Calpain5 expression is decreased in endometriosis and regulated by HOXA10 in human endometrial cells

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Calpains have been implicated in the regulation of apoptosis. Here, we identified Calpain5 as a target of HOXA10 transcriptional regulation in endometrial cells as well as its aberrant regulation in endometriosis. Histologically confirmed biopsies of endometriosis were obtained from 20 women. Eutopic endometrium was collected by endometrial biopsy from 30 controls and from the 20 subjects with endometriosis. First trimester decidual samples were obtained from five subjects at the time of pregnancy termination. Immunohistochemistry was used to identify Calpain5 expression. Calpain5 was expressed in endometrial stromal and glandular cells throughout the menstrual cycle and in decidua. Calpain5 protein expression was decreased in both stromal and glandular cells from women with endometriosis compared with that of fertile controls. Human endometrial stromal and epithelial cell lines were transfected with pcDNA/HOXA10, HOXA10 siRNA or respective controls. Quantitative real-time RT–PCR was performed to determine expression of HOXA10 and Calpain5 in each group. Transfection of HESC cells with an HOXA10 expression construct led to increased Calpain5 expression, whereas transfection with siRNA resulted in decreased expression. In conclusion, Calpain5 expression is regulated by HOXA10. Calpain5 expression was decreased in endometriosis likely as a result of decreased HOXA10 expression. Decreased apoptosis in endometrial cells may promote the development of endometriosis through a pathway involving HOXA10, Calpain5 and caspase.

Keywords: Calpain5; HOXA10; endometrium; endometriosis; apoptosis

Introduction

Calpains are cytoplasmatic calcium-dependent cysteine proteases. The Calpain family has four members whose functions are not fully characterized. Calpains have been implicated in several different cellular activities including the regulation of apoptosis and cell differentiation (Koichi et al., 2004). Mutations in members of the Calpain family have been identified in several human diseases. Limb-girle muscular dystrophy type 2A is seen with mutation of Calpain3 and type II diabetes has been linked to Calpain10 mutation (Koichi et al., 2004). There have been no prior reports implicating Calpain5 in disease.

The mouse and human orthologs of Calpain5 show significant similarity, 87% DNA and 95% protein sequence identity. Calpain5 also shares homology with Caenorhabditis elegans tra-3 (Syntichaki et al., 2002). This suggests a highly evolutionarily conserved function predating sexual reproduction. Calpain5 is expressed in the ovaries, nervous system and placenta (Gonzalez et al., 2006). Regulation of Calpain5 expression has not yet been characterized in the Mullerian tract.

We previously conducted a microarray screen of murine endometrium after transfection with pcDNA/HOXA10 or antisense-HOXA10 (Vitiello et al., 2008). We identified Calpain5 as a putative target of HOXA10 regulation in the mouse. HOXA10 is a transcription factor that is necessary for endometrial receptivity and embryo implantation. Altering the endometrial expression levels of HOXA10 in murine endometrium results in corresponding alterations in embryo implantation rates (Taylor et al., 1998; Bagot et al., 2000). HOXA10 is expressed in human endometrium during the menstrual cycle, where its expression is regulated by estrogen and progesterone (Taylor et al., 1998). Endometrial epithelial and stromal HOXA10 expression levels are up-regulated in the midluteal phase, coincident with the time of implantation (Taylor et al., 1998; Sarno et al., 2005). Here, we investigated the possibility that Calpain5 was expressed in human endometrium and regulated by HOXA10.

Materials and Methods

Tissue collection

Thirty endometrial biopsies, 15 proliferative phase (cycle days 8–12) and 15 mid-secretory phase (cycle days 19–24) were obtained from fertile women at the time of elective tubal ligation as controls. Histologically confirmed biopsies of endometriosis as well as eutopic endometrium from these subjects with endometriosis were obtained from 20 women at the time of laparoscopy also in the mid-secretory phase. All women were in the reproductive age range (24–41 years old), had regular menstrual cycles and were not using hormonal medication within 3 months prior to surgery. Age, body mass index and race did not significantly vary between the groups. First trimester (6–9 weeks gestational age) decidual samples were obtained at the time of elective pregnancy termination. Biopsy specimens were fixed in formalin and processed as described below. Approval was obtained from the Yale University School of Medicine Human Investigations Committee.

