Differentiation of endometrial stromal cells in vitro: down-regulation of suppression of the cell cycle inhibitor p57 by HOXA10?

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Decidualization is a critical step during embryo implantation that is characterized by the differentiation of endometrial stromal cells (ESC) into decidua cells. However, the mechanism of differentiation remains largely unknown. Previously, it has been shown that the null function of homeo box A10 (HOXA10) causes defects in both implantation and decidualization, suggesting that the HOXA10 signalling pathway is likely to be involved in uterine decidualization. In the present study, we determined the expression and subcellular distribution of HOXA10 and its downstream molecule, p57, in ESC during in vitro decidualization induced by a combination of 8-bromo-cAMP and medroxyprogesterone acetate. We demonstrated that the HOXA10 was down-regulated while in contrast, p57 was up-regulated in the process of decidualization. Immunocytochemistry and transient expression of the HOXA10 tagged with green fluorescence protein revealed that there were no differences in the HOXA10 subcellular localization between the induced and non-induced ESC. Our results suggest that the down-regulation of HOXA10 may contribute to increased p57 and that up-regulation of p57 likely plays an important role in ESC differentiation in the process of decidualization. The progesterone receptor pathway may participate in promoting ESC to exit the cell cycle and enter differentiation.

Key words: embryonic implantation/decidua/homeo box A10 protein/human/p57KIP2

Introduction

The initiation of pregnancy represents a successful combination of two independent processes: embryo development and uterine differentiation. The latter involves secretory transformation of the glandular epithelium followed by decidualization of the stromal compartment in the late-luteal phase of the cycle. Several lines of evidence indicate that decidualization is essential for coordinated trophoblast invasion and placenta formation (Christian et al., 2002a). In vitro, decidualization can be induced by progesterone and estradiol (E2), or by cyclic AMP (cAMP) and the other ligands that activate protein kinase A dependent process (Gellersen et al., 2003). In vivo, decidual transformation of some stromal cells occurs around day 23 of the menstrual cycle (Noyes et al., 1975) and continues throughout pregnancy. In addition, the percentage of stromal cells that express prolactin (PRL) in the decidua increases progressively from 9.8% in early pregnancy to 57.8% at full term (Wewer et al., 1985; Wu et al., 1993), but the molecular mechanism underlying the process of decidualization remains poorly understood.

It has been documented that the expression of progesterone receptor A (PR-A), the dominant form in differentiated endometrial stromal cells (ESC), is decreased greatly during the course of the decidualization response (Wang et al., 1994; Koshiyama et al., 1995; Brosens et al., 1999). The decline in PR levels was of functional relevance. Transient expression of either PR-B or PR-A suppresses PRL promoter activity in response to cAMP. Furthermore, the expression of endogenous PRL in response to cAMP plus medroxyprogesterone acetate (MPA) was down-regulated by constitutive expression of PR. So the decrease in cellular PR levels likely determines the onset of decidualization (Brosens et al., 1999).

Homeo box A10 (HOXA10) is expressed in the endometrial glands and stroma of the human uterus through the menstrual cycle and increases dramatically in the mid-luteal phase. The expression of HOXA10 also persists in the decidua of pregnancy. Sex steroid–sex steroid receptor complexes probably bind directly to the cis-elements of HOXA10, regulating its expression (Ma et al., 1998; Taylor et al., 1998). HOXA10 seems to be an essential regulator in endometrial receptivity and decidualization (Bagot et al., 2000; Daftary and Taylor, 2000). At the time of implantation, HOXA10 mediates the progesterone stimulating proliferation of uterine stromal cells. On the other hand, HOXA10 mutations caused stromal cell proliferation defects that were accompanied by quantitative alterations in the expression of one cyclin-dependant kinases inhibitors (CDKIS) gene, p57 (Yao et al., 2003). Since, HOXA10 is one of the downstream genes of the PR, which is down-regulated during the process of decidualization, and p57 is HOXA10 downstream signalling molecule, we characterized the expression of HOXA10 and p57 in the process of ESC differentiation in vitro to determine, whether the PR pathway participates in promoting ESC to exit the cell cycle and go into differentiation.
Materials and methods

