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Inhibiting Uterine PC6 Blocks Embryo Implantation: An Obligatory Role for a Proprotein Convertase in Fertility[1]

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ABSTRACT

Successful embryo implantation involves complex interactions between the embryo and the uterus and is critical in establishing pregnancy. Proprotein convertase (PC) 6 (PC6) is one of the PC endoproteases regulating protein function through posttranslational activation of precursor proteins, including growth and differentiation factors. Here we show that PC6 protein is induced in the uterine stromal cells specifically at the site of embryo attachment during early pregnancy in mice. In vivo blocking of uterine production of PC6 protein using morpholino antisense oligonucleotides in mice resulted in total inhibition of implantation, revealing a vital role for PC6 in modulating the uterus for embryo implantation. Studies in primates (rhesus monkey and human) showed a dramatic upregulation of endometrial PC6 during the phase of uterine receptivity and at implantation, particularly during a critical uterine cell differentiation process termed decidualization. Thus, the current studies have demonstrated that PC6 is an essential molecule in modulating uterine function to support the establishment of embryo implantation. Interestingly, PC6 is one of the PCs identified to be important in processing the coat protein of HIV; inhibition of PCs has been suggested to be an effective approach to reduce HIV transmission. We therefore propose the novel concept that PC6 could be a potential nonhormonal target in the female reproductive tract for dual protection for women, both in preventing pregnancy and reducing HIV infection.

decidua, female reproductive tract, implantation, pregnancy, uterus

INTRODUCTION

Implantation of the embryo into the uterus is critical for establishing a pregnancy. Failure of implantation accounts for 75% of human pregnancy loss occurring before 20 wk of gestation and is a major limiting factor in assisted reproduction [1]. Implantation is a complex and highly coordinated process involving active interactions between the embryo and the uterus. To facilitate implantation, the uterus must undergo considerable morphological and physiological changes, one of which is the decidual reaction involving dramatic proliferation and differentiation of endometrial stromal cells [2–4]. In the mouse endometrium, decidualization begins at early implantation in the subluminal stroma on the antimesometrial side immediately surrounding the mucosal crypt where the embryo attaches, resulting in the formation of the primary decidual zone [2]. With the progression of implantation, decidualization extends to the rest of the stroma, resulting in a broad, secondary decidual zone completely encapsulating the developing embryo with large, polyhedral, and closely packed decidual cells [2]. In nonhuman primates, as in the mouse, significant decidualization is initiated only at implantation [5]. However, in women, decidualization occurs spontaneously during the late secretory phase of the menstrual cycle in perivascular stromal fibroblasts, in anticipation of potential embryo implantation, and progresses to the rest of the stroma with the establishment of pregnancy [3, 4]. Decidualization is critical for implantation and maintaining pregnancy, as decidual cells are proposed to supply nutrition to the growing embryo until the formation of a functional placenta, to shield the mother from the invasive nature of the trophoblast, to protect the embryo from the maternal immune system, and to provide paracrine factors necessary for modulating trophoblast in implantation and placentation [6, 7]. The importance of decidualization has been unequivocally illustrated in a number of genetically modified mice in which decidualization defects lead to implantation failure [4, 8–12].

Proprotein convertase 6 (PC6), also known as PC5 or PC5/6, is one of the proprotein convertases (PCs) that are structurally related to bacterial subtilisins and yeast kexin [13, 14]. Two PC6 isoforms, one soluble (more widely distributed) and one membrane bound (mainly restricted to the intestine), generated by alternative splicing, have been identified in mice [15, 16]. The two isoforms differ only at the C-termini, with the membrane-bound form containing a large Cys-rich region and a transmembrane domain. In the human, only the soluble form has been clearly characterized [17]. The soluble PC6 protein shares 95% identify between the mouse and human, indicating that this protein is well conserved. PCs posttranslationally process growth factors, peptide hormones, proteolytic enzymes, and other inactive precursor proteins by cleaving the polypeptides at basic residues within the general motif of (K/R)-(X)n-(K/R)↓ [14]. This converts the inactive precursor proteins into their biologically active forms and thus regulates their temporal and spatial activation. This converting process also plays critical roles in the fusion and infectivity of HIV virus [18]. The viral envelope glycoproteins are initially synthesized as precursor peptides that are incapable of the required conformational changes for membrane fusion with the targeting

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cell; they must be processed by host-cell enzymes, such as the PCs, for maturation, as the virus itself lacks these enzymes [18]. PC6 is one of the PCs identified to be important for this processing [17, 19]. Inhibition of PCs has been suggested to be an effective approach to reduce HIV infection [18, 20].

We have previously shown that PC6 mRNA is dramatically upregulated in the mouse uterus specifically at the site of embryo attachment [21]. PC6 mRNA was localized predominantly in the stroma at the antimesometrial pole undergoing decidualization [21], implying that PC6 is involved in decidualization. Our preliminary studies indicated that PC6 is also specifically upregulated in decidual cells at the expected time of implantation in the human, suggesting that PC6 exerts similar functions during decidualization in the mouse and human and that mice would be useful to investigate PC6's function in implantation. One approach would be to specifically eliminate PC6 from the uterus. However, an ideal uterine-specific promoter has yet to be characterized, hindering a conditional knockout approach for PC6 in the uterus. We have taken an alternative approach using morpholino antisense oligonucleotides (MO). These have overcome many limitations of regular antisense DNA oligonucleotides and have been successfully applied in several model organisms (e.g., zebrafish) as well as in the mouse to inhibit the translation of specific proteins [22–24].

