A novel protein Depp, which is induced by progesterone in human endometrial stromal cells activates Elk-1 transcription factor

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Decidualization of the endometrial stromal cells (ESC), considered to be stimulated by progesterone and/or cAMP, is crucial for embryo implantation and placentation. In this study, we isolated a novel clone encoding decidual protein induced by progesterone (Depp) from a human ESC cDNA library enriched with progesterone-inducible genes. Depp mRNA was expressed in various human tissues including placenta, ovary and kidney. Increased expression of Depp was observed in endometria during mid- and late-secretory phases and 1st trimester deciduas. In vitro, Depp mRNA was induced in ESC within 30 min of progesterone treatment, which was inhibited by the antiprogestin RU486. Androgen alone also induced Depp expression. Depp increased the level of phosphorylated Erk and activated the Elk-1 transcription factor in human embryonal kidney 293 cells, suggesting that Depp modulates the effects of progesterone during decidualization and in the decidua by affecting gene expression. Elucidation of the biological function of Depp in the endometrium will facilitate our understanding of the molecular mechanisms of decidualization and placental development.

Key words: decidualization/Elk-1/endometrium/MAPK/progesterone

Introduction

The human endometrium is a mucosal tissue comprised of epithelial, stromal and vascular components that undergo synchronous waves of proliferation and differentiation in response to ovarian estrogen and progesterone (Punyadeera et al., 2003). During the proliferative phase, the endometrium undergoes extensive estradiol-induced growth and remodelling. This is followed by secretory changes in response to postovulatory progesterone, and decidualization typically begins during the last week of the cycle. In the event of pregnancy, endometrial mass continues to increase, eventually forming the maternally derived component of the placenta (Noyes et al., 1975). Decidualized endometrial stromal cells (ESC) are phenotypically different from their precursors and produce a variety of proteins, including pro lactin, relaxin, insulin-like growth factor binding protein 1, desmin and components of basal lamina such as laminin and collagen IV (Gellersen and Brosens, 2003; Salamonsen et al., 2003). This remodelling process is critical for implantation of the developing embryo, formation of the placenta and maintenance of pregnancy, and inadequate postovulatory progesterone to support the endometrium has been associated with infertility and recurrent spontaneous abortion (Ginsburg, 1992).

Progesterone exerts its action on progesterone receptor (PR), a member of the steroid receptor superfamily of ligand-activated transcription factors (Kyriakis, 2000). Binding of ligand induces a conformational change in the receptor, resulting in phosphorylation, dissociation from heat shock proteins, dimerization, sumoylation of a subpopulation of the receptor and binding and activation of specific response elements in the promoter region of target genes (Gellersen and Brosens, 2003). cAMP is known to potentiate the PR activity, although the underlying mechanism is not entirely understood. cAMP content in human endometrium is higher during the secretory phase than the proliferative phase (Bergamini et al., 1985).

In an in vitro model of decidualization, human ESC are cultured in the presence of progesterone after estrogen-priming and/or cAMP, and they undergo morphological differentiation and produce decidual proteins such as prolactin (Punyadeera et al., 2003; Tierney et al., 2003). To identify molecules involved in decidualization, we have constructed a human ESC cDNA library enriched with progesterone-induced genes by cDNA subtraction. We have previously demonstrated that expression of TIMP-3 and transglutaminase are induced by progesterone (Higuchi et al., 1995; Fujimoto et al., 1996). In this study, we isolated a novel gene decidual protein induced by progesterone (Depp) from the cDNA library. Depp mRNA was induced in less than 30 min by progesterone in ESC, and activated Elk-1 transcription factor by, at least partly, increasing phosphorylation of MAPK/Erk.

Materials and methods

Patients and samples

Human endometrial tissues (n = 22) were obtained from patients who underwent hysterectomies for the treatment of uterine myoma. A portion of each endometrial specimen was examined histologically and dated as described (Fujimoto et al., 1996). First trimester deciduas (n = 4) were obtained from patients who had undergone legal elective abortions. The tissue specimens from which RNA was extracted were immediately frozen in liquid nitrogen and stored at −80°C. The project was approved by the Faculty of Medicine,
**Results**

