Steroid hormone regulation of *Clca3* expression in the murine uterus

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Abstract

Progesterone (P4) and its cognate receptor, the progesterone receptor (PGR), have important roles in the establishment and maintenance of pregnancy in the murine uterus. In previous studies, using high-density DNA microarray analysis, we identified a subset of genes whose expression is repressed by chronic P4-PGR activation in the uterus. The *Clca3* gene is one of the genes whose expression is the most significantly downregulated by P4 and PGR. In the present study, we performed real-time RT-PCR and *in situ* hybridization to investigate the regulation of *Clca3* by P4 and determine the pattern of expression of *Clca3* in the uterus during early pregnancy. This analysis shows that *Clca3* mRNA transcripts were detected in the luminal and glandular epithelium of the pseudopregnant uterus at day 0.5 and that the expression

of *Clca3* was not detected after day 3.5. P4 represses *Clca3* mRNA synthesis in the luminal epithelial and glandular epithelial cells of the uterus in ovariectomized wild-type mice, but not in *Pgr* knockout (PRKO) mice. Conversely, estrogen (E2) induces *Clca3* expression in the luminal epithelium and glandular epithelium, and this induction was repressed by P4 in the murine uterus. Analysis of the promoter region of *Clca3* by *in silico* and transient transfection analysis in HEC-1A cells identified the regulation of *Clca3* by estrogen receptor- α (ESR1) within the first 528 bp of 5'-flanking region of the *Clca3* gene. Our studies identified *Clca3* as a novel downregulated gene of PGR that is a direct target of E2 regulation.

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Introduction

The uterus consists of heterogeneous cell types that undergo dynamic changes to support embryo development and implantation. These phenomena are primarily dependent on coordinated interactions mediated by progesterone (P4) and estrogen (E2). E2 stimulates proliferation of both the uterine epithelial and stromal cells in neonatal mice. However, this proliferative action of E2 is restricted to epithelial cells in the adult mouse uterus (Martin *et al.* 1973b, Huet-Hudson *et al.* 1989). In contrast, while P4 inhibits E2-mediated proliferation of the luminal and glandular epithelial cells, P4, alone or in conjunction with E2, leads to uterine stromal cell proliferation (Martin *et al.* 1973a, Huet-Hudson *et al.* 1989, Paria *et al.* 1993).

The ovarian steroid hormone progesterone (P4) is an essential regulator of reproductive events associated with all aspects of the establishment and maintenance of pregnancy (Clarke & Sutherland 1990, Lydon *et al.* 1995). The physiologic affects of P4 are mediated through progesterone receptors (PGRs) that are expressed as two isoforms, PGR-A and PGR-B, that arise from the same gene (Mulac-Jericevic *et al.* 2000, Conneely *et al.* 2002). PGR is a transcription factor belonging to the nuclear receptor superfamily (Evans 1988, O'Malley & Conneely

1992, Tsai & O'Malley 1994). The fertility defects exhibited by the PRKO mice unequivocally demonstrated the critical importance of P4 and its receptor in establishment and maintenance of pregnancy (Lydon *et al.* 1995, 1996).

Although the impact of the P4-PGR axis on murine uterine function has been extensively investigated, only a few P4-PGR-regulated genes have been identified. These include genes encoding amphiregulin (*Areg*) (Das *et al.* 1995), histidine decarboxylase (*Hdc*) (Paria *et al.* 1998), homeo box A10 (*Hoxa10*) and homeo box A11 (*Hoxa11*) (Lim *et al.* 1999), calcitonin (*Calca*) (Kumar *et al.* 1998, Zhu *et al.* 1998), calbindin-D9K (Nie *et al.* 2000), Indian hedgehog (*Ihh*) (Takamoto *et al.* 2002), hypoxia-inducible factor 1 (*Hif1a*; Daikoku *et al.* 2003) and immune-responsive gene 1 (*Irg1*) (Cheon *et al.* 2003). These target genes have been identified by testing candidate genes (Das *et al.* 1995), by differential library screening (Zhu *et al.* 1998) and by DNA microarray approaches (Cheon *et al.* 2002, Takamoto *et al.* 2002). The advent of the last named, high-density DNA microarray technology, has immensely improved the ability to identify PGR-regulated genes in the uterus.