Here we report that PC6 is critical for embryo implantation. Inhibition of its uterine production during early pregnancy in vivo completely blocked stromal cell decidualization and prevented embryo implantation in mice. PC6 expression is also closely associated with decidualization in human and rhesus monkey endometrium at around the expected time of implantation, as well as in an in vitro human decidualizing cell model. Taken together, these results suggest that inhibition of endometrial PC6 production and/or action in the human would prevent the maternal decidual response and hence block implantation and pregnancy. Given the role of PC6 in HIV infection, we propose the novel concept that this protein could be used as a target in developing woman-centered dual contraceptives to protect women against unwanted pregnancies and HIV infection.

MATERIALS AND METHODS

Anti-PC6 Antibody Production and Western Blot Analysis

An anti-PC6 antibody was generated in sheep against a peptide DYDLSHAQSTY, corresponding to residues 117–127 of mouse PC6 (accession no. BAA02143) and representing a large part of a peptide previously used for antibody production [16]. Specific IgG was affinity purified using HiTrap N-hydroxysuccinimide-activated High Performance (HP) affinity columns (Amersham Pharmacia, Piscataway, NJ). Total uterine proteins (15 μg/lane) extracted as previously described [25] were analyzed by Western blot using the above PC6 antibody (25 μg/ml) and a horseradish peroxidase-conjugated donkey-anti-sheep IgG (1:20 l000; Silenus Laboratories, Hawthorn, Australia).

Mouse Studies

Swiss out-bred mice were housed and handled according to the Monash University animal ethics guidelines on the care and use of laboratory animals. All studies were approved by the Animal Ethics Committee at the Monash Medical Centre, Melbourne, Australia. Normal pregnant mouse tissues were obtained as previously described [26]. The morning of finding a vaginal plug was designated as Day 0 of pregnancy. The following MO sequences were used: (i) anti-PC6 MO: 5′-GCGGTTCCCCCAGTCCCAGTCCATG-3,′ targeted in the ATG start codon region of mouse PC6 mRNA (accession no. D12619) to block synthesis of PC6 protein; (ii) an irrelevant control MO: 5′-CCTCTTACCTCAGTTACAATTTATA-3′ supplied as a standard control MO from Gene Tools LLC (Philomath, OR); (iii) MO against mouse calcium binding protein d9k: 5′-TGCAGGAGACTTCTCAGCACATT-3′ and (iv) anti-HtrA3 MO: 5′-CCTGCATCGCGGAGAGGCGGCGGC-3′. All MOs were synthesized by Gene Tools LLC and supplied combined with partially complementary DNA molecules as carriers for delivery into cells. For each treatment, 10 μl MO stock solution (2 mM), 10 μl H_2O, and 10 μl weak-base delivery reagent ethoxylated polyethylenimine (Gene Tools) were mixed, vortexed for 30 sec, and incubated for 20 min at room temperature before use. The dose was determined according to previous studies [23, 27].

To administer MOs in the uterine horn, the mice were anesthetized as previously described [26] and an incision (<1 cm) was made into the lower abdomen at the apex of the triangle arch to locate the uterus. The MO solution was slowly injected into the uterine lumen through a 26s-gauge high-performance liquid chromatography needle inserted just below the utero-tubal junction, and the needle was held in place for a further 30 sec to prevent reflux. The incisions were then closed using surgical clips.

To examine the uterine penetrance of the MOs and cross-contamination between the two horns, fluorescein isothiocyanate (FITC)-labeled control MO (FITC-MO) was injected into one horn and unlabelled MO into the contralateral horn of a nonpregnant (NP) mouse. The mice were killed 48 h later, uteri dissected and frozen in Optimal Cutting Temperature compound (Tissue Tek, Elkhart, IN) and 5-μm cross-sections analyzed under a fluorescence microscope at 488 nm.

To inhibit PC6 protein production, anti-PC6 MO was injected into one horn and control MOs into the contralateral horn on pregnancy Day 3.5 or 4.5 (n = 4 for each day). Additional controls were provided by further experiments in which two alternative molecules both upregulated during early pregnancy (calbindin d9k [n = 4] and HtrA3 [n = 5]) were targeted. In some cases, one of the horns was not touched to confirm that the procedure itself does not affect implantation. After 48 h of treatment, the uteri were examined for implantation sites and corresponding ovaries for corpora lutea. The uteri were then fixed in 10% buffered formalin (pH 7.6) for immunohistochemistry. The data were analyzed using Student *t*-test; $P < 0.05$ was considered statistically significant.

Primate Uterine Tissue Collection and Processing

Rhesus monkeys (*Macaca mulatta*) were obtained as described [25]. Tissues examined included uterus at 1 day before (ov-1, n = 3), 5 days after (ov+5; n = 3), 10 days after (ov+10; n = 3), and 15 days after ovulation (ov+15; n = 2) across the menstrual cycle and implantation sites from Day 16–35 pregnant monkeys (n = 5). All experimental work was approved by the Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences.

Human endometrial tissues during menstrual (Days 1–4, n = 4), proliferative (Days 5–14, n = 6), early secretory (Days 16–19, n = 5), midsecretory (Days 20–24, n = 8), and late secretory (Days 25–28, n = 5) phases of the menstrual cycle were obtained as described [25]. Early pregnant (6–8 wk) decidua and placental tissues (n = 5) were collected from women undergoing elective surgery. Approval was by the Human Ethics Committee at Monash Medical Centre, Melbourne, and the women gave informed consent.