Primary ESC culture

The ESC from normal proliferative endometrial tissues were isolated from normal cycling women by endometrial biopsy at the time of diagnostic laparoscopy because of fallopian tube obstruction. Histological examination of the endometrium was normal. This study was approved by Tongji Hospital Research and Ethics Committee, and patient consents were obtained before biopsy. The tissues were collected in Earle’s buffered saline containing 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen Corporation, Grand Island, NY, USA), washed twice in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Invitrogen Corporation, Grand Island, NY, USA), minced and enzymatically digested with 0.1% collagenase (Sigma Chemical Co., St Louis, MO, USA) for 40 min at 37°C. After centrifugation at 400 g for 5 min, the pellet was resuspended in the maintenance medium, a mixture of DMEM/F12 and 10% Fetal bovine serum (FBS) (Invitrogen Corporation, Grand Island, NY, USA). ESC were separated from epithelial cells and cultured as described previously (Ryan et al., 1994).

Decidualization by MPA and 8-bromo-cAMP in vitro

Confluent ESC were cultured in phenol red-free DMEM/F12 (Invitrogen Corporation, Grand Island, NY, USA) without serum for 24 h. To induce decidualization, the media was changed to phenol red-free DMEM/F12 containing 2% dextran-coated charcoal-treated FBS (DCC-FBS) (Hyclone Company Logan, UT, USA) with 0.5 mM 8-bromo-cAMP (8-Br-cAMP) and 10−6 M MPA (Sigma Chemical Co., St Louis, MO, USA) for 1, 2 and 4 days, respectively (D1, D2 and D4). The non-induced cells (control) were maintained in phenol red-free DMEM/F12 with 2% DCC-FBS for 1 and 4 days, respectively (C1 and C4). All experiments were carried out at the third cell passage.

PRL assay

The culture medium was centrifuged at 800 g for 10 min. The PRL was determined using a chemiluminescence assay (Bayer Corporation). The sensitivity of the assay was 0.3 ng/ml.

RNA isolation and RT–PCR

Total RNA was extracted using the Total RNA isolation reagent (TRI reagent, Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer’s instructions. Four micrograms of total RNA from each sample was denatured at 70°C for 5 min and chilled rapidly on ice. The RNA was then reverse transcribed in a 30 μl reaction mixture containing 6 μl 5× RT buffer, 1 μl 10 mM dNTP, 1 μl 0.5 μg/ml Oligo (dT)15, 0.5 μl 50 μM ribonuclease inhibitor, 1 μl 200 U/ml Moloney murine leukemia virus transcriptase (Promega Corporation, Madison, WI, USA), reaction condition: 37°C for 60 min; 95°C for 5 min in a Biometra T Gradient Thermocycler. After RT, 2 μl of cDNA was amplified in 50 μl PCR mixture containing 10 μl PCR buffer 5×, 1 μl of each primer pair 1 μM of primer, 5 μl Taq polymerase 0.5 μU (Promega Corporation, Madison, WI, USA) and 10 pmol/μl of each primer pair 1 μM. The primers were specific for HOXA10 (5′-GCCCTTCAGGACGCAAAG-3′, 5′-AGGTCGACGCTGCGCTAACTCTA-3′), for p57 (5′-CGTGATCCGTTTCTGGCTGG-3′, 5′-TCTGTTGGCTCATTATTG-3′), and for G3PDH (5′-GGTGCGGTACAAGGTCGCTCG-3′, 5′-TCTCCGACGCGCTTCCACAC-3′). The PCR reaction was performed as follows: 94°C for 30 s, at different annealing temperatures for 1 min and 72°C for 1 min 20 s, followed by incubation at 72°C for 10 min. Annealing temperatures for HOXA10, p57 and Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were 60°C, 56°C and 57°C, respectively. The number of cycles were 26, 30 and 25, respectively. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining under UV light.