We recently used oligonucleotide microarrays to identify the genes with expression regulated by the P4-PGR axis in the mouse uterus (Jeong *et al.* 2005).

PRKO and wild-type mice were ovariectomized and then treated with 1 mg P4 or vehicle (sesame oil) every 12 h. Groups of mice were killed 4 h after the first P4 injection (acute treatment) or 4 h after the fourth injection of P4 (chronic treatment). Using this methodology, we identified several genes regulated by PGR in the uterus in response to P4 (Jeong *et al.* 2005). Interestingly, we found that the expression of *Clca3* (chloride channel calcium-activated 3) mRNA was suppressed significantly by P4 and PGR.

The calcium-activated chloride channels (CLCA) family appears to mediate a calcium-activated chloride conductance in a variety of tissues, including epithelium (Evans & Marty 1986, Huang *et al.* 1993, Arreola *et al.* 1996), smooth muscle (Amedee *et al.* 1990, Clapp *et al.* 1996), skeletal muscle (Hume & Thomas 1989) and neurons (Barnes & Hille 1989, Hallani *et al.* 1998). Six members of this family have been identified, cloned and partially characterized in the mouse: *Clca1* (Gandhi *et al.* 1998, Romio *et al.* 1999), *Clca2* (Lee *et al.* 1999), *Clca3* (Komiya *et al.* 1999), *Clca4* (Elble *et al.* 2002), *Clca5* and *Clca6* (Abdel-Ghany *et al.* 2003, Beckley *et al.* 2004, Evans *et al.* 2004). Structural analysis indicates significant similarities among different CLCA family members, including protein sizes of 902–943 amino acids and four or five transmembrane regions (Pauli *et al.* 2000, Jentsch *et al.* 2002). However, the tissue and cellular distribution patterns appear to be unique to each protein.

Clca3 (also termed gob-5) has been identified in goblet cells throughout the intestinal tract by *in situ* hybridization (Komiya *et al.* 1999). The *Clca3* transcript was also detected by Northern blot in the murine trachea and uterus without identification of the respective cell types. However, the function and regulation of *Clca3* have not been reported to date, and the biologic processes in which it is involved are unclear. In this study, we analyzed the spatiotemporal expression and regulation of *Clca3* in the response to P4 and E2 in early pregnant uterus.

Materials and Methods

Animals and tissue collection

Mice were maintained in the designated animal care facility at Baylor College of Medicine according to the institutional guidelines for the care and use of laboratory animals. Thirty-six wild-type and 12 PRKO mice at 6 weeks of age were ovariectomized. Two weeks later, ovariectomized wild-type mice were injected with one of the following: vehicle (sesame oil), P4 (1 mg/mouse), E2 (0.1 µg/mouse) or P4 plus E2. The injections were at 0 h and at 12-h intervals thereafter, the animals being killed at 4, 16, and 40 h (wild-type) or 4 and 40 h (PRKO) ($n=3$ animals per genotype per treatment). Hormone injection was repeated every 12 h for the 16- and 40-h samples to prevent the effect of hormone degradation by metabolism. The mice were anesthetized with Avertin

(2,2,-tribromoethyl alcohol; Sigma-Aldrich) and killed by cervical dislocation under anesthetic at 4, 16 or 40 h (4 h after fourth injection) to collect the uteri. Multiple-timed pseudopregnant female mice were achieved by treating female mice with a superovulatory regimen of gonadotropin and mating with vasectomized male mice. Hormonal induction of ovulation was used to synchronize the cycle of mice for multiple-timed pregnant females. Briefly, wild-type females were administered 5 IU pregnant mare's serum gonadotropin (VWR Scientific Products, West Chester, PA, USA), followed 48 h later by 5 IU human chorionic gonadotropin (Organon, West Orange, NJ, USA), and placed with a vasectomized male mouse. The morning of vaginal plug was designated as day 0.5. Uterine tissues were flash frozen at the time of dissection and stored at -80°C for RNA or fixed with 10% (v/v) formalin for *in situ* hybridization.