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**Plasmids and siRNA**

HOXA10 cDNA was cloned into the EcoRI site of pcDNA3.1(+) (Invitrogen, CA, USA). pcDNA3.1(+) without the HOXA10 insert was used as a control (Invitrogen). SiGenome duplex HOXA10 siRNA (cat# B-002000-UB-015) and control non-targeting siRNA (cat# D-001210-02) were purchased from Dharmacon (Dharmacon, IL, USA).

**Cell culture**

The human endometrial stromal cell line HESC and the human endometrial adenocarcinoma cell line Ishikawa were generous gifts of Charles J. Lockwood and Richard Hochberg, respectively (Yale University). HESC and Ishikawa cells were maintained in a phenol-red-free DMEM and MEM, respectively (Sigma, St Louis, MO, USA). Each was supplemented with 10% charcoal-stripped calf serum, 1% penicillin/streptomycin and 1% sodium pyruvate.

**Transient transfection of human cell lines**

HESC, grown to 60–70% confluence, were transfected using TransIT-LT1 Minus (Madison, WI, USA) with either pcDNA3.1(+) or HOXA10 (0.4 µg for 6-well plates, 12 µg for 10 cm dish) or HOXA10 siRNA (20 µM for 6-well plates, 60 µM for 10 cm dish), using empty pcDNA3.1(+) or non-specific siRNA as respective controls. Ishikawa, grown to 45–55% confluence, were transfected using lipofectamine 2000 (Invitrogen) with either pcDNA3.1(+) or HOXA10 (0.4 µg for 6-well plates, 12 µg for 10 cm dish) or HOXA10 siRNA (20 µM for 6-well plates, 60 µM for 10 cm dish), using empty pcDNA3.1(+) or non-specific siRNA as respective controls. Forty eight hours post-transfection, total RNA and protein were isolated. All transfection experiments were conducted in triplicate.

**Real-time RT–PCR**

Quantitative real-time RT–PCR (qRT–PCR) was performed using the Light-cyke SYBR Green RT–PCR kit from Roche. The total RNA of 1 µg was reverse transcribed in 20 µl of reaction mixture containing 10 mM of dATP, dCTP, dGTP and dTTP; 20 pmol oligo(dT); 40 U/µl ribonuclease inhibitor, 10 U/µl avian myeloblastosis virus-reverse transcriptase and 10× AMVRT buffer for 30 min at 61°C. PCR amplification of HOXA10 was performed for 45 cycles of 95°C for 2 s; 65°C for 5 s; 72°C for 18 s. PCR amplification of β-actin as a control was performed for 45 cycles of 95°C for 2 s; 61°C for 5 s; 72°C for 18 s. PCR amplification of Calpain5 was performed for 45 cycles of 95°C for 2 s; 63°C for 5 s; 72°C for 18 s. The HOXA10 and Calpain5 primers have been previously described (Smith and Taylor, 2007).

**Western blot**

Ishikawa (i) or HESC (ii) were incubated with either (i) rabbit anti-caspase-3 polyclonal antibody, which recognizes the active form of caspase-3 (9661; 1:5000), obtained from Cell Signaling Technology, Inc. (USA), (ii) anti-Calpain5 as described above or (iii) goat polyclonal actin antibody (Santa Cruz) dilution 1:1000, all at 4°C, followed by secondary antibody for 1 h at room temperature in PBS/T%1% powdered milk. The membrane was then washed twice in TBS for 5 min at room temperature and immersed in a horse-radish peroxidase color developer buffer (Bio-Rad Laboratories) for 30 min. Photographs were taken immediately after color development.

**Statistics**

Quantitative PCR results were compared using t-test. H scores were compared using ANOVA on ranks with post hoc test to distinguish individual differences.