Western blot analysis

Cells in 50 ml flasks were washed twice with phosphate-buffered saline (PBS) and lysed on ice in a lysis buffer (50 mmol/l Tris–HCl (pH8.0), 150 mmol/l NaCl, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, 1% detergent NP-40, 0.02% sodium azide and freshly added protein inhibitors 10 μg/ml phenylmethylsulphonyl fluoride and 1 μg/ml aprotenin). Solid cellular debris was removed by centrifugation at 1200 g for 5 min. Protein concentration was measured by Coomassie Brilliant Blue G-250 assay. Protein samples (45 μg for each) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDSPAGE) (12%) and were transferred onto nitrocellulose membranes using a Bio-Rad electroblot apparatus. Non-specific binding sites were blocked in 5% non-fat dry milk in 0.05% PBS-Tween. The following primary antibodies were used: anti-human HOXA10 polyclonal antibody (Santa Cruz, CA, USA), (1:150, 4°C overnight) and anti-human p57 monoclonal antibody (NeoMarkers Corporation, Westhingham Drive, Fremont, CA, USA; 1:100, at room temperature for 2 h). Secondary antibodies: rabbit peroxidase-conjugated anti-goat IgG, or goat peroxidase-conjugated anti-mouse IgG (Zhongshan Biotechnology Co., Ltd, Beijing, PRC; 1:2000, at 37°C for 1.5 h). Anti-actin antibody (Zhongshan Biotechnology Co., Ltd, Beijing, PRC) was used as a loading control (1:500). Protein bands were visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, PO, USA).

Indirect immunofluorescence

The isolated ESC were seeded and grown on coverslips to confluence and then treated as described in the materials. The cells were fixed in acetone at −20°C for 10 min, permeabilized by incubation for 5 min in 0.5% Triton X-100/PBS at room temperature and then blocked by 10% Rabbit serum/PBS for 30 min at room temperature. The slides were incubated with HOXA10 antibody (1:100) overnight at 4°C in a moist chamber. After three washes in PBS, bound antibodies were detected using second FITC-conjugated antibodies (Zhongshan Biotechnology Co., Ltd, Beijing, PRC) at 1:100 dilution for 30 min at 37°C. Staining was visualized on a Nikon fluorescent microscope to see the intensity and subcellular location of fluorescence of HOXA10.

Plasmid construction and transient transfection

The HOXA10 cDNA (a generous gift from C.Largest; University of California VA Medical Center, San Francisco, CA, USA) was inserted into the EcoRI site of vector pEGFPC1. The correct clone was confirmed by digestion with NheI and sequencing. ESC grew to 95% confluence in 24-well plates and were transfected with HOXA10-GFP. One microtitre of lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) mixed with 0.8 μg of plasmid DNA. Four hours after transfection, the cells were induced by 8-br-cAMP plus MPA for 4 days as described previously. The cells maintained in 2% DCC-FBS were used as control. The subcellular location of HOXA10 was visualized under the fluorescent microscope.

Statistical analysis

Each experiment was performed at least in triplicate and repeated at least three times on different specimens. The results were expressed as the mean ± SD. Two way analysis of variance was performed on the cells treated with 8-br-cAMP plus MPA for 1, 2 and 4 days (D1, D2 and D4, respectively), followed by SNK post hoc test. The difference between the treated and control cells (C1 and D1, C4 and D4) were tested by paired t-test. P < 0.05 was considered significant.

Results

Morphological appearance and PRL secretion during differentiation of ESC

The non-induced cells retained a fibroblast-like appearance. However, in the presence of 0.5 mM 8-br-cAMP plus 10−6 M MPA for 4 days, the cells were transformed into large polygonal cells with enlarged nuclei and increased amounts of cytoplasm, resembling decidua cells in vivo. As the cells proceeded to differentiate, their borders became less distinct because of the extracellular matrix formation (Figure 1). The PRL levels were low after 2 days of treatment, but increased dramatically thereafter. The untreated cells secreted very low levels of PRL. These results indicate that the treated ESC were successfully decidualized in vitro (Figure 2).
HOXA10 expression during differentiation of ESC

The HOXA10 mRNA level progressively declined after the treatment of ESC with phenol red-free DMEM/F12 supplemented with 2% DCC-FBS (control) or treated with 8-br-cAMP plus MPA as described in Materials and methods.