Quantitative real-time PCR

RNA was extracted from uterine tissues with the RNeasy total RNA isolation kit (Qiagen). Expression levels of *Clca3* mRNA were measured by real-time RT-PCR TaqMan analysis with the ABI Prism 7700 Sequence Detector System according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Prevalidated probes and primers for *Clca3* and 18S RNA were purchased from Applied Biosystems. RT-PCR was performed with One-Step RT-PCR Universal Master Mix reagent and TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. Standard curves were generated by serial dilution of a preparation of total RNA isolated from whole mouse uterus. All real-time PCR was done with the three independent RNA sets. mRNA quantities were normalized against 18S RNA with ABI rRNA control reagents. Statistical analyses used one-way ANOVA followed by Tukey's *post hoc* multiple range test with the Instat package from GraphPad (San Diego, CA, USA). *P* values of <0.05 were considered statistically significant, and values of <0.01 highly significant. In the figures, S.E.M. is indicated in all bar graphs.

In situ hybridization

The protocol for *in situ* hybridization was essentially as described previously by Simmons *et al.* (1989). Uterine tissues were fixed in 10% (v/v) formalin. After overnight fixation at room temperature, tissues were dehydrated through a series of ethanol and then processed for paraffin embedding. Paraffin sections were mounted onto poly-L-lysine-coated slides (VWR Scientific Products, West Chester, PA, USA), and used for *in situ* hybridization. The riboprobes were generated by *in vitro* transcription of amplified DNA products containing the T7 polymerase promoter sequence flanking the desired

Figure 1 The expression pattern of *Clca3* from P4-treated wild-type (WT) and PRKO uteri by real-time RT-PCR. Total RNA used for the RT-PCR assays was prepared from wild-type or PRKO uteri that were treated with P4 or vehicle (Veh; sesame oil) for 40 h. The results represent the mean $\pm$ S.E.M. of three independent RNA sets. ** $P < 0.01$.

nucleotide primer sequence, using ^{35}S -UTP (Promega). Slides were incubated for 7 min at room temperature in Proteinase K (20 $\mu\text{g}/\text{ml}$) in a buffer containing 50 mM Tris and 5 mM EDTA (pH 8). Slides were then acetylated with acetic anhydride, dehydrated and exposed to either denatured antisense or sense probes in hybridization buffer (50% (v/v) formamide, 10% (w/v) dextran sulfate, 5 Denhardt's solution, 300 mM NaCl, 5 mM EDTA (pH 8), 20 mM Tris (pH 8) and 0.05 mg/ml yeast tRNA). Hybridization was performed at 55 °C overnight in a humidity chamber containing 5 SSC and 50% (v/v) formamide. Hybridized slides were exposed to 20 $\mu\text{g}/\text{ml}$

RNase A for 30 min at 37 °C. Slides were washed in 50% (v/v) formamide, 2 SSC and 100 mM 2-mercaptoethanol, followed by 2 SSC at 55 °C for 30 min, dehydrated in a graded series of ethanol in 0.3 M ammonium acetate, and exposed to Biomax MR film overnight (Kodak). The following morning, slides were dipped in autoradiography emulsion (Amersham) and placed at 4 °C in a light-proof box for several days. After development, slides were counterstained with hematoxylin.

Construction of *Clca3*-luciferase-expression vectors

The 891 bp (−907 to −17) of 5'-flanking region of the *Clca3* gene were PCR-amplified from mouse genomic DNA as a template, using the 5'-PCR primer, 5'-GAA GACCAAAAGGATGAAAATGAC-3', and the 3'-PCR primer, 5'-GGGAAGCTTTGGAAAGGGCTGGGTG TAGAAG-3'. The resulting PCR product was subcloned into the pCR II TA TOPO cloning vector (Invitrogen). The 891 bp *Clca3* fragment was liberated from the pCR II TA TOPO vector and subcloned into the luciferase gene in the pGL3 Basic vector (Promega).

Two deletion constructs, 528 bp (−544 to −17) and 319 bp (−335 to −17) promoter, were generated via PCR with the same 3' PCR primer and two different 5' PCR primers: −544 (5'-GATGCTGAGGAGAAATGTGGA GTT-3'), and −335 (5'-TGAGGAACCAGATTAG GAT-3'). The resulting PCR product was subcloned into the luciferase gene in the pGL3 basic vector (Promega).

