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Endothelin Type A Receptor (ET_A) Expression Is Regulated by *HOXA10* in Human Endometrial Stromal Cells

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Abstract

Endothelin type A receptor (ET_A) is a member of the superfamily of G protein-coupled receptors. Our laboratory conducted a microarray screen that identified ET_A as target of *HOXA10* transcriptional control in endometrium. Here, we confirm *HOXA10*-regulated ET_A expression in endometrium. Endometrial biopsies were obtained from fertile reproductive-age individuals, and first trimester decidual samples were obtained at the time of elective termination. Immunohistochemistry (IHC) was used to identify ET_A protein in endometrium as well as first trimester decidua. ET_A was expressed in endometrial stromal cells throughout the menstrual cycle. ET_A was also highly expressed in first trimester decidual cells. The regulatory relationship between *HOXA10* and ET_A was established by transient transfection analysis. The human endometrial stromal cell line (HESC) and the human endometrial epithelial cell line (Ishikawa) were transfected with pcDNA/*HOXA10*, *HOXA10* small interfering RNA (siRNA), or respective controls. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to determine expression levels of *HOXA10* and ET_A in each group. ET_A gene expression increased 9-fold ($P < .05$) after pcDNA/*HOXA10* transfection of HESC. ET_A was not regulated by *HOXA10* in Ishikawa cells. We conclude that ET_A is expressed in normal endometrium and decidua. Expression of this receptor is regulated by an essential mediator of endometrial receptivity, *HOXA10*. ET_A may enhance the proliferative potential of endometrial cells in a manner similar to that seen in vascular smooth muscle cells. ET_A likely acts as a molecular mechanism by which *HOXA10* promotes stromal cell growth and prostaglandin production in both the implantation window and decidua.

Keywords

ET_A; *HOXA10*; endometrium; decidua

INTRODUCTION

The proteins endothelin-1 (ET-1), -2 (ET-2), and -3 (ET-3) comprise a family of 21-amino acid peptides with potent vasoactive properties.¹ In addition, endothelins promote proliferation of vascular smooth muscle and other cells. While originally described as a product of endothelial cells, the endothelins are expressed in multiple epithelial cell types, including endometrial epithelial cells.² They function through binding 2 G protein-coupled receptors, endothelin type A receptor (ET_A) and endothelin type B receptor (ET_B). The ET_A

receptor has a high specificity for ET-1, while ET_B binds all 3 endothelins with equipotent affinity. ET_A and ET_B receptor expression has also been described in the human endometrium. ET_A is expressed primarily in the stroma throughout the menstrual cycle while ET_B is expressed in the glands in the secretory phase and menstrual stroma.³⁻⁵ Endothelin-1 is believed to promote both endometrial vasoconstriction at menses through its action on endometrial vasculature and also the subsequent proliferation seen during endometrial regeneration through a direct effect on endometrial stromal cells.⁶ The ET_A receptor is also expressed in decidua, where it mediates prostaglandin production from first trimester cells.⁵

We previously conducted a microarray screen of murine endometrium after transfection with pcDNA/HOXA10 or antisense-HOXA10.⁷ We identified ET_A as a putative target gene of *HOXA10* regulation in the mouse uterus. *HOXA10* is a transcription factor that is necessary for endometrial receptivity and embryo implantation. Altering the endometrial expression levels of *HOXA10* in murine endometrium results in corresponding alterations in embryo implantation.^{8,9} *HOXA10* is also expressed in human endometrium, where its transcription is regulated by estradiol and progesterone.^{9,10} Endometrial epithelial *HOXA10* expression levels are upregulated in the mid-luteal phase and stromal upregulation is required for normal decidualization.^{8,9}

Here, we characterize the expression of ET_A in the human endometrium and in the decidua. We also demonstrate the regulation of endometrial ET_A expression by *HOXA10*.

