Progestin-induced heart and neural crest derivatives expressed transcript 2 is associated with fibulin-1 expression in human endometrial stromal cells

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Objective: To investigate whether heart and neural crest derivatives expressed transcript 2 (HAND2) regulates fibulin-1 (FBLN1) expression during decidualization of human endometrial stromal cells (ESCs).
Design: In vitro experiment.
Setting: Research laboratory.
Patient(s): Twenty-four patients undergoing hysterectomy for benign reasons.
Intervention(s): ESCs were cultured with various progestins (medroxyprogesterone acetate [MPA], norethisterone, levonorgestrel, dienogest, and P), E_2, dexamethasone, and/or 8-bromoadenosine 3′, 5′-cyclic monophosphate (8-Br-cAMP). HAND2 and FBLN1 were silenced by small interfering RNA technology.
Main Outcome Measure(s): HAND2 and FBLN1 expression levels were assessed by real-time polymerase chain reaction and Western blot analysis.
Result(s): MPA or E_2 + MPA increased HAND2 mRNA levels in ESCs in a time- and dose-dependent manner, and this stimulatory effect was blocked by RU-486 (P receptor antagonist). HAND2 was increased by E_2 + MPA earlier than FBLN1. Simultaneous MPA and 8-Br-cAMP treatment synergistically enhanced HAND2 mRNA levels. P and all the progestins significantly increased HAND2 mRNA levels, whereas E_2, 8-Br-cAMP, or dexamethasone alone had no effect. Silencing of HAND2 expression significantly reduced FBLN1 expression, whereas FBLN1 silencing had no effect on HAND2 expression.
Conclusion(s): These results suggest that progestin-induced HAND2 contributes to FBLN1 expression in human ESCs. (Fertil Steril® 2013;99:248–55. ©2013 by American Society for Reproductive Medicine.)
Key Words: Endometrial stromal cells, decidualization, FBLN1, HAND2, progestin

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The human uterine endometrium undergoes cyclic proliferation and differentiation controlled by a sequential, carefully timed interplay of the ovarian steroid hormones, E_2 and P, during the menstrual cycle. P plays a central role in reproduction, mediating ovulation, embryo implantation, uterine growth, and maintenance of pregnancy (1). Problems with implantation and placental development are clinically important. In fact, a postovulatory P level inadequate to prepare this important biological event in the endometrium is associated with infertility and recurrent spontaneous abortion. Cellular responses are predominantly mediated by the progesterone receptor (PR), a member of the superfamily of ligand-inducible transcription factors. Decidualization is a process that occurs in the human

Received April 10, 2012; revised and accepted August 28, 2012; published online October 1, 2012.
H.C. has nothing to disclose. H.O. has nothing to disclose. T.T. has nothing to disclose. A.N. has nothing to disclose. K.Y. has nothing to disclose. H.K. has nothing to disclose.
This work was supported in part by Grants-in-Aid 23592425 and 23592426 for Scientific Research from the Ministry of Education, Science, and Culture, Japan.
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Fertility and Sterility® Vol. 99, No. 1, January 2013 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2012.08.056

endometrium in response to ovarian steroid hormones. Many studies have addressed the molecular mechanisms underlying decidualization in an in vitro human endometrial stromal cell (ESC) model because these cells express functional PR and estrogen receptor (ER) (2–6).

To investigate the molecular mechanisms underlying decidualization, we attempted to identify the genes induced by progestin in ESCs in a 3-day culture (7). Microarray analysis showed fibulin-1 (FBLN1) up-regulation after medroxyprogesterone acetate (MPA) treatment of human ESCs. FBLN1 is an extracellular matrix (ECM) scaffolding protein that binds to many ECM proteins, including fibronectin, laminin-1, fibrinogen, nidogen, and the proteoglycans aggrecan and versican (8). FBLN1 also plays an essential role in tissue remodeling, affecting cell adhesion, migration, proliferation, and differentiation (9). ECM remodeling is central to preparing the endometrium for receptivity and is known to be under hormonal influence (10–12). Indeed, FBLN1 transcript levels in the human endometrium are higher during the secretory phase than during the proliferative phase (13, 14). FBLN1 in human endometrial tissues is expressed in the glandular epithelium during the proliferative phase, and this expression is switched to the stroma during the secretory phase (14). These results show FBLN1 as an important molecule that mediates P action in human ESC differentiation toward implantation. However, the mechanism underlying induction of FBLN1 expression by MPA remains unclear.