Immunohistochemistry

Five-micron sections were subjected to immunohistochemistry as previously described [25] with the following modifications: (i) the nonspecific binding was blocked with a blocking buffer containing high-salt TBS (0.3M NaCl in 50 mM Tris, pH 7.6), 0.1% Tween, 12% rabbit serum, 2% human serum, and 6% fetal calf serum. The primary antibody (sheep anti-PC6 antibody [4 μg/ml] or preimmune sheep IgG for negative control) was incubated in the blocking buffer at 4°lC for 24 h and washed with high-salt TBS plus 0.6% Tween. The secondary antibody was a biotinylated rabbit anti-sheep IgG (1:200 dilution; Vector Laboratories, Burlingame, CA). The relative expression level among different phases of the menstrual cycle both in monkey and human endometrium was assessed by the intensity of the staining; zero represented no stain and 3 (human) and 4 (monkey) represented maximum staining. Results were presented as mean ± SEM. The data were analyzed using Student *t*-test, $P < 0.05$ was considered statistically significant.

In Vitro Decidualization

Fibroblast cells were isolated from human term decidual tissues [28], cultured to confluence [29], and passaged twice. The cells were decidualized with cAMP [30] for 11 days and then either lysed for reverse tran-

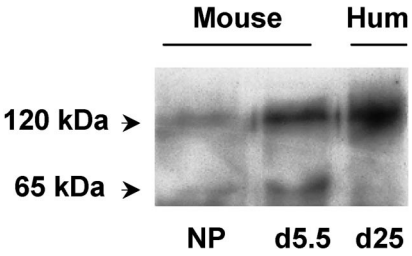

FIG. 1. Western blot analysis of PC6 protein in a NP uterus and a Day 5.5 implantation site of mouse, and a human endometrium (Menstrual Cycle Day 25; Hum).

scription-polymerase chain reaction (RT-PCR) or Western blot analysis. For RT-PCR analysis, total RNA was isolated using an Rneasy Minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed as previously described [21], except 300 ng total RNA was reverse transcribed in a 20-μl reaction, 4 μl and 1 μl of which were used for subsequent PCR amplification of PC6 and 18S, respectively. Thirty and 24 cycles were used to amplify PC6 and 18S, respectively. Primers for PC6 analysis (expected product of 545 base pair [bp]) were 5′-GATCGGTGCACTGAAGGACTA-3′ and 5′-CCAGCATTCGCACTCCTC-3′. Primers for 18S amplification (expected product of 187 bp) were 5-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′. The PCR products were analyzed by electrophoresis, the band intensities determined by Gel Doc 2000 system (Bio-Rad, Hercules, CA), and the level of PC6 expression normalized against that of 18S.

RESULTS

Endometrial PC6 Is Induced in Decidual Cells at Embryo Implantation in Mice

We first examined whether PC6 protein is induced at implantation as is its mRNA. We produced an affinity-purified sheep polyclonal antibody against a synthetic peptide common to both isoforms of mouse PC6. Western blot using this antibody detected bands around 120 kDa, representing pro (126 kDa) and mature (116 kDa) forms of soluble PC6 in mouse uterus and human endometrium (Fig. 1), as previously published in other systems [31]. A faint band at 65 kDa representing a C-terminally truncated form was also seen in the mouse as reported [31] (Fig. 1). This data confirmed the specificity of the antibody and indicated that only the soluble PC6 protein is expressed in the uterus. A much higher level was detected in mouse implantation sites on Day 5.5 of pregnancy (vaginal plug = Day 0) compared with the NP state (Fig. 1), indicating that uterine PC6 protein is induced at implantation. This antibody also recognizes human PC6, as corresponding bands around 120 kDa were similarly detected in a human endometrial sample (Menstrual Cycle Day 25) (Fig. 1).

We then determined the spatial and temporal expression of PC6 protein in the mouse uterus during early pregnancy, especially at implantation. Immunoreactive PC6 was below detection in NP uteri (Fig. 2A); whereas at the expected time of embryo contact with the uterus on Day 4.5 of pregnancy, PC6-positive signals were detected specifically in the subluminal stromal cells adjacent to the implanting embryo at the antimesometrial pole, which form the primary decidual zone (Fig. 2, B and C). The luminal epithelium in direct contact with the embryo and the embryo, including the trophoblast, remained negative (Fig. 2, C and D). The expression was detected predominantly in the primary decidual cells immediately surrounding the embryo during the time of active implantation on Day 5.5 of pregnancy (Fig. 2E). The identity of the decidual cells was confirmed by