MATERIAL AND METHODS

Tissue Collection

Ten endometrial biopsies (4 proliferative phase and 6 secretory phase) were obtained from fertile women as controls. All women were of reproductive age, had regular menstrual cycles, and had not used hormonal medication within 3 months of surgery. Five first trimester decidual samples were obtained at the time of elective pregnancy termination. Written informed consent was obtained from all participants. Approval was obtained from the Yale University School of Medicine Human Investigations Committee.

Plasmids and Small Interfering RNA

HOXA10 complementary DNA (cDNA) was cloned into the EcoRI site of pcDNA3.1(+) (Invitrogen, California). pcDNA3.1(+) without the *HOXA10* insert was used as a control (Invitrogen). SiGenome duplex *HOXA10* small interfering RNA (siRNA; cat# B-002000-UB-015) and control nontargeting siRNA (cat# D-001210-02) were purchased from Dharmacon, Lafayette, Colorado.

Cell Culture

The human endometrial stromal cell line (HESC), human endometrial adenocarcinoma cell line (Ishikawa), and primary human decidual cells were generous gifts of Charles J. Lockwood, Richard Hochberg, and Susan Richman, respectively (all of Yale University). Cells were maintained in a phenol red-free Dulbecco's Modified Eagle Medium (DMEM), Ham's F-12, and minimal essential medium (MEM), respectively (Sigma, St Louis, Missouri). Each was supplemented with 10% charcoal-stripped calf serum, 1% penicillin/streptomycin, and 1% sodium pyruvate.

Transient Transfection of Human Cell Lines

Human endometrial stromal cell lines, grown to 60% to 70% confluence, were transfected using TransIT-LT1 Mirus (Madison, Wisconsin) with either pcDNA3.1(+)/*HOXA10* (0.4 µg

for 6-well plates, 12 µg for 10-cm dish) or *HOXA10* siRNA (20 µmol/L for 6-well plates, 60 µmol/L for 10-cm dish), using empty pcDNA3.1(+) or nonspecific siRNA as respective controls. Ishikawa cells, grown to 45% to 55% confluence, were transfected using lipofectamine 2000 (Invitrogen) with either pcDNA3.1(+)/*HOXA10* (0.4 µg for 6-well plates, 12 µg for 10-cm dish) or *HOXA10* siRNA (20 µmol/L for 6-well plates, 60 µmol/L for 10-cm dish), using empty pcDNA3.1(+) or nonspecific siRNA as respective controls. Forty-eight hours posttransfection, total RNA was isolated. All transfection experiments were conducted in triplicate.

Real-Time Polymerase Chain Reaction

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the Lightcycle SYBR Green RT-PCR kit from Roche, Nutley, NJ. One microgram of total RNA was reverse transcribed in 20 µL of reaction mixture containing 10 mmol/L each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP); 20 pmol oligo (dT); 40 U/µL ribonuclease inhibitor, 10 U/µL avian myeloblastosis virus–reverse transcriptase (AMVRT), and 10× AMVRT buffer for 30 minutes at 61°C. Polymerase chain reaction amplifying *HOXA10* was performed for 45 cycles at 95°C for 2 seconds, 65°C for 5 seconds, and 72°C for 18 seconds. Polymerase chain reaction amplifying β-actin as a control was performed for 45 cycles at 95°C for 2 seconds, 61°C for 5 seconds, and 72°C for 18 seconds. Polymerase chain reaction amplifying ET_A was performed for 45 cycles at 95°C for 2 seconds, 65°C for 5 seconds, and 72°C for 18 seconds. The *HOXA10* and ET_A intron spanning primers were selected using the primer selection program Primer3 developed by the Whitehead Institute for Biomedical Research. The primers selected yield a 211 bp reaction product from messenger RNA (mRNA) and a 1389 bp DNA reaction product from DNA, which allowed control for possible DNA contamination.