**Isolation of Depp cDNA**

From the subtracted cDNA library enriched with progesterone-induced genes, we isolated one clone whose expression was markedly induced when ESC were cultured with progesterone (Figure 1A). Northern blot analysis of three more cases of human ESC confirmed that this gene is inducible by progesterone (Figure 1B). We named this gene Depp and determined the full cDNA sequence by using 5' and 3' rapid amplification of cDNA ends (RACE). The Depp cDNA was 2114-nucleotide long (GenBank accession number: AB022718) with a single open reading frame encoding 212 amino acids (Figure 1C). A search with the predicted Depp amino acid sequence against PROSITE PROFILE, but not PROSITE PATTERN, matches the dedicated software packages available online, PSORT-II (http://psort.nibb.ac.jp/), Predotar (http://www.inra.fr/predotar/french.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/). To search the motif libraries, PROSITE PATTERN and PROSITE PROFILE, for motifs in a protein query sequence, we used MOTIF: Searching Protein and Nucleic Acid Sequence Motifs (http://motif.genome.jp/) in GenomeNet (http://www.genome.jp/).

**Immunofluorescence staining**

HEK293 cells were transfected with pmKit-neo plasmids containing Depp cDNA fused in frame to HA cDNA. As a control, vector alone was transfected. HEK293 cells were replated on chamber slides after transfection, fixed with PBS containing 4% paraformaldehyde for 30 min, then rendered permeable with PBS containing 0.2% Triton X-100 for 30 min at room temperature. After blocking nonspecific antibody-binding sites with bovine serum albumin, the cells were incubated with rabbit polyclonal anti-HA antibody (BabCO, Richmond, CA, USA). Bound antibodies were detected with a goat anti-rabbit secondary antibody conjugated to FITC (Dako Japan, Kyoto, Japan). Chromosomal DNA was visualized by staining with blue-fluorescent 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Inc., Eugene, OR, USA).

**Analysis of effects on signal transduction pathways**

The effects of Depp on signal transduction pathways were analysed by using PathDetect In Vivo Signal Transduction Pathway Reporting Systems (Stratagene, La Jolla, CA, USA). To analyse the effect on Elk-1 pathway, plasmids pFA2-Elk-1 (50 ng), pFR-Luc (GAL4-luciferase reporter, 1 µg), pmKit-neo containing human Depp cDNA (1000 or 100 ng) or vector alone (900 or 1000 ng) and pRl-Tk that directs expression of Renilla luciferase (200 ng) were transfected onto HEK293 cells in a 60 mm dish, and luciferase activities were measured 24 h later using a Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan). The pFA2-Elk-1 expresses a chimerical Elk-1 protein fused to GAL4 DNA-binding domain that will be phosphorylated if the upstream signal transduction pathway is activated. For activator protein 1 (AP-1) and γ-activated sequence (GAS) pathways, cis-reporter plasmids pAP-1-Luc and pGAS-Luc (1 µg), respectively, were cotransfected with pmKit-neo expressing Depp or vector alone (1 or 2 µg) and pRl-Tk (200 ng) onto HEK293 cells, and analysed as above. The Mann–Whitney U-test was used for statistical evaluation of all the results. Differences of P < 0.05 were considered statistically significant.

**Cell culture**

Human 293 cells, a permanent epitheloid line of primary human embryonal kidney (HEK) transformed by adenovirus DNA (Graham et al., 1977), were obtained from ATCC (CRL-1573, Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Grand Island, NY, USA) at 37°C under an atmosphere of 5% CO2 in air. DNA transfection was performed using the calcium phosphate method as described (Sakurai et al., 2004). The human ESC were purified from the proliferative phase endometrium and cultured as described (Higuchi et al., 1995; Fujimoto et al., 1996). Initially, the purified fraction contained 95% stromal cells, but after culturing for more than 3 days, the ratio of stromal cells was more than 99%. To examine the effects of ovarian steroids on Depp expression, ESC were cultured in the presence of progesterone (10 nM, 100 nM or 1 µM) (Sigma, St. Louis, MO, USA), 17β-estradiol (10 nM) (Wako Pure Chemical Co. Ltd., Osaka, Japan), testoster (10 nM) (Sigma), progesterone (1 µM) plus RU486 (1 µM) and/or ethanol for 9 days. The culture media were changed every 2 days.

**Construction of a subtracted cdNA library and analysis of the genes**

Total RNA was extracted from ESC purified from one specimen and cultured for 12 days in the presence or absence of progesterone (1 µM). Polyadenylated [poly(A)+] RNA was prepared using a Messagemaker Reagent Assembly (Life Technologies, Rockville, MD, USA). After the synthesis of a double-stranded cDNA from each poly(A)+ RNA using a Time Saver cDNA Synthesis kit (Pharmac, Piscataway, NJ, USA), we constructed the subtracted cdNA library as described previously (Fujimoto et al., 1996). The cDNA sequence of each clone was determined using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

To predict the subcellular localization of the identified protein, we used the dedicated software packages available online, PSORT-II (http://psort.nibb.ac.jp/), Predotar (http://www.inra.fr/predotar/french.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/). To search the motif libraries, PROSITE PATTERN and PROSITE PROFILE, for motifs in a protein query sequence, we used MOTIF: Searching Protein and Nucleic Acid Sequence Motifs (http://motif.genome.jp/) in GenomeNet (http://www.genome.jp/).