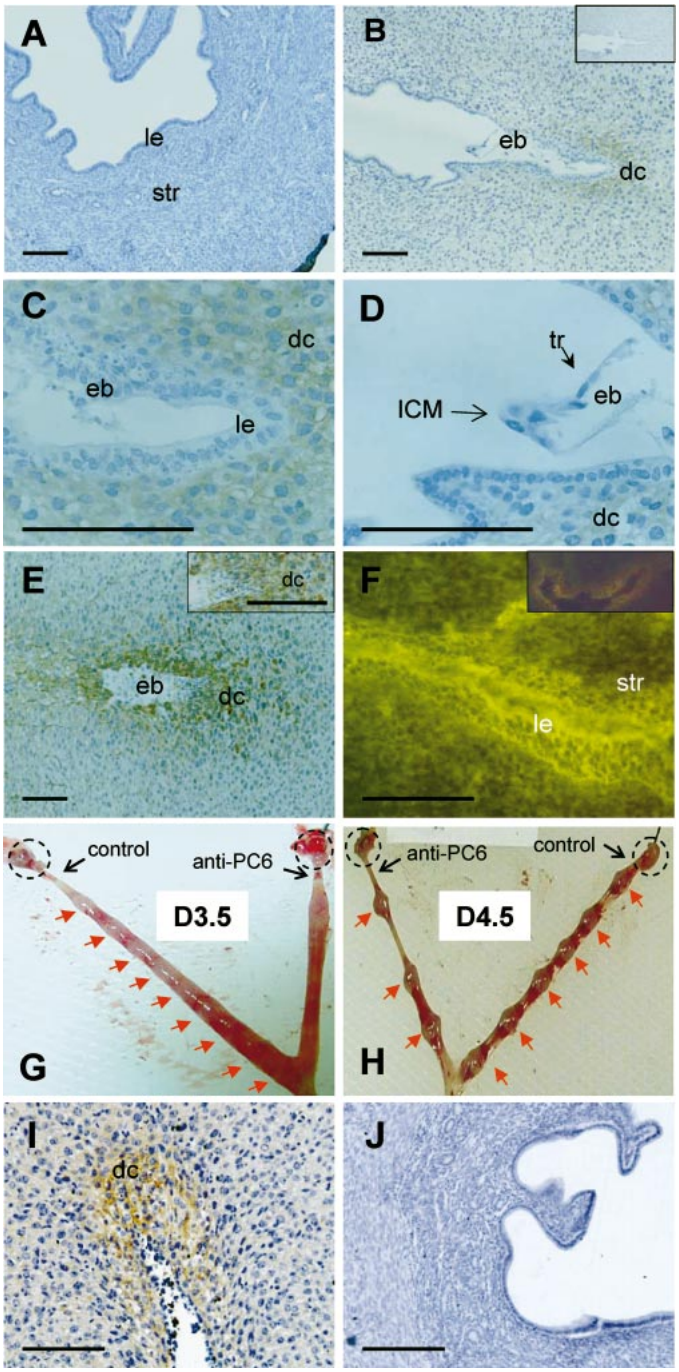

FIG. 2. Immunolocalization of PC6 and effects of anti-PC6 MO on implantation. (**A**–**E**) Representative photomicrographs showing temporal and spatial immunoreactive PC6 expression in mouse uterus. **A**) Nonpregnant, (**B,**) Day 4.5 implantation site, insert showing a serial section using preimmune IgG as the primary antibody for negative control, (**C** and **D**) higher power of (**B**) showing positive staining in primary decidual cells (**C**), and negative staining in the embryo and uterine epithelium (**D**). (E) Day 5.5 implantation site, insert showing a higher power image. **F**) Fluorescence micrographs of uteri following intrauterine administration of FITC-MO or unlabelled control MO (insert). **G**–**H**) Examples of uteri after intrauterine administration on Day 3.5 (**G**) or 4.5 (**H**) with anti-PC6 or control MO (treatment for each horn is specified). Red arrows highlight the implantation sites, black circles mark the ovaries. **I**, **J**) Photomicrographs showing immunoreactive PC6 in the uterus after treatment with control MO (**I**) (decidualization and PC6 protein clearly detected) or anti-PC6 MO (**J**) (no decidualization or PC6 protein detected) on Day 3.5. dc, decidual cells; eb, embryo; ICM, inner cell mass; le, luminal epithelium; str, stroma; tr, trophoblast. Scale bars = 400 μm.

TABLE 1. Effects of anti-PC6 MO on embryo implantation in mice.*

Day of injection		Implantation sites	Corpora Lutea
Day 3.5	Control	4.5 ± 1.6	5.0 ± 1.5
	Anti-PC6	0.0 ± 0.0†	4.6 ± 0.9
Day 4.5	Control	5.6 ± 1.5	5.6 ± 1.5
	Anti-PC6	4.0 ± 1.5	4.0 ± 1.5

* Numbers of implantation sites and corpora lutea in mice when treated by intrauterine injection of either control or anti-PC6 MO on Day 2.5 or Day 3.5 and examination 48 h later (n = 4 for each day). Data are expressed as mean ± SEM and analyzed using Student *t*-test.
† $P < 0.05$ compared with control on same day.

positive staining for desmin (data not shown). These results demonstrate that the induction of PC6 protein in decidualizing endometrial stromal cells is associated with the very early development of the primary decidua in the mouse at the time of embryo implantation.

Blocking Uterine PC6 Induction In Vivo Leads to Implantation Failure in Mice

To determine the importance of uterine PC6 in embryo implantation, we administered antisense MO into the mouse uterine lumen during early pregnancy to inhibit the induction of PC6 protein. We first examined whether MO could reach stromal cells expressing PC6 by injecting FITC-labeled irrelevant control MO into one horn and unlabelled MO into the contralateral horn of a NP mouse. Cross-sections of the uteri were examined after 48 h and strong green fluorescence representing cellular uptake of FITC-MOs was detected in the luminal epithelium and, to a lesser extent, in the underlying stroma (Fig. 2F). This demonstrated that MO could reach the target cells in vivo. Only low autofluorescence was observed in the contralateral horn (Fig. 2F, insert), indicating that MO transfer between the two horns was minimal.

