treatment leads to derepression of *PHGDH* expression as predicted. Treatment with excess *HOXA10* had less effect on expression of *PHGDH*, likely reflecting some repression by the endogenous *HOXA10* expressed by Ishikawa cells. The endogenous level of *HOXA10* in these cells is higher than that seen in the proliferative phase endometrial epithelial cells. Conversely, in stromal cells there is a low level of *PHGDH* expression in both proliferative and secretory phases.

Employing HESC cells, we demonstrate that siRNA treatment had minimal effect; these data imply that co-repressors may govern *PHGDH* expression even in the absence of *HOXA10*. Additionally, excess *HOXA10* expression leads to further suppression. Such transcriptional regulation would explain the persistently low expression in stromal cells. These findings support an additional repressive mechanism whereby *PHGDH* expression is limited significantly during the implantation window and that the degree of enzyme synthesis repression varies within this timeframe. It appears that overexpression of *HOXA10* gene product affords additional *PHGDH* repression, leading to the near absence of expression seen in the secretory phase.

In vivo we demonstrate that in human cycling endometrium tissue, *PHGDH* expression is relative high in the proliferative phase in glandular cells and lower in the secretory phase. *HOXA10* represses endometrial *PHGDH* expression. *PHGDH* expression and therefore function were inhibited by *HOXA10* in the secretory phase in human cycling endometrium, a phase in which endometrial cells growth is suppressed. In the

proliferative phase, *HOXA10* expression decreases and *PHGDH* expression was derepressed facilitating endometrial cell growth. *In vitro*, we show that *PHGDH* expression is similarly regulated by *HOXA10* expression in isolated primary human endometrial cells. *PHGDH* was downregulated by *HOXA10* overexpression in primary HESC, and upregulated by *HOXA10* repression in primary human endometrial epithelial cells.

This dynamic regulation of *PHGDH* gene expression by *HOXA10* provides for specific cellular metabolic profiles to meet the changing needs of the local environment. Specifically, heightened levels of the

enzyme provide a potential nondietary and nonhepatic source of serine and glycine at a time in the reproductive cycle when protein production is necessary for proliferation and generation of a receptive endometrium. Linking the regulation of endometrial receptivity and cellular metabolic demands through a single transcription factor assures synchrony of these events. Data presented here contribute to defining the molecular signature of uterine receptivity during early mouse implantation. Further characterization of these genes and their role in implantation may assist to elucidate the complex molecular and physiologic events that govern reproductive success.

Materials and Methods

Plasmids, oligonucleotides and siRNA

HOXA10 cDNA was cloned into the *EcoRI* site of pcDNA3.1(+) (Invitrogen). PcDNA3.1(+) without the *HOXA10* insert was used as a control (Invitrogen). Two 30-mer phosphothioate-protected oligodeoxyribonucleotides were synthesized by the W M Keck Oligonucleotide Laboratory at Yale University; one oligonucleotide complementary to the start of translation of *Hoxa10/HOXA10* corresponding to nucleotides 44–73 (GenBank accession number L08757): 5'-CTCTCCGAGCATGACATTGTTGTGGGATAA-3' (antisense); the second has the same nucleotide composition but a random sequence and was used as a missense control: 5'-TGCTGCTAGGATCGTTCAAGTGTATCACGA-3'. The efficacy of the *HOXA10* expression vector and the *HOXA10* antisense molecule have been tested and validated as previously described

Figure 6 (A) *HOXA10* protein expression in human cell lines and primary human endometrial cells. (a) Western blot results show that pcDNA/*HOXA10* transfection induced *HOXA10* protein expression in A2780 cells. (b) *HOXA10* protein expression was increased after transfection with *HOXA10* and decreased after transfection with *HOXA10* siRNA in HESC. (c) Similarly, *HOXA10* protein expression was increased after transfection with *HOXA10* and decreased after transfection with *HOXA10* siRNA in Ishikawa cells. (d) *HOXA10* protein expression was increased by pcDNA3.1/*HOXA10* transfection and was decreased by *HOXA10* siRNA transfection in primary human endometrial cells. (e) *HOXA10* protein expression was increased after transfection with pcDNA3.1/*HOXA10* and decreased after transfection with *HOXA10* siRNA in primary human epithelial cells. (B) *HOXA10* regulated *PHGDH* protein expression in human cell lines and primary human endometrial cells. (a) Western blot results show that *PHGDH* protein expression was not changed in A2780 cells after pcDNA3.1/*HOXA10* and *HOXA10* siRNA. (b) pcDNA/*HOXA10* transfection decreased *PHGDH* protein expression and *HOXA10* siRNA transfection increased *PHGDH* expression in HESC cells. (c) Western blot results also show that *PHGDH* protein expression was increased by *HOXA10* siRNA transfection and decreased by *HOXA10* expression in Ishikawa cells. (d) Similarly, western blot results show that *PHGDH* protein expression was increased by *HOXA10* siRNA transfection and decreased by *HOXA10* expression in primary human endometrial cells. (E) *PHGDH* protein expression was increased by *HOXA10* siRNA transfection and decreased by *HOXA10* expression in primary human epithelial cells.