**Analysis of gene expression**

Northern blot analysis was performed as described (Higuchi et al., 1995; Fujimoto et al., 1996). Total RNA was extracted from cultured human cells using Trizol Reagent (Life Technologies) according to the manufacturer’s protocol. RNA from various human tissues was a kind gift from Dr. Manabu Fukumoto. Briefly, either 10 or 15 µg per lane of total RNA were electrophoresed in a 1.0% agarose-formaldehyde gel and transferred to nylon membranes (Hybond-N+, Amersham Biosciences Corp., Arlington, IL, USA) and cross-linked by UV light. The membranes were incubated with a prehybridization solution (Rapid Hyb, Amersham Biosciences Corp.) for 30 min at 65°C and hybridized with the labelled probes in the same solution. The Depp cDNA corresponding to the coding sequence was amplified by PCR and radiolabelled with [α-32P]dCTP using the random primer technique. 18S ribosomal RNA cDNA was also radiolabelled with [α-32P]dCTP for normalization. After hybridization and stringent washing, autoradiography was performed using intensifying screens at −80°C for 48–72 h.

For RT–PCR, each RNA sample isolated from human tissues and cultured cells was treated with Deoxyribonuclease I Amplification Grade (Life Technologies) to remove contaminating DNA before amplification. First-strand cDNA was then synthesized in a volume of 20 µl containing 1 µg of total RNA, 0.5 µl of oligo (dT) 18 primer, 10 pmol of dNTP, 5 IU of ReverTra Ace (Toyobo, Japan) and first-strand synthesis buffer (Toyobo). Synthesis of cDNA was performed at 37°C for 10 min, 42°C for 1 h and 99°C for 5 min. PCR amplification was performed using 1 µl of first-strand cDNA as a template. As a negative control, RNA sample was incubated in first-strand synthesis buffer (Toyobo) without the enzyme and used for PCR amplification. Depp cDNA was amplified using the Depp cDNA-specific sense (5′-ATAGAGGTCCGGGTTCTGCTC-3′) and antisense (5′-GGTCCAGTCTCATGATCACCGG-3′) primers. As a control, β-actin cDNA was amplified using the β-actin cDNA-specific sense (5′-CCGCAAAAGACCTGTACGCCA-3′) and antisense (5′-TGAGCTTTGGGAGAGACTGG-3′) primers. PCR was performed by 30 cycles of incubation at 94°C for 1 min, 59°C for 1 min and 72°C for 2 min in the GeneAmp PCR system 2400 (Applied Biosystems). The PCR product was electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide and examination using a UV transilluminator.

Western blot analysis was performed as described (Sakurai et al., 2004). Mouse monoclonal antibodies used were anti-phospho p44/42 MAPK/Erk antibody (Cell Signaling Technology, Beverly, MA, USA), anti-phospho stress-activated protein kinase (SAPK) c-Jun N-terminal kinase (JNK) antibody (Boehringer Mannheim Biochemica, Mannheim, Germany), anti-GFP antibody (Sigma) and anti-β-actin antibody (Chemicon International, Temecula, CA, USA). Polyclonal antibodies used were rabbit anti-p44/42 MAPK/Erk antibody (Cell Signaling Technology), anti-phospho p38 antibody (Cell Signaling Technology), and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Dako, Kyoto, Japan). Band intensities were quantified by densiometric scanning of the film.
The nucleotide sequence demonstrated the presence of the t-SNARE coiled-coil homology domain motif, and predicted that Depp is a nuclear protein with reliability of 94.1% using the Reinhardt’s method for cytoplasmic/nuclear discrimination.

The BLAST search of mouse expressed sequence tags (ESTs) identified an IMAGE clone 1924729 as a possible homologue of human Depp. The nucleotide sequencing indicated that it encodes a 205-amino acid protein 58.5% identical to human Depp. Asterisks indicate residues common to both sequences. t-SNARE coiled-coil homology domain motifs are boxed. Second peroxisomal targeting signals are underlined.