We then injected specific anti-PC6 MO, designed to target the translation initiation site of PC6 mRNA sequence and thus to inhibit the synthesis of PC6 protein, into the uterine lumen on Day 3.5 (before the detection of any PC6 protein induction) or Day 4.5 (induction of PC6 protein already detectable) of pregnancy. The uteri were examined for the number of implantation sites 48 h later. Administering anti-PC6 MO on Day 3.5, 12–18 h before the expected induction of PC6 protein, completely inhibited implantation (Fig. 2G, Table 1); whereas injection on Day 4.5, when PC6 protein expression is already initiated in the primary decidual zone, had no significant effect on the number of implantation sites (Fig. 2H, Table 1). The control MO, when injected at similar concentration to that used for anti-PC6 MO, did not significantly affect the number of implantation sites when injected on either day (Fig. 2, G and H; Table 1), except minor alterations in embryo spacing were occasionally observed with injection on d3.5. The mean number of corpora lutea found on the two ovaries in each mouse was not significantly different (Table 1), indicating that normal ovulation occurred in all cases.

The specificity of anti-PC6 MO resulting in total inhibition of implantation when administered on Day 3.5 was further confirmed by treating the uterine horns with two additional unrelated MOs in the same manner as for anti-PC6 MO. First, as reported previously [23], no effect on the number of implantation sites was observed when we injected MO against calcium binding protein 9k, a molecule upregulated in uterine luminal epithelium at the time of implantation, on Day 3.5 of pregnancy. Second, when we injected MO against HtrA3, another molecule upregulated during early pregnancy in the mouse uterus [32], no significant impact on implantation was achieved (data not shown).

We next determined the efficacy of anti-PC6 MO in inhibiting in vivo PC6 production. The horns treated on Day 3.5 with control MO showed normal immunoreactive PC6 protein in the primary decidual cells (Fig. 2I), whereas the horns treated with anti-PC6 MO showed neither PC6 protein nor decidual cells (Fig. 2J), demonstrating that anti-PC6 MO specifically inhibited PC6 protein production and blocked decidualization. No significant difference in uterine gross morphology or PC6 expression was observed between the two horns treated on Day 4.5 (data not shown). This indicates that treatment on Day 4.5 did not affect PC6 production, possibly because the MOs administered at this time could not reach the deep stroma to affect the further expression that had already been initiated in the subluminal region. Taken together, these data demonstrate that induction of PC6 in the uterus during early pregnancy is necessary for stromal decidualization, especially the formation of primary decidua, and is obligatory for embryo implantation in the mouse.

PC6 Expression Is Associated with Implantation in Primates, Including the Human

To determine whether uterine PC6 is associated with decidualization and embryo implantation in primates, we initially performed RT-PCR analysis of a few samples and confirmed the expression of PC6 mRNA in rhesus monkey and human endometrium (data not shown). We then examined the precise spatial and temporal expression pattern of PC6 mRNA and protein in the endometrium across the menstrual cycle and early pregnancy in these two primate species. In all cases, a similar pattern was obtained for mRNA and protein and only immunostaining of the PC6 protein is shown.

For the rhesus monkey, we examined the expression in the uterus 1 day before ovulation (ov-1) and 5 days (ov+5), 10 days (ov+10), and 15 days (ov+15) after ovulation, and implantation sites between Days 16–35 of pregnancy. During the menstrual cycle, PC6 was detected primarily in the glandular epithelium with no expression in the stroma (Fig. 3, A–C), but the glandular expression was significantly upregulated at ov+10 (Fig. 3D). This time coincides with the anticipated time of implantation in a conception cycle, termed window of implantation, indicating that PC6 is associated with uterine preparation for implantation. In pregnant uteri, expression was also detected in the glands, but the most intense staining was now detected in the stromal decidual cells at the implantation sites (Fig. 3, E and F), with the adjacent trophoblast shell and the cell column being negative (Fig. 3E). No expression was detected in other trophoblast cells except a low level in syncytial trophoblast (Fig. 3, E and G). Cell identities were confirmed with positive staining for desmin in decidual cells and cytokeratin in trophoblasts (data not shown).

In the human endometrium, PC6 expression was detected in the glandular epithelium in all phases of the menstrual cycle (Fig. 4, A–C), but strong staining was detected in the stroma only in the late secretory phase in the decidual cells, as decidualization occurs spontaneously at this time (Fig. 4, C and D). Glandular expression exhibited a significant increase ($P < 0.05$) from the early to midsecretory phases