Transient transfection assay

HEC-1A cells were seeded in 24-well plates and cultured according to ATCC recommendations until they were

Figure 2 The localization pattern of *Clca3* transcription by *in situ* hybridization in the P4-treated uterus. Tissue sections from wild-type uteri were treated with P4 or vehicle (Veh; sesame oil) for 4 and 40 h. Nuclei were lightly counterstained with hematoxylin. Magnification $\times 20$. Scale bar = 100 or 50 μm (inset). LE, luminal epithelium; GE, glandular epithelium; ST, stroma cells; Myo, myometrium.

Figure 3 The localization pattern of *Clca3* transcription by *in situ* hybridization in the PRKO mice. Tissue sections from wild-type (WT) or PRKO uteri were treated with P4 or vehicle (Veh; sesame oil) for 40 h. Nuclei were lightly counterstained with hematoxylin. Magnification $\times 20$. Bar=100 μ m. LE, luminal epithelium; GE, glandular epithelium; ST, stroma cells; Myo, myometrium.

approximately 60–70% confluent. Cell cultures were maintained in media containing charcoal-stripped fetal calf serum (FCS). The cells were transfected with Superfect (Qiagen) with 1 μ g reporter gene, and 10 or 50 ng estrogen receptor alpha (*Esr1*) or estrogen receptor beta (*Esr2*). Cells were treated with either E2 (10^{-8} M), or

vehicle (ethanol) for 24 h. Cells were harvested for luciferase assay 24 h after addition of the E2 or ethanol. Cell extracts were prepared after 24-h transfection and assayed by a luciferase reporter system (Promega). Protein concentration was used to correct for differences in transfection efficiencies. The data represent two indepen-

Figure 4 The expression pattern of *Clca3* from E2- or E2 plus P4-treated uteri by real-time RT-PCR. Total RNA used for the RT-PCR assays was prepared from wild-type mice treated with P4, E2, E2 plus P4, or vehicle (Veh; sesame oil) for 4, 16 and 40 h. The results represent the mean $\pm$ S.E.M. of three independent RNA sets. * $P<0.05$; ** $P<0.01$.

Figure 5 The localization pattern of *Clca3* transcription by *in situ* hybridization in (A) E2- and (B) E2 plus P4-treated uteri. Tissue sections from wild-type uteri were treated with E2, E2 plus P4, or vehicle (sesame oil) for 4, 16 and 40 h. Nuclei were lightly counterstained with hematoxylin. Magnification $\times 20$. Bar = 100 μ m. LE, luminal epithelium; GE, glandular epithelium; ST, stroma cells; Myo, myometrium.

dent experiments, each performed in triplicate. Statistical analyses were by one-way ANOVA followed by Tukey's *post hoc* multiple-range test with the Instat package from GraphPad. *P* values of <0.05 were considered statistically significant and values of <0.01 highly significant. The S.E.M. is indicated in all bar graphs.

Results

Downregulation of the Clca3 expression by P4 in the uterus

We previously identified *Clca3* as a potential chronic P4- and PGR-regulated gene in the murine uterus by DNA microarray analysis (Jeong *et al.* 2005). In this study, we analyzed the P4 and PGR regulation of *Clca3* in the murine uterus. For further determination of the reliance of *Clca3* expression on PGR function, ovariectomized wild-type and PRKO mice were injected with P4 or vehicle (sesame oil). Uteri were collected from the mice after 4 h (acute treatment) or 40 h (chronic treatment) of hormone treatment, and the expression of *Clca3* was investigated by real-time RT-PCR. As shown in Fig. 1, the mRNA transcript of *Clca3* was detected in the uteri of wild-type ovariectomized mice. However, the *Clca3* transcript was significantly decreased after chronic treatment with P4 in the wild-type mice (10%). To establish

whether the P4 regulation of *Clca3* is PGR dependent, we analyzed the expression of the *Clca3* in the uteri of wild-type and PRKO mice. After chronic P4 treatment, downregulation of the *Clca3* transcription was not detected in the PRKO mice (Fig. 1). We also did not detect any change of *Clca3* expression in the wild-type mice after the acute P4 treatment, although a significant effect was seen at 16 h in the wild-type mice. These results were consistent with our microarray data identification of *Clca3* gene as a late responsive gene. These results show that *Clca3* mRNA expression is downregulated by both P4 and PGR in chronic treatment, but not in acute treatment.