Recent reports have demonstrated that heart and neural crest derivatives expressed transcript 2 (HAND2) plays a key role in uterine receptivity and is increased in the mouse uterus during decidualization (15, 16). HAND2 is a transcription factor required for the growth and development of the heart, branchial arches, and limb buds (17). In uterine tissue–specific HAND2 knockout mice, continued induction of paracrine mitogenic mediators in the stroma maintains epithelial proliferation and stimulates E-induced pathways, resulting in impaired implantation (15). These results suggest that HAND2 expression in the stroma is a critical regulator of the uterine stromal-epithelial communication that directs proper steroid regulation conducive to the establishment of pregnancy. Furthermore, HAND2 plays an important role in decidualization (16). However, the precise molecular and cellular mechanisms that regulate HAND2 expression in the human endometrium are not completely understood.

We hypothesized that HAND2 regulates FBLN1 expression during decidualization. We aimed to determine whether progestins and/or E_2 direct the effects of HAND2 mRNA expression in human primary cultured ESCs. We then silenced HAND2 or FBLN1 with small interfering RNA (siRNA) technology and measured these expression levels by real-time polymerase chain reaction (PCR) and Western blot analysis to investigate their functional roles in the action of female sex steroids on the endometrium.

MATERIALS AND METHODS

Tissue Collection and Culture of Cells

This study was approved by the Institutional Review Board of Kansai Medical University. All human tissues were obtained with informed consent from all patients and in accordance with the Declaration of Helsinki. Human endometrial tissues were obtained from 24 patients in the proliferative phase, aged 41–47 years, with regular menstrual cycles, who underwent hysterectomies for the treatment of myoma uteri without hormone therapy. ESCs were purified by the standard enzyme digestion method as described elsewhere (18). ESCs were cultured in DMEM/F-12 medium supplemented with 10% fetal calf serum (FCS) (HyClone), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37°C under a humidified atmosphere of 5% CO_2 in air. The culture medium was replaced 30 minutes after plating to reduce epithelial cell contamination. The percentage of vimentin-positive cells in confluent ESCs was more than 99% by immunohistochemical staining as described elsewhere (19).

Steroid Hormones Treatment and Reagents for ESCs

After passage 0–1 when ESCs were nearly confluent, cells were trypsinized and replated in six-well plates (1×10^6 cells/well) for real-time PCR analyses. To remove the effect of endogenous steroid hormones, cells were cultured until confluent and then the medium was replaced with phenol red-free DMEM/F-12 supplemented with 10% dextran-coated charcoal stripped (DCS)-FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). After 48 hours, ESCs were washed and cultured in DCS-FCS supplemented medium containing E_2 (10^{-8} mol/L; Wako Pure Chemical Co. Ltd.), P (10^{-7} mol/L), MPA (10^{-11}, 10^{-9}, and 10^{-7} mol/L), norethisterone (NET; 10^{-7} mol/L), levonorgestrel (LNG; 10^{-7} mol/L), RU-486 (PR antagonist; 10^{-6} mol/L), dexamethasone (DEX; 10^{-7} mol/L), 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; 0.5 mmol/L; Sigma-Aldrich Corp.), dienogest (DNG; 10^{-7} mol/L; Mochida Pharmaceutical Co.), and/or ethanol as vehicle control. The culture medium was changed every 3 days. Each experiment was repeated at least 3 times with different cell preparations.

RNA Extraction and Real-Time PCR Analysis

Total RNA was isolated from cultured ESCs using an RNeasy Minikit (Qiagen GmbH) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SYBR green I nucleic acid Gel Stain (Roche, Diagnostics GmbH) as described elsewhere (14). Elongation factor-1α (EF-1α) as an internal control is valid as reference "housekeeping" gene for transcription profiling, which is also used for real-time PCR experiments (20, 21). Real-time PCR efficiency (E%) for amplification of each gene was calculated using the following formula: $E\% = [-1 + 10^{(-1/\alpha)}] \times 100$, where α is the slope of the corresponding amplification plot (21). Forward (F) and reverse (R) primers used in this study were as follows: HAND2, 5′-AGAGGAAGAAGGAGCTGAACGA-3′ (F) and 5′-CGTCCGGCCTTTGGTTTT-3′ (R); EF-1α, 5′-TCTG GTTGGAATGGTGACAACATGC-3′ (F) and 5′-AGAGCTTCA CTCAAAGCTTCATGG-3′ (R); FBLN1, 5′-GGAGCAGTGCTGC CACAG-3′ (F) and 5′-AGCACCTCTTCACAAATGTG-3′ (R); PRL, 5′-ATTCGATAAACGGTATACCCATGGC-3′ (F) and 5′-TT GCTCCTCAATCTCTACAGCTTTG-3′ (R); 18S, 5′-CGGCTACCA