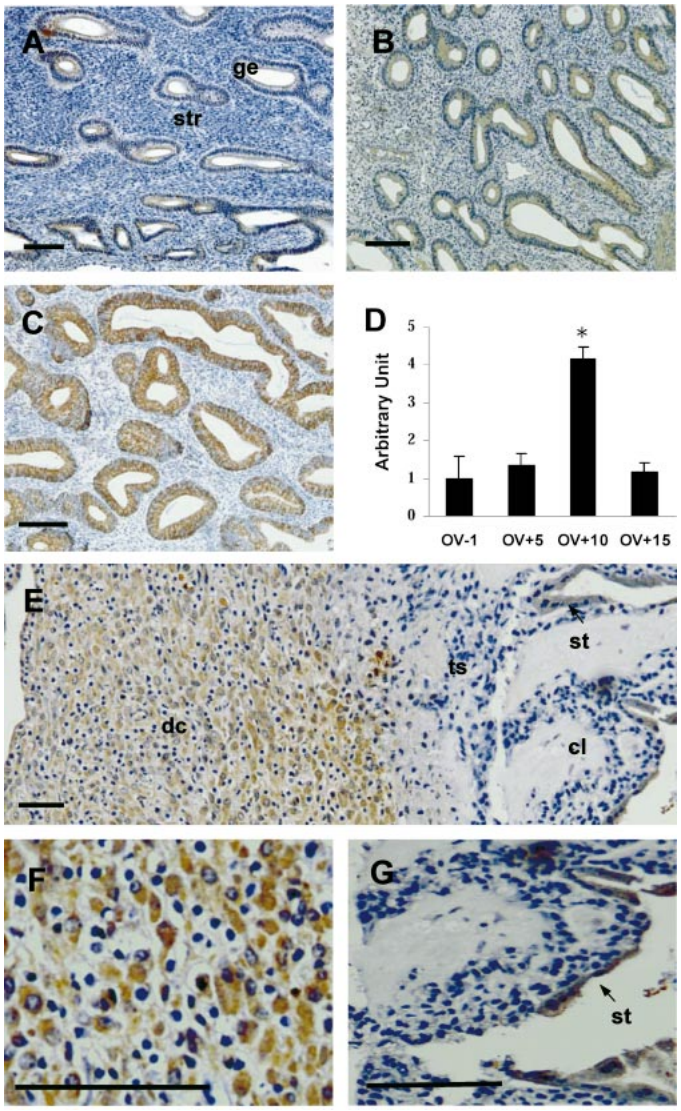

FIG. 3. PC6 protein in rhesus monkey endometrium throughout the menstrual cycle and during early pregnancy. (**A**–**C**, **E**–**G**) Micrographs showing immunostaining during the cycle (**A**–**C**) and at implantation sites during early pregnancy (**E**–**G**). **A**) ov-1, (**B**) ov+5, (**C**) ov+10. **D**) Mean density of glandular staining during the menstrual cycle (means ± SEM, * $P < 0.05$ for ov+10 compared with other stages). **E**) Implantation sites, (**F**) maternal decidua, and (**G**) trophoblast cells at the implantation sites. cl, trophoblast cell column; st, syncytial trophoblast; ts, trophoblast shell. Other labels as in Figure 2. Scale bars = 400 μm.

(Fig. 5A), indicating that, as in the monkey, glandular PC6 may play a role in uterine preparation for receptivity. Stromal expression was maximal in the late secretory phase in the decidual cells (Fig. 5B), indicating that PC6 is associated with stromal cell decidualization in the human during the menstrual cycle.

The endometrial expression of PC6 during the first trimester of pregnancy was essentially similar to that seen in the monkey. PC6 was detected in the glands (data not shown), but most intense staining was again detected in the uterine decidual cells (Fig. 4E). Expression was also observed in the syncytial trophoblast, but the level was relatively low (Fig. 4F). These results suggest that endometrial PC6 expression is associated with embryo implantation, particularly with stromal cell decidualization at implantation, in both rhesus monkey and human endometrium.

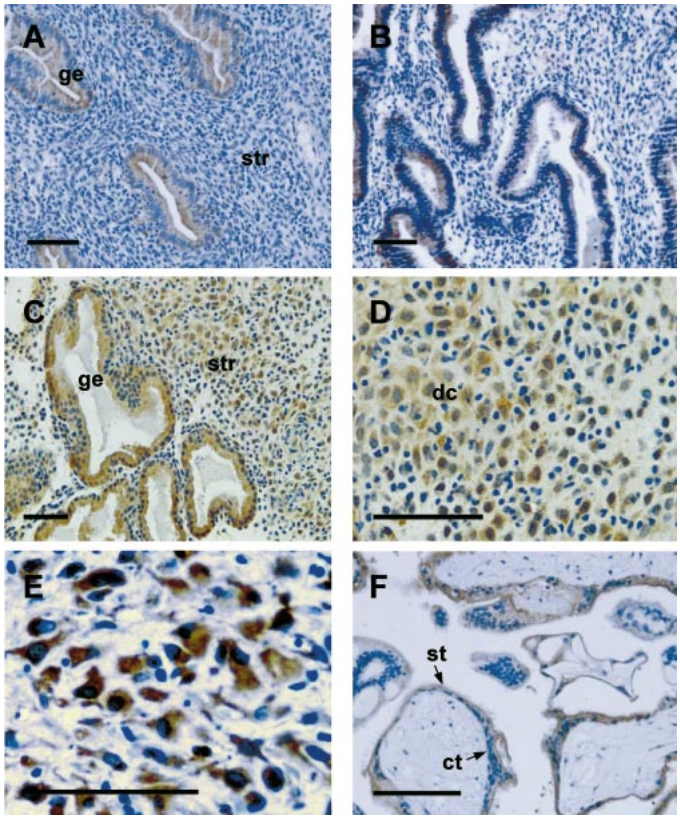

FIG. 4. Representative micrographs showing immunostaining for PC6 throughout the menstrual cycle (**A**-–**D**) and during early pregnancy (**E**, **F**). **A**) Proliferative, (**B**) early secretory, and (**C**) late secretory phase, (**D**) highlights strong staining in the decidual cells in the late secretory phase. During early pregnancy, strong staining was detected in the maternal decidua (**E**) with no significant signals in trophoblast cells except low levels in syncytial trophoblasts (**F**). Labels as in Figure 3. Scale bars = 400 μm.