Subcellular localization and immunoreactive intensity of HOXA10 during differentiation of ESC

Transient transfection showed that green fluorescence protein (GFP) was localized in both the cytoplasm and the nucleus (Figure 4 Panel A). GFP-tagged HOXA10 was mainly localized in the nucleus (Figure 4 Panel B). No differences in the pattern of HOXA10-GFP location were observed between the non-induced and MPA/8-br-cAMP induced cells. By immunocytochemistry, we detected endogenous HOXA10 expression in the nucleus of both induced and control cells. The intensity of fluorescence was more prominent in the non-induced cells (Figure 4 Panel C) than induced cells (Figure 4 Panel D). The immunocytochemical data were consistent with the results of RT–PCR and Western blot analysis.

p57 expression during differentiation of ESC

p57 mRNA was barely detectable in treated ESC for 1 day (D1) under our RT–PCR conditions. Then it increased progressively. p57 mRNA expression was not statistically different between the induced and control cells on the first day (D1 and C1). Then it became markedly up-regulated and reached statistical difference compared to the control (C4) after 4 days of exposure (D4). Similar to mRNA, p57 protein was also up-regulated in the process of decidualization. Therefore, increased expression of p57 may be a character of ESC decidualization (Figure 5).

Discussion

Proper development of an organism requires an integration of cell-cycle exit and differentiation pathways (Zhang et al., 1998). The proliferation of cells and their progression through the cell cycle are regulated by the sequential activity of various cyclin-dependent kinases (CDKS; Reed et al., 1994; Chellappan et al., 1998; Pavletich, 1999; Massague et al., 2000). The enzyme activity of CDKS depends on physical interaction with one of the cyclin proteins. The well-known regulators of mammalian cell proliferation are the three D-type cyclins (D1, D2 and D3). The D-type cyclins accumulate during the G1 phase and their association with CDK4 and CDK6 is particularly important to form holoenzymes that facilitate cells to enter the S-phase. CDK activity can be negatively regulated by a group of proteins termed as CDKIS. CDKIS levels, similar to cyclin levels, vary during the cell cycle, thus contributing to the timing of cyclin–CDK activation (Arellano and Moreno, 1997). The CDKIS are divided into two families, the INK4 and CIP/KIP. The CIP/KIP family includes three structurally related members, p21, p27 and p57 (Lee et al., 1995, 1997; Luo et al., 1995; Matsuoka et al., 1995). Over-expression of these inhibitors attenuates the cell proliferation response.

The uterus provides a unique and dynamic physiological model in which cellular proliferation, differentiation and apoptosis occur during pregnancy. Existing data suggest that cell-cycle regulatory molecules play potential roles in the uterus during steroid hormonal stimulation (Geum et al., 1997; Prall et al., 1997), reproductive cycle (Shiozawa et al., 1998) and trophoblast differentiation (Bamberger et al., 1999). However, little is known regarding cell-cycle molecules that participate in ESC proliferation and differentiation.

The CDK4 and CyclinD3 are expressed in the endometrium at the site of the embryo following the onset of implantation on day 5 of pregnancy in mice (Tan et al., 2002). Then they become down-regulated in the primary decidua zone (PDZ) at the implantation site.
Figure 3. Expression of HOXA10 mRNA and protein during decidualization of ESC. Total RNA (A) and protein (B) samples were prepared from human ESC after culture for 1, 2 and 4 day(s) (D1, D2, D4, respectively) in the presence of MPA plus 8-br-cAMP (D1, D2, D4, respectively). Non-induced cells maintained in 2% DCC-FBS were also harvested at 1 day and 4 days (C1, C4, respectively). The level of mRNA for HOXA10 was calculated as a ratio of the densitometric readings for HOXA10 to the corresponding G3PDH readings. The level of protein for HOXA10 was calculated as a ratio of the densitometric reading for HOXA10 to the corresponding β-actin. The results are presented in the bar diagram. The results shown above are representative of three separate experiments.