(Bagot *et al.* 2001). SiGenome duplex *HOXA10* siRNA (cat# D-006336-01) and control nontargeting siRNA (cat# D-001210-02) were purchased from Dharmacon (Chicago, IL, USA).

In vivo gene transfection and tissue microarray

Nulliparous reproductive age female and male CD1 mice were obtained from Charles River (Wilmington, MA, USA). Three animals were used for each test condition. Mice were mated and examined every 12 h until the detection of a vaginal plug. Twenty-four hours after plug detection, a laparotomy was performed. The mice first were anesthetized with 0.1 ml/10 g of a xylazine (10 mg/kg)/ketamine (100 mg/kg) mixture given by i.p. injection. Each uterine horn was injected with 25 µl of a pre-incubated DNA/lipofectamine (Bagot *et al.* 2000, Vitiello *et al.* 2008). Liposome-mediated gene transfection was used to deliver either pcDNA-HOXA10/pcDNA3.1(+) vector ($n=3$) or an enzyme-resistant *Hoxa10* antisense or missense oligonucleotide to the murine uterus ($n=3$). A phosphorothioate-DNA-oligonucleotide (CTCTCCGAGCATGACATTGTTGTGGGATAA) complementary to the translational start site of the *Hoxa10* transcript (nucleotides 44–73) was used in addition to a missense oligonucleotide of the same nucleotide composition but in scrambled order. The peritoneum was closed separately from the skin incision employing a running 3-0 vicryl stitch. Forty-eight hours post-laparotomy, the uterus was excised and total uterine RNA was isolated using Trizol (Invitrogen) and RNA-easy kit (Qiagen) according to manufacturer's directions. Purified RNA was analyzed using the Affymetrix mouse genome 430 2.0 array which probes for 39 000 gene products. Gene Spring 6.0 was used to perform a two-sided *t*-test and genes were classified according to functional ontologies. Those candidates demonstrating statistically significant changes in expression profiles were selected for further assessment. These experiments were conducted in accordance with an approved protocol issued by the Yale Animal Care and Use Committee.

Immunohistochemistry

Endometrial samples were collected from 20 normally cycling reproductive age women (age 28–40, proliferative phase, $n=10$; secretory phase, $n=10$) not using hormonal therapy, throughout the menstrual cycle under a Human Investigation Committee approved protocol. Formalin-fixed paraffin-embedded tissues were cut into 5 µm thick sections and mounted on coated slides. Slides were de-paraffinized and rehydrated through a series of xylene and ethanol washes, followed by permeabilization in 95% cold ethanol. After a 5 min rinse in distilled water, an antigen-presenting step was performed by steaming the slides in 0.01 mM sodium citrate buffer for 20 min, followed by removal of the staining jar from the steam chamber and cooling for 20 min. Slides were rinsed for 5 min in PBS with 0.1% Tween20 (PBST20), and sections were circumscribed with a hydrophobic pen. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min followed by a 5 min PBST wash. Nonspecific binding was blocked with 1.5% normal horse serum in PBST for 1 h at room temperature. Slides were incubated in the primary antibody, goat polyclonal HOXA10 antibody at a dilution of

1:200, or polyclonal PHGDH antibody at a dilution of 1:150 overnight at 4 °C. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): HOXA10 (sc-17159), PHGDH (sc-16222). Normal goat IgG (Santa Cruz Biotechnology) was used as a negative control. Biotinylated secondary antibodies were purchased from Vector Laboratories (Vector; Burlingame, CA, USA). Horse anti-goat secondary antibody (3.5 µg/ml) for HOXA10 and PHGDH was applied for 1 h at 4 °C. Slides were washed in 1×PBST, incubated in ABC Elite (Vector) for 15 min at room temperature, washed in 1×PBST, and incubated for 5 min in diaminobenzidine (DAB; 400 mg/ml; Vector). A 15 s exposure to hematoxylin was used as a counterstain. For each individual primary antibody, all slides were processed simultaneously. Slides were rehydrated through 3 min ethanol and xylene washes and mounted with Permount. Expression and localization differences were evaluated and scored on an Olympus BX 40 light microscope by two different observers blinded to the tissue origin using the H-SCORE system (Lessey *et al.* 1994, Sharpe-Timms *et al.* 2000). The H-SCORE, representing levels of staining intensity and distribution, is calculated using the following equation: $H\text{-SCORE} = \sum Pi (I + 1)$, where I is the intensity of staining with a value of 1, 2 or 3 (weak, moderate or strong respectively), and Pi is the percentage of stained cells for each intensity, varying from 0 to 100%. *H*-score data were analyzed by Mann–Whitney rank sum test.