PC6 Expression in Human Stromal Cells During In Vitro Decidualization

To further confirm that PC6 is induced during human stromal cell decidualization, we examined PC6 expression in an in vitro decidualization model using isolated human endometrial fibroblasts in the presence of cAMP [33]. Decidualization was confirmed by an 800-fold induction of prolactin secretion in cells treated with cAMP compared with the control (data not shown). The expression of PC6 mRNA during decidualization was determined by RT-PCR analysis. A band of expected size of 545 bp corresponding to PC6 transcript was detected in all cells (Fig. 6A), but the level was significantly increased in cells treated with the decidualization stimulus cAMP compared with the control (Fig. 6, A and B). This demonstrated that PC6 was upregulated during decidualization of human endometrial cellsin vitro. This upregulation was further confirmed by Western blot analysis, where a clear increase in PC6 protein level, mainly at 120 kDa, was observed in cells treated with cAMP compared with the control (Fig. 6C). These results demonstrate that, as in the mouse, PC6 is induced during decidualization of human endometrial stromal cells in vitro.

DISCUSSION

We have demonstrated that, in mice, endometrial PC6 is vital for the maternal stromal decidual response to an implanting embryo at implantation. This is supported by the following findings: (i) endometrial PC6 protein is induced

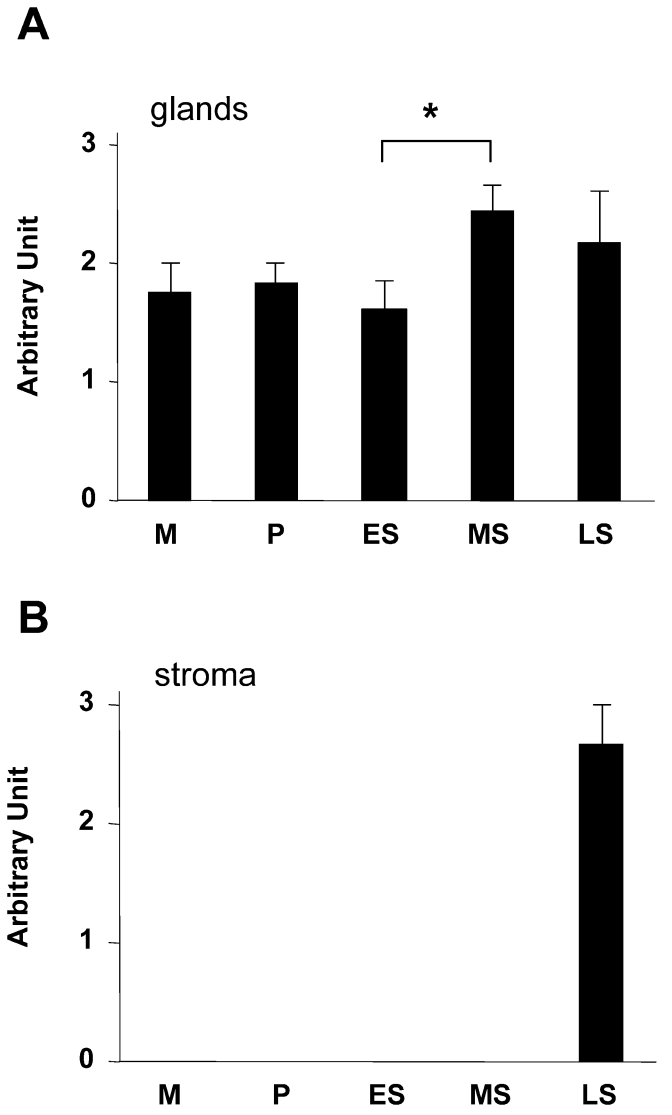

FIG. 5. Mean density (± SEM) of PC6 immunostaining in glands (**A**) and stroma (**B**) across the menstrual cycle. The increase of glandular staining from early secretory (ES) to mid secretory (MS) is significant (* $P < 0.05$). M, menstrual; P, proliferative; ES, MS, and LS, early, mid, and late secretory phases, respectively.