To analyze the spatial expression of *Clca3* by P4 in the uterus, we performed *in situ* hybridization in the P4-treated uterine samples. Uterine sections were hybridized with a 569 bp, 35 S-labeled antisense RNA probe containing sequences from *Clca3* cDNA. As shown in Fig. 2, using the antisense *Clca3* probe, we observed a hybridization signal in the luminal and glandular epithelium cells of the uterine section obtained from vehicle-treated wild-type uterus. The observed signal was not uniform but indicated distinct regions of signal intensity in the luminal and glandular uterine epithelium. These signals were not detected in the chronic P4-treated, wild-type samples. To establish whether the P4 downregulation of the *Clca3* gene is mediated through PGR, ovariectomized

PRKO and wild-type female mice were treated with P4 or vehicle. After chronic P4 treatment, the uteri were collected, and *in situ* hybridization was performed. As expected, hybridizing signals for *Clca3* transcripts were detected in the P4-treated PRKO uterine sections (Fig. 3). These results indicate that PGR is essential for *Clca3* gene downregulation in luminal and glandular epithelium cells.

Regulation of *Clca3* gene by E2 and P4 in the uterus

P4 antagonizes E2 actions, such as the stimulation of proliferation of the epithelial cells in the mouse uterus (Martin *et al.* 1973b, Huet-Hudson *et al.* 1989). To determine whether P4 antagonizes the effect of E2 in *Clca3* expression, we treated ovariectomized female mice with E2 or E2 plus P4 for 4, 16, or 40 h. Real-time RT-PCR analysis was performed with uterine RNA after E2 or E2 plus P4 treatment. As shown in Fig. 4, P4 repressed *Clca3* mRNA levels 16 and 40 h after treatment. The expression of *Clca3* mRNA was increased 3·65-, 5·52- and 87·02-fold 4 and 16 h after E2 treatment and after 40 h of chronic E2 treatment respectively. The results suggest that E2 induces the expression of *Clca3* in the uterus. Figure 4 also shows that treatment of mice with E2 plus P4 significantly inhibited the induction of *Clca3* by E2. These results indicate that the expression of *Clca3* is induced by E2, but the induction of mRNA expression by E2 is inhibited by P4 in the uterus.

Next, we analyzed by *in situ* hybridization the spatial expression of the *Clca3* transcription in the uterus after E2 treatment. As we expected, *Clca3* mRNA was weakly detected in the luminal and glandular epithelial cell at 4 h (Fig. 5A). The signal of *Clca3* was increased at 16 h, and then a significant induction of *Clca3* mRNA was observed in the luminal epithelium and glandular epithelium cells at 40 h. To determine the spatial regulation between E2 and P4 in the *Clca3* expression, we performed *in situ* hybridization on the E2 plus P4-treated uteri (Fig. 5B). The weak epithelial signals were detected at 4 h, and then the signals were increased at 16 h. However, we could not detect any signal at 40 h in uterine sections. These results suggest that P4 is a downregulator of *Clca3* expression and E2 is a positive regulator in the luminal and glandular epithelium.

The expression of the *Clca3* gene during early pregnancy

The above analysis identified the pharmacologic regulation of *Clca3* by the ovarian steroids E2 and P4. We next established the expression profile of *Clca3* mRNA in mouse uteri during early pregnancy with real-time RT-PCR to measure levels of *Clca3* and compare the expression to two other uterine P4-responsive genes, amphiregulin (*Areg*) (Das *et al.* 1995) and patched

Figure 6 The expression pattern of *Clca3* by real-time RT-PCR in pseudopregnancy. The expression level of *Clca3* (A), *Areg* (B) and *Ptch1* (C) were measured in uteri of pseudopregnancy. Total RNA used for the RT-PCR assays was prepared from pseudopregnant uteri. The results represent the mean $\pm$ S.E.M. of three independent RNA sets. ** $P < 0.01$.

homolog 1 (*Ptch1*) (Takamoto *et al.* 2002) from pseudopregnant samples. As shown in Fig. 6A, the strong expression of *Clca3* detected on day 0·5 declined sharply on days 1·5 and 2·5 (5·59% and 1·38% respectively) and was undetectable after day 3·5. The levels of P4 were elevated on day 2·5 during early pregnancy, as shown by the induction of P4-responsive genes *Areg* and *Ptch1* (Fig. 6B and C respectively). These results suggest that strong downregulation of *Clca3* expression occurs in the uterus on day 2·5 of gestation, presumably in response to P4.