HOXA10:

Sense: 5'-GCCCTTCCGAGAGCAGCAAAG-3'

Antisense: 5'-AGGTGGACGCTGCGGCTAATCTCTA-3'

ET_A:

Sense: 5'-TCCTCA ATC TCT GCG CTC TCA-3'

Antisense: 5'-ATC TCA ATG GCT GTG ACC AAT G-3'

The Roche Light Cycler monitored the increasing fluorescence of PCR products during amplification. A quantitative standard curve was then created. Quantification of samples was determined with the Roche Light Cycler and adjusted to the quantitative expression of β-actin from these same samples. 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. Three independent experiments were conducted at each concentration.

Immunohistochemistry

ET_A was evaluated by immunohistochemistry (IHC) using a rabbit polyclonal antibody to ET_A (Abcam, Inc., Cambridge, MA). The specimens were embedded in paraffin and serial 5-mm sections obtained. The sections were deparaffinized in xylene and ethanol. Endogenous peroxidase was blocked with 3% H₂O₂. After a 45-minute incubation with 1.5% normal goat blocking serum, the sections were incubated overnight at 4°C with primary antibody (anti-ET_A 1:300). The sections were then incubated with biotinylated goat anti-rabbit secondary antisera for 60 minutes, avidin and biotinylated peroxidase

(Vectastain; Vector Laboratories, Burlingame, California) for 45 minutes, and diaminobenzidine (400 mg/mL) for 45 seconds. Hematoxylin and Li_2CO_3 were used for counterstaining. One hundred consecutive cells in 5 nonadjacent microscopic fields were evaluated for staining to quantify the expression of each cell-type specific marker.

Statistics

Quantitative PCR results were compared using the *t* test.

RESULTS

ET_A Is Expressed in Human Endometrium and Decidua

In normal human endometrium, IHC results demonstrate that ET_A was expressed in endometrial stromal cells throughout the menstrual cycle (Figure 1A-C). ET_A was expressed at high levels within the cytoplasm of subset of stromal cells. No significant variation was observed in ET_A expression throughout the menstrual cycle in either glands or stroma.

ET_A Expression Persisted in the Decidua of Pregnancy

The first trimester decidua demonstrated high levels of ET_A expression (Figure 1D). ET_A was also more highly expressed in first trimester decidua compared to proliferative or secretory phase endometrium.

ET_A Expression Is Regulated by HOXA10

To determine whether ET_A was regulated by *HOXA10*, we performed transient transfection with a *HOXA10* expression construct or *HOXA10* siRNA. Successful transfection and increased/decreased *HOXA10* expression was confirmed using the real-time PCR (Figure 2). Real-time PCR demonstrated that pcDNA3.1/*HOXA10* transfection increased ET_A mRNA expression 9-fold compared to that obtained using empty pcDNA as a control in HESC ($P < .05$). ET_A mRNA expression was decreased 50-fold after *HOXA10* siRNA transfection compared to control siRNA transfection in HESC ($P < .05$). Transfection with either pcDNA3.1/*HOXA10* or *HOXA10* siRNA did not alter ET_A expression in Ishikawa cells, suggesting that ET_A was not regulated by *HOXA10* in glandular epithelium.

DISCUSSION

The endothelins are a family of proteins with vasoactive properties. Endothelin-1 was originally isolated from porcine aortic endothelial cells; subsequently, 2 other isoforms, ET-2 and ET-3, have been described.^{11,12} Endothelins are also produced from nonendothelial cell types, including cardiac myocytes, cells of the adrenal zona glomerulosa, follicular cells, and Sertoli cells. The endothelins in general, and in particular ET-1, also appear to play a role in mitogenesis of smooth muscle cells and fibroblasts.⁶ These actions are mediated by the binding of endothelins to 2 pharmacologically distinct G protein-coupled receptors, ET_A and ET_B. The ET_A receptor has high specificity for ET-1 (about 100 times that of ET-2 and -3 isoforms), while the ET_B receptor binds all 3 endothelins with equal affinity.¹³