Cell culture

The HESC were a generous gift of Dr Charles J Lockwood (Yale University; Krikun *et al.* 2004); the human endometrial adenocarcinoma cell line (Ishikawa) and human ovarian cancer cell line (A2780) were generous gifts of Dr Richard Hochberg and Dr Gil Mor respectively (Yale University). Primary HESC and primary human endometrial epithelial cells were isolated from human endometrial tissues (Zhang *et al.* 1995). Previously we have demonstrated that these cells consist of 97% epithelial cells using Factor VIII, cytokeratin, 3C10, and vimentin as markers of endothelial cells, epithelial cells, macrophages and stromal cells, respectively (Taylor *et al.* 1998). HESC and primary HESC were maintained in a phenol-red-free DMEM, Ham's F-12 (Sigma), supplemented with 10% charcoal-stripped calf serum, 1% penicillin/streptomycin, and 1% sodium pyruvate. Ishikawa and primary human endometrial epithelial cells were maintained in MEM (Sigma), supplemented with 10% charcoal-stripped calf serum, 1% penicillin/streptomycin and 1% sodium pyruvate. Passage 2–4 of primary human endometrial stromal and epithelial cells was used. A2780 cells were cultured in RPMI 1640 (Invitrogen), supplemented with 10% charcoal-stripped calf serum and 1% penicillin/streptomycin, 1×nonessential amino acid and 1% sodium pyruvate.

Transient gene transfection in human cell lines

HESC and primary HESC, grown to 60–70% confluence, were transfected using TransIT-LT1 Mirus (Madison, WI, USA) with either pcDNA3.1(+)/HOXA10 (0.4 µg for 6-well plate, 12 µg for 10 cm dish) or *HOXA10* siRNA (20 µM for 6-well plate, 60 µM for 10 cm dish), using empty pcDNA3.1(+)

or nonspecific siRNA as respective control. Ishikawa cells, A2780 cells and primary human endometrial epithelial cells, grown to 45–55% confluence, were transfected using lipofectamine 2000 (Invitrogen) with either pcDNA3.1(+)/HOXA10 (0.4 µg for 6-well plate, 12 µg for 10 cm dish) or HOXA10 siRNA (20 µM for 6-well plate, 60 µM for 10 cm dish), using empty pcDNA3.1(+) or nonspecific siRNA as respective control. After 4 h, the media were changed and cells were incubated for an additional 20 h in OPTI-MEM. OPTI-MEM I Reduced Serum Medium (Invitrogen), without serum or antibiotics, was used during transfection. Forty-eight hours post-transfection, total RNA and protein were isolated. All transfections were performed in triplicate.

Real-time PCR

qRT-PCR was performed using iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad). RNA was reverse-transcribed for 30 min at 42 °C. PCR for HOXA10 was performed for 45 cycles at 95 °C for 15 s; 61 °C for 20 s; 72 °C for 25 s. PCR for PHGDH was performed for 45 cycles of 95 °C for 15 s; 60 °C for 20 s; 72 °C for 25 s. PCR for control β-actin was performed for 45 cycles of 95 °C for 15 s; 60 °C for 20 s; 72 °C for 25 s. HOXA10 and β-actin primers were used as previously described (Du *et al.* 2005). PHGDH primers were 5'-AACTTCTTCCGCTCCCATTT-3' (forward) and 5'-GTCATCAACGCAGCTGAGAA-3' (reverse). Expression was adjusted to the expression of β-actin from the same sample. Melting curve analysis was conducted to determine the specificity of the amplified products and to ensure the absence of primer-dimer formation. All products obtained yielded the predicted melting temperature. Analysis of relative gene expression data used $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). The C_t values were converted to the term 2^{-C_t} . Group means were evaluated by *t*-test ($n \geq 3$). Differences of $P < 0.05$ were considered significant.

Western blot

Whole cell protein was extracted from A2780 cells, HESC, Ishikawa cells as well as primary human endometrial stromal and epithelial cells using Nuclear Extract Kit (Activemotif, Carlsbad, CA, USA) according to manufacturer's protocol. Equal amounts of protein (60 µg for both HOXA10 and PHGDH) were electrophoresed through 4–15% polyacrylamide gels (Bio-Rad) at 160 V for 70 min and transferred onto Immobilon-P nitrocellulose membranes (Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100 V for 1 h. After incubation in blocking buffer (1 × PBS, 0.2% Tween 20, 5% milk), the membrane was incubated individually with either goat polyclonal HOXA10 antibody (sc-17159) (Santa Cruz Biotechnology) dilution 1:200, or polyclonal PHGDH antibody (sc-16222) (Santa Cruz Biotechnology) dilution 1:200 overnight at 4 °C. Alternatively the membranes were incubated with either goat polyclonal actin antibody (sc-1615) (Santa Cruz Biotechnology) dilution 1:1000, mouse β-tubulin MAB (sc-8035) (Santa Cruz Biotechnology) at a dilution of 1:1000, at room temperature for 1 h. After washing, the membranes were incubated for 1 h with biotinylated horse anti-goat secondary antibody or goat

anti-mouse secondary antibody (Vector) diluted (3.5 µg/ml) in the blocking buffer. The membranes were incubated in ABC Elite (Vector), and then stained by DAB (Vector). Densitometry was performed using Image J version 1.43a (NIH, Bethesda, MD, USA) and normalized to actin expression. Group means were evaluated by *t*-test ($n = 3$). Differences of $P < 0.05$ were 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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