Figure 4. Subcellular localization and immunoreactive intensity of HOXA10 during differentiation of ESC; (A) fluorescence microscopy of ESC transiently transfected with GFP showed cytoplasm and nucleus location; (B) ESC transiently transfected with HOXA10-GFP showed mainly nucleus location; (C) the immunoreactive intensity and subcellular localization of endogenous HOXA10 in non-induced ESC; (D) endogenous HOXA10 in ESC induced with 8-br-cAMP plus MPA.
in the afternoon of day 5 with concomitant expression of p21, which supports the notion that cell proliferation activity of CDK4/CyclinD3 ceases with the development of the PDZ (Tan et al., 2002). Furthermore, the expression of CDK4 and CyclinD3 in decidualizing stroma outside the PDZ in the afternoon of day 5 is again consistent with their role in proliferation of the stromal at the secondary decidua zone (Tan et al., 2002). Data shown above demonstrate that CyclinD3/CDK4 promotes ESC proliferation and that low expression of CyclinD3/CDK4 and persistent expression of p21 facilitate ESC exit from the cell cycle. Indeed, it has been shown that the expression of p21 during development strongly correlates with terminally differentiated cells (Parker et al., 1995).

However, implantation and embryo development are apparently normal in p21 null mice (Deng et al., 1995). It is possible that other functionally similar inhibitory molecules may compensate for p21 deficiency. Our investigation suggested that p57 might be such a molecule. In the undifferentiated state, ESC expresses low levels of p57. However, p57 is up-regulated progressively in the process of differentiation. In vivo, p57 was strongly expressed in decidual cells, cytotrophoblasts, intermediate trophoblasts and villous stromal cells, which confirmed our observation (Fukunaga, 2002). However, they did not compare differentiated ESC with undifferentiated ESC. p57 has been shown to be a potential inhibitor of several G1 cyclin/CDK complexes. Its over-expression leads to cell-cycle arrest in vitro.

Figure 5. Expression of p57 mRNA and protein during decidualization of ESC. Total RNA (A) and protein (B) samples were prepared from ESC after culture for 1, 2 and 4 day(s) (D1, D2, D4, respectively) in the presence of MPA plus 8-br-cAMP. Non-induced cells maintained in 2% DCC-FBS were also harvested at 1 day and 4 days (C1, C4, respectively). The level of mRNA for p57 was calculated as a ratio of densitometric readings for p57 compared to the corresponding G3PDH. The level of protein for p57 was calculated as a ratio of densitometric reading for p57 to the corresponding β-actin readings. The results are presented in the bar diagram. The results shown are representative of three separate experiments. P > 0.05, P < 0.05.

Figure 6. A proposed model for ESC exiting the cell cycle and going into differentiation.
in G1 phase (Matsuoka et al., 1995). Human p57 protein, like p21, contains a proliferation control cell nuclear antigen-binding domain within its C-terminus that can prevent DNA replication (Watanabe et al., 1998). The 310 helix region of p57, but not of p21 or p27, was indispensable for the inhibition of cyclinA/CDK2 and cyclinE/CDK2 complex (Hashimoto et al., 1998). p57 is considered a critical terminal effector of signal transduction pathways that controls cell differentiation (Lee et al., 1995; Matsuoka et al., 1995; Zhang et al., 1998). Indeed, using multiple mutant mice, it was reported that CDKI, p57 and p21 function mutually exclusively to control cell-cycle exit and differentiation of lens fibre cells, placental trophoblasts and myoblasts (Zhang et al., 1998; Zhang et al., 1999).