Figure 7 The localization pattern of *Clca3* transcription by *in situ* hybridization during pseudopregnancy. The mice were killed to harvest uteri at indicated pseudopregnancy. Nuclei were lightly counterstained with hematoxylin. Magnification $\times 20$. Bar = 100 μm . d, day; LE, luminal epithelium; GE, glandular epithelium; ST, stroma cells; Myo, myometrium.

Next, we analyzed by *in situ* hybridization the spatial expression profile of the *Clca3* transcription in the uterus from pseudopregnant uterine samples (Fig. 7). Consistent

with the real-time RT-PCR results, the *Clca3* mRNA transcripts were strongly expressed in luminal and glandular epithelial cells at day 0.5. However, the specific

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A

tgtatccattttttatatgaacgtgtactttgaatcagctagataaggatttaaacctctttttaatacttcatctgcaattgtttcat -991
ttggaaaatactaattcagttgttatgagctgaatgtcaaagagaatggcagacaaaggaatgatgcctttgtctatctgaagacc -901
aaaaggatgaaaatgacttccaagcaatttgaaatcagcagcaatggcagaccactttgggcttctctctaaagtttgtgaagcttgaaa -811
gaatttatggggtagcagagccaccttaatttatgtttcagagtggcataaatttcagcaaaagatttcatgttgtgtggtttaactttac -721
tgactgtatagaagctgtggggtagagaagcaaaagtgggggttaggggaactgggggaagtttgtcttgcaaaatacaatgaaagtttacc -631
gtcctctctcaactggggactacttagtgacatctgaattactcgtgatgctttgttagTgtgtatggaatgaaagagcatcccgatgac -541
ctgagggaaaattgtggagttcaccgaagagaacaaactggccaagatctctacagaaactaaagcttctcttaaatgcataatgtcaagc -451

ERE
tggaaagggtgggggttcagggggtggagatggggtGGTCAgTGTCCctgactagacaaatatttgaagtaaacagatactccaattggacaa -361
cctgctttccctggatgagtgtttctGaggaaccaagattaggattctccacccttgctcaaacacctggtgcagccaggggcaaacccaa -271
cattccctttaattagAaacacttaaaagtacagtttggtttttaaacacacttcctcttatattgtaggcaaatgtggtaaaqaacca -181

^{1/2}ERE
t t t a a g g g t t a a a g g c c t a t a a t a a a t c a c c t c c a g t a a g t t g a g a a a c c a c a t c t a c c t t g t g t g t g t g t g t g t g -91

tgtgtgtgtgtgtgtgtgtgtgtgtatgtgt**TATATATA**tttgagtaaagacacttctacaccagccctttccaga**GGGAAAGCTGCAGG**-1

ATGGAATCTTTGAAGAGTCCTGTCTTCCTCTTGATCCTCCACCTTCTGGAAGGAGTTCTGAGTGAGTCCCTCATCCAACCTGAACAACAAC +90

B

Mouse TGGGTGGGTCAGTGTCCCTG
Rat TGGGTGGGTCAGTGTCCATA
Human TGGGTGGTTGGTGTCCATG
Cow TGGGTGGGTCGGTGTCCCTG
Dog TGGGTGGGTCAGTATCCCTG

C

D

hybridization signal was not observed in the epithelial cells of uterine sections obtained from day 2.5. These results further confirm that *Clca3* transcription is repressed in the luminal and glandular epithelial cells of pregnant uteri. Moreover, these spatial patterns of *Clca3* expression are similar to the ones observed in the uteri of ovariectomized mice in response to P4 treatment. These results suggest that the repression of *Clca3* may be important during early pregnancy.

Transcriptional regulation of the *Clca3* promoter by estrogen

To investigate the molecular mechanisms underlying *Clca3* gene expression regulation by P4 and E2, we examined the *Clca3* promoter and 5'-flanking region for potential sequences that are recognized by PGR and estrogen receptor (ESR). Although no palindromic PRE (progesterone response element) could be identified in the 2 kb 5'-flanking region, this region contains one palindromic ERE (estrogen response element) and one half-ERE, the most proximal of which is present within the -420 (Fig. 8A). This palindromic ERE of *Clca3* was shown to be conserved in mouse, rat, man, cow and dog (Fig. 8B).