Expression of Depp in various tissues

Northern blot analysis revealed that Depp transcripts of 2.3 kb in length were expressed in various tissues including the placenta, ovary and kidney (Figure 2A). A faint 3.0 kb band was also observed in several tissues. RT–PCR analysis confirmed that Depp is expressed in human placenta and kidney (Figure 2B). The HEK293 cell line derived from human embryonic kidney also expressed Depp.

To determine the localization of Depp within a cell, Depp was fused to hemagglutinin (HA)-tagged Depp. Twenty hours after transfection, the cells were fixed and stained with a rabbit polyclonal antibody directed against human Depp. Detection of HA-Depp was performed using a secondary antibody conjugated to a fluorescein isothiocyanate (FITC) analogue. Immunostaining revealed that Depp was present in the nucleus and cytoplasm, mainly in the nucleus (Figure 2C).

Induction of Depp mRNA by progesterone

The induction of Depp mRNA by progesterone in ESC was dose-dependent and was observed at 10 nM progesterone (Figure 3A). To determine the specificity of the effects, we cultured ESC for 9 days in the presence of progesterone alone or in combination with RU486, estrogen or testosterone. As shown in Figure 3A, the effect of progesterone was inhibited by the presence of RU486. Androgen alone induced Depp mRNA expression. Estrogen slightly induced Depp mRNA expression and did not affect the induction by progesterone.

The induction of Depp mRNA was induced in ESC in less than 30 min, reaching a high level at 2–4 h and then the level declined till 3 days. At 6 days of incubation with progesterone, the level was again increased and continued to be high during the observation period (Figure 3B and C). Essentially similar biphasic pattern of Depp mRNA induction by progesterone was observed in other samples as well (data not shown).

We next analysed the Depp mRNA levels in the uterus endometria in the proliferative and secretory phases. Progesterone acts on the estradiol-primed endometrium to induce decidualization after the mid-secretory phase (Punyadeera et al., 2003). As shown in Figure 4, the Depp mRNA levels varied in the endometria of secretory phase, but tended to be high after the mid-secretory phase. The level was higher in the endometria during the secretory phase than the proliferative phase. The level was high in all first trimester decidua specimens examined.

Effects of Depp on signal transduction pathways

The MAPK pathway regulates many transcription factors, including c-fos, jun-B and Elk-1 (Buchwalter et al., 2004). We analysed the effect of Depp on the signal transduction pathways using HEK293
cells. Although HEK293 cells were derived from the human embryonal kidney and not from ESC, it expresses Depp under physiological conditions (Figure 2B). As shown in Figure 5A, Depp induced luciferase activity in HEK293 cells transfected with activator plasmids for Elk-1 reporting system, indicating that Depp directly or indirectly increased the amount of phosphorylated Elk-1. Depp did not activate signal transduction pathways leading to AP-1 or GAS (Figure 5B, data not shown). Because Elk-1 is a direct target of activated p38, SAPK/JNK and mitogen-activated protein kinase (MAPK)/Erk (Buchwalter et al., 2004), we analysed the effects of Depp on phosphorylation of these MAPks. As shown in Figure 5C, the level of phosphorylated Erk was increased in HEK293 cells overexpressing Depp. Depp did not affect the level of phosphorylated SAPK/JNK or phosphorylated p38. These results suggest that Depp increases phosphorylation of Erk, leading to activation of Elk-1.

Discussion

By subtractive cDNA hybridization, we have demonstrated that the expression of a novel gene Depp is induced by progesterone in human ESC. After deposition of the Depp cDNA sequence to GenBank, a cDNA sequence encoding a 213-amino acid protein similar to Depp (3 amino acid difference) was reported as fasting induced gene (FIG, GenBank accession number: AB025244.1), and both Depp and FIG are included in the UniGene Cluster Hs.93675 as Chromosome 10 open reading frame 10 (C10orf10). The deduced amino acid sequence of mouse Depp
reported herein is identical to that of RIKEN full-length enriched library, clone: 8430408G22 (GenBank accession number: AK078806).

Northern blot analysis revealed that Depp is expressed in many human tissues, especially in placenta, pancreas and ovary, being consistent with the expression profile suggested by EST counts (UniGene Cluster Hs.936757). As predicted from the sequence analysis with PSORT-II, the transduced Depp protein localized mainly in the nucleus in HEK293 cells. Recently, a single Pro-Pro-Pro-Ser-Asp (PPPSP) motif has been shown to activate the Wnt pathway, and Wnt signalling stimulates phosphorylation of the PPPSP motif, which creates an inducible docking site for axin (Tamai et al., 2004). Although Depp has a PPPSP motif, we could not communoprecipitate Depp with axin in HEK293 cells (T. Masuda and J. Fujita, unpublished data). Whether Depp affects Wnt signalling remains to be determined.