However, why does p57 become up-regulated during decidualization? cAMP can inhibit proliferation of normal fibroblasts, smooth muscle cells, lymphoid cells, neuronal cells and glial cells (Hollenberg and Cuatrecasas, 1973; Nilsson and Olsson, 1984; Blomhoff et al., 1988; Mark and Storm, 1997; Dugan et al., 1999). In some cells cAMP blocks the mitogenic effects of growth stimulatory factors by up-regulating p27 expression and preventing CDK4 activation (Kato et al., 1994; L’Allemaen et al., 1997; Fukumoto et al., 1999). Recently, cAMP has been reported to inhibit proliferation of orotic vascular smooth muscle cells by inducing p53 and p21 expression (Hayashi et al., 2000). However, so far whether cAMP can induce p21 expression in ESC needs further investigation, and there is no report that cAMP can induce p57 expression in vitro.

On the other hand, full decidual transformation requires down-regulation of PR-A and PR-B, activation of protein kinase A and activation of other potential decidua specific transcription factors (Christian et al., 2002a, b). In general, the PR-A isoform is transcriptionally less active than PR-B (Christian et al., 2002a, b). However, down-regulation of the PR-B isoform leads PR-A to become the dominant form during decidualization (Brosens et al., 1999). The critical role of PR-A is demonstrated by the lack of a decidual response in PR-A deficient mice (Mulac-Jericevic et al., 2000). However, PR-A isoform is also down-regulated during the course of decidualization (Wang et al., 1998; Brosens et al., 1999). Maintenance or elevated PR-A or PR-B inhibits decidualization (Brosens et al., 1999). Decidualization in vivo also coincides with a dramatic reduction in the expression of classical PR-dependent genes. For instance, PR regulates the expression of neutral endopeptidase gene by directly binding to the multiple consensus progesterone response elements in the 5′-flanking region. However, neutral endopeptidase expression decreases significantly in those cells undergoing decidualization in the late secretory phase of the cycle (Head et al., 1993). Our investigation proved that HOXA10, another PR directly regulated molecule, was also down-regulated during differentiation. The other experiment suggested that over-expression of the HOXA10 increased IGFBP-1 promoter activity in ESC, but not in decidual cells. HOXA10 suppressed the PR enhanced activities (Gao et al., 2002). The experiment showed indirectly that HOXA10 should be down-regulated during ESC differentiation in accordance with our results.

Hox genes are considered to act as local regulators of cell proliferation during development (Duboule, 1995). At the time of implantation, HOXA10 mediates progesterone stimulated proliferation of uterine stromal cells. The HOXA10-deficient female mouse presented a stromal cell proliferative defect and p57 transcripts were increased by 6.6 ± 1.8-fold (Yao et al., 2003). It still remains unclear whether HOXA10 regulates p57 expression directly, but the cis-acting elements, TTAT, of HOXA10 do exist in the core promoter of p57 (−864 to −867 region of p57 gene, Gene Bank Accession No. D64137). These data imply that the up-regulation of p57 during ESC differentiation is probably attributed to the suppression of HOXA10.

Current knowledge on the distinct or overlapping roles of various cell-cycle molecules during the onset of stromal cell decidualization is still limited. Based on our finding, a model is proposed to describe how ESC exit the cell cycle and enter differentiation (Figure 6). This model needs a number of additional observations. During ESC differentiation, the factors such as LH, CRH, RLX, PGE2, etc. activate the second messenger cAMP, leading to an up-regulation of p21. On the other hand, PR and HOXA10 are down-regulated during decidualization, which further enhances the over-expression of p57. p21 and p57 suppress the activity of the CDK4/CyclinD3 complex and other CDK/cyclin complexes and then ESC exit the cell cycle. Thereafter, by the coordinated interaction of the PR pathway and cAMP pathway, the ESC starts to express decidua-specific genes, leading to ESC differentiation (Gellerson and Brosens, 2003).

In conclusion, our experiment demonstrated that HOXA10 was down-regulated while p57 was up-regulated in the process of decidualization. These suggest that the PR pathway may participate in promoting ESC to exit the cell cycle and enter differentiation.

Acknowledgements

We thank Dr Yungpin Lu, Dr Fuxian He and Dr Ling Xi for their help in obtaining endometrial tissue and laboratory techniques. We especially thank Dr Xiaoli Tian for reviewing the manuscript.

References