In order to determine whether the estrogen receptor directly regulates the expression of *Clca3*, cotransfection assays were performed in HEC-1A endometrial adenocarcinoma cells, with 891 bp of the 5' flanking region of the *Clca3* promoter fused to the luciferase reporter, as described in Materials and methods. This region contains all putative ERE motifs. HEC-1A cells were transfected with the 891 bp promoter and different amounts of expression vectors containing *Esr1* and *Esr2* (10 and 50 ng). The data were represented as fold-induction where the value of luciferase activity for the construct with no treatment was set to 1.0 (Fig. 8C and D). Under these conditions, it was shown that *Esr1*, and not *Esr2*, activated, in a dose-dependent manner, the 891 bp promoter of *Clca3* in HEC-1A cells (Fig. 8C). We could not detect regulation of the *Clca3*-luciferase reporter when cotransfected in HEC-1A cells with expression vectors for *Pgr-A* or *Pgr-B* (data not shown).

For further identification of the region of *Esr1* regulation of the *Clca3* promoter, cotransfection experiments in HEC-1A cells were conducted with deletion fragments of

the *Clca3* promoter fused to the luciferase reporter. These reporters contained 528 and 319 bp of the *Clca3* promoter. The fragment containing 891 and 528 bp of the promoter region showed enhanced luciferase activity by E2. However, deletions to -335 bp, which eliminated the only full ERE site, resulted in 58% loss of E2 induction of luciferase activity. These results suggest that the conserved ERE region of *Clca3* is important for *Esr1* regulation.

Discussion

P4 plays important roles through its nuclear receptors in the uterine functions associated with the establishment and maintenance of pregnancy (Conneely & Lydon 2000, Conneely *et al.* 2001, 2002, Conneely & Jericevic 2002). Studies of the mouse model carrying a null mutation of the *Pgr* gene (PRKO) showed essential roles that these receptors play in P4-mediated response (Lydon *et al.* 1995, 1996). Thus, the identification of P4-regulated pathways in the uterus is crucial for understanding the impairments that underlie the complex phenotype of PRKO mice. In previous studies, we have identified P4-regulated genes by PRKO and microarray analysis (Jeong *et al.* 2005). Two time points (4 and 40 h) were used to identify early-responsive genes (direct targets) and late-responsive genes (indirect targets). *Clca3* was the strongest repressed gene as a potential indirect target of P4 regulation. In the present study, we provide clear evidence that *Clca3* is a novel late-responsive gene of PGR regulation in the murine uterus.

The analysis of *Clca3* in mouse uterus shows that the gene is expressed in the luminal and glandular compartments of the endometrium. The expression of this gene is not uniform throughout the epithelial compartments, but shows focal regions of intensity. This may reflect a cellular specificity to the expression of this gene. Although there is little information on the expression of cell specific markers in uterine epithelial cell types, *Clca3* may represent a means of detecting subpopulations of uterine epithelial cell types.

Our results indicate that the expression of *Clca3* is stimulated by E2 and repressed by P4. The expression studies during pregnancy show that *Clca3* is highest at day 0.5, and its expression decreases as pregnancy progresses, when P4 levels are rising. This decrease in *Clca3* is

Figure 8 Transcriptional regulation of the *Clca3* promoter by estrogen. (A) Nucleotide sequence of the mouse *Clca3* promoter. Nucleotides are numbered from the ATG site (+1) of the gene. The transcription initiation site is denoted by arrow. Exon 1 sequence is shown in boldface, and putative ER binding sites are boxed and in boldface. (B) Alignment of conserved ERE region for mouse, rat, human, cow and dog *Clca3*. The conserved ERE of the *Clca3* gene was deduced from the University of California (Santa Cruz) Genome Browser (<http://genome.ucsc.edu>). The boxed regions indicate sequences in the promoter that are conserved ERE in the five species. (C) Transient transfection assays of the *Clca3* promoter in HEC-1A cells. Luciferase reporter plasmids containing *Clca3* promoter 891 bp fragments were transiently transfected into HEC-1A cells with *Esr1* or *Esr2*. (D) Transient transfection assays of serial deletion of *Clca3* promoter in HEC-1A cell lines. The 528 and 319 bp promoter fragments were subcloned into the polylinker region of the promoterless luciferase reporter vector pGL3-basic. The promoter deletion/luciferase reporter constructs were cotransfected with *Esr1*. These data represent two independent experiments, each performed in triplicate. Values shown are $\pm$ S.E.M. Student's *t*-test was used for statistical analysis. **P*<0.05; ***P*<0.01. +: 10 ng/well; ++: 50 ng/well; Et-OH, ethanol (vehicle); E2, 17 β -estradiol.