Endothelin-1 has also emerged in recent years as an essential modulator of reproductive function, where it is involved in contraction of seminiferous tubes, inhibition of follicular cells, propagation of the luteolytic process, and endometrial receptivity.¹⁴⁻¹⁶ The action of ET-1 in the corpus luteum is linked to progesterone-induced ET_A expression; ET_A mRNA levels are upregulated in the luteal phase, allowing ET-1 to bind to ET_A receptors present on corpus luteal cells.¹⁷ ET_A subsequently regulates blood flow via its expression on smooth

muscle cells, where ET-1 binding elicits an increase in intracellular Ca^{2+} and inositol and subsequent vasoconstriction.^{18,19}

In the human uterus, ET-1 is expressed in vascular endothelium, stroma as well as glandular and luminal epithelium.²⁰⁻²³ The mRNA for both ET_A and ET_B receptor subtypes are expressed as well.⁶ Our results, demonstrating the presence of ET_A in human endometrium, corroborate these data. The ET_A receptor may regulate a number of endometrial functions including menstruation, mitogenesis, and the release of other paracrine agents.^{6,24} Here, we have shown that *HOXA10* regulates ET_A receptor expression in endometrium. In addition, *HOXA10* expression is greatest in decidua, a tissue that expresses more ET_A than proliferative or secretory phase endometrium, suggesting a regulatory role for *HOXA10* in decidual ET_A receptor expression in vivo.

While stromal/decidual expression of ET_A is regulated by *HOXA10*, expression in epithelial cells is not highly regulated, likely due to the limited role that these cells play in early pregnancy. Stromal/decidual cells normally proliferate in early pregnancy; *HOXA10*-deficient mice demonstrate diminished stromal and decidual cell proliferation. *HOXA10* action may be mediated, at least in part, through its regulation of ET_A in the decidua and the known effects of ET_A on cell proliferation.

HOX genes are transcription factors that regulate a vast number of downstream target genes; in particular, *HOXA10* regulates endometrial gene expression necessary for embryo implantation and viability.^{7,25-28} In the uterus, specific targets of *HOXA10* include genes that are important for decidualization such as insulin-like growth factor binding protein 1 (IGFBP-1), *EMX2*, prolactin, and the Prostaglandin E receptor subtype EP2 and subtype EP4.²⁹⁻³⁴ Here, we demonstrate that *HOXA10* also regulates ET_A , likely promoting endometrial receptivity and decidualization; ET_A activation specifically promotes proliferation as well as vascular adaptation of the endometrium to decidualization and pregnancy.

Activation of ET_A is known to induce prostaglandin production in decidua.^{35,36} *HOXA10* directly induces expression of prostaglandin receptors as well as prostaglandin production through ET_A . This leads to the establishment of a system that has the coordinated ability to both produce and respond to prostaglandins. ET_A receptor is in a position to stimulate prostaglandin production and simultaneously prostaglandin receptors are also expressed. Thus, the *HOXA10*-mediated developmental pathway establishes all of the conditions needed to respond to prostaglandins, however, limits this response to a biologically appropriate one that requires input from ET-1.

In conclusion, we report for the first time the regulation of expression of ET_A by *HOXA10* in endometrial cells. We also show the expression of this protein during the menstrual cycle and in first trimester decidua. ET_A likely functions via a molecular mechanism by which *HOXA10* promotes stromal cell activation in a manner similar to that seen in vascular smooth muscle cells.

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Figure 1. Immunohistochemistry (IHC) demonstrating expression of endothelin type A receptor (ET_A) in the female reproductive tract. A, Negative control. B, Proliferative endometrium. C, Secretory endometrium. D, First trimester decidua.

Figure 2.

Endothelin type A receptor (ET_A) and HOXA10 messenger RNA (mRNA) expression after transient transfection of human endometrial stromal cell line (HESC) with pcDNA/HOXA10 or pcDNA/control (top) and with small interfering RNA (siRNA)/HOXA10 and siRNA/control (bottom). Note difference in the scale of the Y axis. **P* < .05 compared to no control-treated cells.