Many proteins are known to be under the control of progesterone. Popovici et al. (2000) have screened 588 genes in human ESC stimulated with progesterone or cAMP and observed marked up-regulation of cytokines, growth factors and nuclear transcription factors. Kao et al. (2002) have further analysed 12,686 genes in human ESC during the window of implantation (peak estrogen and progesterone levels) and found 156 significantly up-regulated genes. Previously, we have found the induction of transglutaminase and TIMP-3 mRNA in human ESC after 6 h and 6 days, respectively, of culture in the presence of progesterone (Higuchi et al., 1995; Fujimoto et al., 1996). In this study, induction of Depp mRNA was observed in less than 30 min after incubation with 100 nM progesterone. The induction was biphasic, namely, the expression level increased until 2–4 h, decreased until 3 days, then increased again and remained high at 6–12 days after incubation with progesterone. Expression of prolactin and morphological transformation of ESC occurs after 6–9 days of culture with progesterone under the present conditions (Higuchi et al., 1995). In vivo, the expression of Depp in the cycling human endometrium was higher during the mid- and late-secretory phase than the proliferative phase (Figure 4). The Depp mRNA expression varied among the secretory phase samples, but consistently high in the first trimester decidua. Whether this observation reflects the biphasic induction of Depp mRNA by progesterone observed in vitro and is physiologically relevant are presently unknown.

The induction of Depp by progesterone was inhibited by the antiprogestin RU486. Progesterone exerts its activities mainly through binding to its cognate receptor (Gellerson and Brosens, 2003; Punyadeera et al., 2003). Although progesterone can activate both PR and glucocorticoid receptor (GR) at high concentrations, and RU486 can inhibit both GR and PR, the finding that Depp was induced by 10 nM progesterone suggests that the induction is PR mediated. Recently, Depp has been identified by a microarray as one of the genes up-regulated during cAMP-induced decidualization of ESC (GES accession number GDS286, Edgar et al., 2002), which is consistent with a notion that Depp is involved in decidualization. Androgen receptor (AR) and PR are members of the nuclear receptor superfamily, and act as hormone-inducible transcription factors that bind specific DNA elements as homodimers (Kyriakis, 2000; Khorsazianadeh and Rastinejad, 2001). The DNA elements that bind AR and PR share the common sequence 5′-AGAACANNNTGTTCT-3′, known as the consensus progesterone/androgen response element (PRE/ARE). As both AR and PR recognize the same sequence, it is likely that the same genes are regulated when either AR or PR are present in a given cell. Therefore, the fact that Depp mRNA was induced by androgen as well as progesterone raises the possibility that the PRE/ARE is present in the Depp gene. Although no consensus PRE/ARE is present in the 2 kb sequence 5′-to the putative Depp transcription initiation site (data not shown), further study is necessary because even 5′-GGGACAAAACCTTTTCT-3′ has been reported to function as the PRE/ARE (Matsui et al., 2002).

The transcription factor Elk-1 is a component of the ternary complex that binds the serum response element found in the c-fos and other immediate early gene promoters (Buchwalter et al., 2004). Elk-1 is a target of activated mitogen-activated protein (MAP) kinases, Erk1/2, SAP/JNK and p38. Erk cascade responds to growth factors and mitogens, whereas the JNK and p38 cascades are triggered by cytokines and stress. In this study, Depp increased the level of phosphorylated Erk, which phosphorylates and activates Elk-1 in HEK293 cells. The relevance of this finding made in non-ESC to the process of decidualization is not warranted, but it may explain the finding that progesterone modestly stimulates Erk group MAP kinases (Weigel and Zhang, 1998). Erk is also known to phosphorylate PR on Ser294 to mediate ligand-dependent PR degradation (Kyriakis, 2000). Paradoxically, this down-regulation is coupled to transcriptional hyperactivity of PR in human breast cancer cells (Shen et al., 2001). Whether Depp affects the stability and activity of PR in ESC remains to be determined.

In this study, we have demonstrated that progesterone immediately induces Depp mRNA in human ESC in vitro and that Depp affects the signalling pathway leading to activation of Elk-1 in HEK293 cells. Further investigation is required to determine whether Depp mediates the effects of progesterone during decidualization by regulating gene expression and what roles Depp plays in the first trimester decidua. The knowledge will facilitate our understanding of the molecular mechanisms of decidualization and placental development.

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