opposite to the expression of genes stimulated by P4, as well as the expression of *Pgr* (Tan *et al.* 1999, Takamoto *et al.* 2002), validating *in vivo* physiologic repression of this gene by P4. *In silico* analysis of the 10 kb region flanking 5' to the proximal promoter region of *Clca3* could not detect any conserved PRE. However, one conserved ERE was detected at -418 ~ -407, and one half site was detected at -176 ~ -171 of the 5' region of the mouse gene (detection by TESS (Transcription Element Search System; www.cbil.upenn.edu/~tess/)). The consensus ERE motif is a palindromic repeat of the sequence GGTC A separated by three nucleotides (Klinge 2001). These palindromic repeats are separated by one nucleotide, but conserved in five different species, including mouse, rat, man, cow and dog (Fig. 8B). We show significant E2 induction of the 891 and 528 bp promoter reporter construct, while induction was significantly reduced in the 319 bp promoter-reporter construction. Even though the 319 bp promoter fragment did not contain a full ERE consensus site, E2 induction was still observed, albeit significantly reduced. This residual regulation by E2 may be through activation of the ERE half-site at -176 ~ -171, or it may be through a mechanism independent of DNA binding (Aronica *et al.* 1994, Tesarik & Mendoza 1995, Le Mellay *et al.* 1997, Simoncini *et al.* 2004). Interestingly, the 0.5 kb region displays higher luciferase activity than the 1.0 kb full-length promoter region. This region may contain potential repressive elements between -543 and -1006, relative to ATG. The above results demonstrate that E2 regulates, through its receptor, *Clca3* transcription.

These results suggest that this estrogen response in *Clca3* may serve as a direct docking site for the estrogen receptor and provide direct regulation by estrogen. Since no conserved PRE was found, repression of PGR may be through an indirect mechanism. Since P4 inhibits the expression of *Esr* in the uterine epithelium, the expression of *Clca3* may be by P4 inhibiting *Esr* expression. However, this does not exclude other transcription factors being inhibited by P4.

Clca3 has been identified in goblet cells of the intestinal tract by *in situ* hybridization (Komiya *et al.* 1999). Increasing evidence suggests that members of the CLCA family play a role in diseases with epithelial secretory dysfunction (Hashimoto *et al.* 2004). By use of the murine model of bronchial asthma, *Clca3* was found to be a key regulator of the induction of mucus overproduction. In this model, antisense *Clca3* therapy effectively suppressed the asthma phenotype, whereas *Clca3* overexpression exacerbated the condition (Nakanishi *et al.* 2001). Changes in MUC1 glycoform expression have been related to a phase of the menstrual cycle and endometrial receptivity in a number of studies (Meseguer *et al.* 1998, Lagow *et al.* 1999, Aplin *et al.* 2001). Failure of embryo implantation was associated with an abnormal endometrial expression of MUC1 mucin and retention of PGR, particularly in epithelial

cells (Horne *et al.* 2005). Downregulation of *Clca3* by P4 during early pregnancy may be a means of initiating the decrease in mucus production by the endometrial epithelial cells in the uterus. The decrease in mucus production may initiate the window of receptivity for embryo attachment and implantation.

In summary, this study demonstrated *Clca3* mRNA to be expressed in murine uterine luminal and glandular epithelial cells, but not in stroma cells in ovariectomized mice. The levels of this mRNA in the uterine luminal and glandular epithelial cells were significantly downregulated by P4 and upregulated by E2. E2-mediated induction was inhibited by P4 when the ovariectomized mice were treated with E2 and P4. The expression of *Clca3* was turned off on day 1.5 of pregnancy. These results suggest that steroid hormonal regulation of *Clca3* may be important in the uterus of mouse during early pregnancy.

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