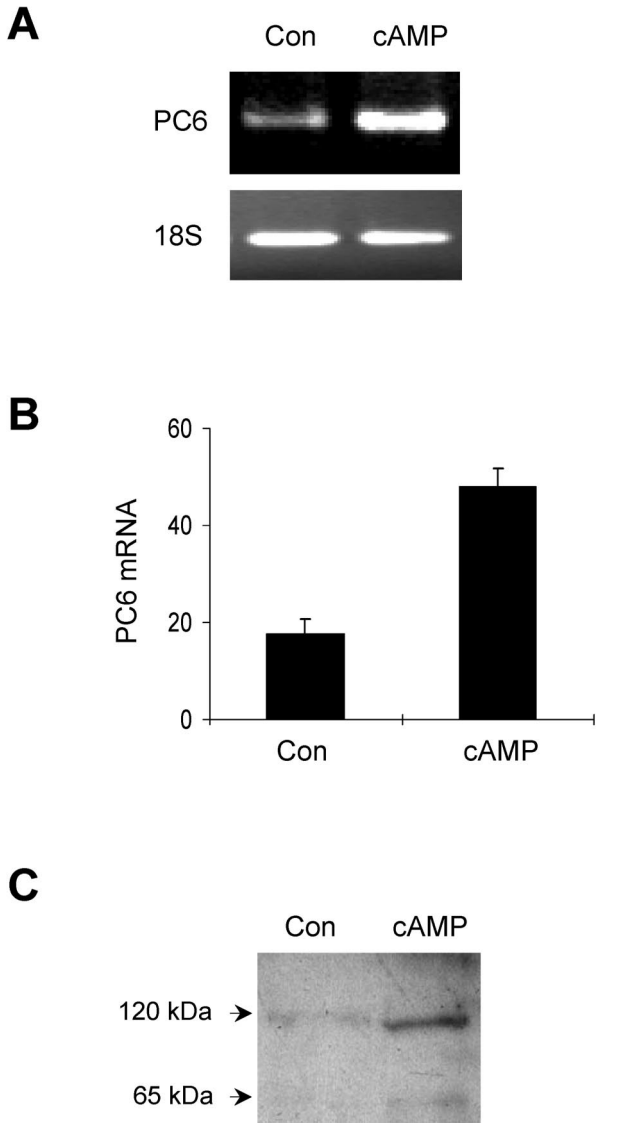

FIG. 6. PC6 expression during human stromal cell decidualization in vitro. **A**) RT-PCR amplification of PC6 mRNA and 18S in control cells (Con) and in cells treated with the decidualizing stimulus cAMP. **B**) Semiquantitative analysis of PC6 mRNA expression in control and decidualized cells. Data are presented as band intensities normalized against 18S. **C**) Western blot showing a higher level of PC6 protein in cAMP-treated cells than in control cells.

specifically in decidual cells, particularly in the primary decidual zone at implantation, but is undetectable in other cells, including the implanting embryo. (ii) Specific inhibition of this induction at a very early stage (Day 3.5) using anti-PC6 MO in vivo blocked decidualization and prevented implantation. We have also established that PC6 is likewise associated with decidualization in the primate endometrium in vivo. In both rhesus monkey and human, strong PC6 expression is detected in the maternal decidual cells at the fetal-maternal junction at the implantation sites during early pregnancy. PC6 is also induced in decidual cells during the late secretory phase of the menstrual cycle in the human in anticipation of implantation. Induction of PC6 also occurs in a human in vitro decidualization model. Taken together, these findings demonstrate that endometrial PC6 plays critical roles in modulating uterine decidualization to facilitate embryo implantation.

Endometrial stromal cell decidualization is a prerequisite for successful implantation in mammals with hemochorial placentation, including most rodents and primates [7]. The physiological importance of decidual cells in ensuring appropriate endometrial functions to support pregnancy is beyond doubt. While the exact molecular mechanisms of decidualization are still largely unknown, it is clear that the ovarian hormone, progesterone, triggers the estrogen-primed endometrium to undergo decidualization [34–36]. It is now understood that locally expressed factors and activation of the cAMP pathway integrate hormonal inputs and confer cellular proliferation and differentiation in decidualization [4]. The complexity of decidualization has been highlighted by the large number of molecules, including cytokines, growth factors, receptors, proteases, nuclear transcription factors, and cell-cycle regulators, identified to be induced or downregulated during decidualization using microarray analysis [33, 37]. Among these molecules, a number of proteins, including insulin-like growth factors, epidermal growth factors, transforming growth factor-β family proteins, vascular endothelial growth factors, and integrins,

are dramatically upregulated [33, 37]. Intriguingly, these molecules are synthesized as inactive precursor proteins and they exert their actions only after conversion into their mature forms. Importantly, the maturation of these molecules is executed through controlled proteolytic cleavage by PC6 [13, 38–40]. Based on our findings, we propose that PC6 functions through the activation of molecules essential for decidualization; thus, PC6 plays an important regulatory role during this process. Evidence is now emerging that PC6 is indeed critical in decidualization, as attenuation of its production in a human in vitro cell model results in decidualization arrest (Okada et al., unpublished data).

In addition, glandular PC6 expression is significantly upregulated at the expected time of implantation during the menstrual cycle in both the monkey and human endometrium, suggesting that PC6 is also involved in regulation of uterine glandular products in preparation for implantation in primates. This is in accordance with published work on other genes, such as interleukin 11, that are strictly expressed in decidual cells in mice but found also in the endometrial glands during the menstrual cycle in primates [29], reflecting a more complex preparation of the endometrium for implantation in primates compared with mice.

The type I membrane protein of HIV virus is synthesized as a single polypeptide precursor protein gp160 and posttranslationally cleaved into subunits gp120 and gp41 [41]. This cleavage is a prerequisite for viral infectivity, as inhibition of this step would disrupt the HIV life cycle [41], suggesting that this cleavage step can be a potential target for intervention [42]. The proteases responsible for this cleavage are the host cell PCs, including PC6; thus, PCs are considered potentially important targets to control HIV infection, and several studies have been initiated to develop PC inhibitors for this purpose [43, 44]. Sexually transmitted HIV/AIDS in women is through the vagina and cervix. Based on our findings, it is exciting to envisage the possibility that attenuating the production and/or function of PC6 simultaneously in (i) the endometrium to block embryo implantation and (ii) the vagina/cervix to reduce HIV infection, may serve as a novel strategy to provide dual protection for women. It is feasible to simultaneously target vagina/cervix/uterus with anti-PC6 substances via the vagina, as small molecules administered into the vagina/cervix can readily reach the uterus due to a uterine first-pass effect [45, 46]. Alternatively, substances administered systemically (orally or by injection or implants) could be used, provided they were targeted to the reproductive tract. If proven, this would have a significant impact on the reproductive health of women and the current HIV/AIDS epidemic.

Our studies in mice provide the proof of principle of the contraceptive effect of blocking PC6. Our demonstration that the temporal and spatial expression of PC6 is similar between the human and rhesus monkey endometrium suggests that the rhesus monkey could provide a useful primate model to test the efficacy and the route of administration of anti-PC6 substances to prevent both pregnancy and HIV infections.

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