**Results**

**Calpain5 is expressed in human endometrium and decidua**

In normal human endometrium, IHC demonstrated that Calpain5 was expressed in endometrial glandular epithelial cells and stromal cells throughout the menstrual cycle (Fig. 1A–C). Calpain5 was also highly expressed in first trimester decidua. Calpain5 expression was localized within the cytoplasm of stromal cells and endometrial glandular epithelial cells. No significant variation was observed in Calpain5 expression throughout the menstrual cycle in the glands. Calpain5 expression was minimally increased in stroma. Expression increased in first trimester decidua (Fig. 1D). Figure 2 demonstrates that the average H score was slightly increased, but not significantly in stromal cells in the secretory phase, but did increase in decidua. The level of Calpain5 mRNA in proliferative and secretory endometrium as well as in decidua was assayed by real-time RT–PCR. Calpain5 expression was increased in the secretory phase and greatly increased in decidua compared with the proliferative phase (Fig. 1D).

**Calpain5 expression is regulated by hoxa10**

To determine if Calpain5 was regulated by HOXA10, we performed transient transfection with a constitutive HOXA10 expression construct or HOXA10 siRNA. Successful transfection and increased/decreased
HOXA10 expression was confirmed using the real-time PCR (Fig. 3). Real-time PCR demonstrated that pcDNA3.1/HOXA10 transfection increased Calpain5 mRNA expression 11-fold in HESC compared with that obtained using empty pcDNA as a control ($P$, 0.05). Calpain5 mRNA expression was decreased 23-fold after HOXA10 siRNA transfection compared with control siRNA transfection in HESC ($P$, 0.05). Calpain5 was regulated by HOXA10 in stromal cells. Transfection with either pcDNA3.1/HOXA10 or HOXA10 siRNA did not alter Calpain5 expression in Ishikawa cells suggesting that Calpain5 was not regulated by HOXA10 in these human endometrial endocarcinoma cells (data not shown).

**Calpain5 expression was decreased in endometriosis**

Calpain5 expression was decreased in the ectopic endometrium obtained from women with endometriosis, a tissue that has previously been shown to have diminished HOXA10 expression (Browne and Taylor, 2006) (Fig. 4A). IHC results were quantified using the $H$ score results. Results obtained using endometrium from normal fertile controls were compared with those obtained from women with the diagnosis of endometriosis. In the eutopic endometrium of women with endometriosis, there was a significant decrease in Calpain5 protein expression compared with fertile controls ($P$, 0.05). Similarly, Calpain5 expression was decreased in endometriosis.
in the ectopic endometriosis, there was an equivalent decrease in Calpain5 expression in both stromal and glandular cells; levels of Calpain5 expression in either the eutopic or ectopic endometrium were ~50% of that seen in fertile controls (Fig. 4B). These results were confirmed using real-time RT–PCR, and western blot, that both also showed a similar decrease in Calpain5 mRNA and protein, respectively, in endometriosis compared with endometrium obtained from controls (Fig. 4C and D).

To determine if decreased Calpain5 expression affected apoptosis, activated caspase-3 expression was determined by western analysis. Figure 4D demonstrates significantly decreased activated caspase-3 protein expression in endometrium obtained from women with endometriosis.

**Discussion**

Calpain5 is a member of the cytoplasmic cysteine protease family, a group of Ca\(^{++}\) regulated enzymes. The Calpain family has four members whose functions are not well characterized. Calpains in general, and in particular Calpain5, have been implicated in multiple cellular processes including cellular differentiation and apoptosis (Koichi *et al.*, 2004). Two observations that have implicated Calpain in apoptosis include the expression of Calpain during cell death and the inhibition of apoptosis by various Calpain inhibitors (Bao *et al.*, 2002). Calpains have been implicated in the activation of caspase-induced apoptosis in neurons, breast and ovary (Bozyczko-Coyne *et al.*, 2001; Bao *et al.*, 2002). However, Calpains have also been demonstrated to have an unusual role in the activation of caspase-independent apoptosis during platelet activation (Houwerzijl *et al.*, 2005). Mitochondria are believed to have an important role in Calpain activation of caspase-independent pathway by the release of apoptosis inducing factor (Artus *et al.*, 2006). Caspase-independent apoptosis pathways are important mechanisms for triggering a response to cytotoxic agents or other death stimuli when the caspase-mediated routes fail (Artus *et al.*, 2006). Calpain5 is expressed in multiple human tissues, including the uterus and placenta (Waghray *et al.*, 2004). The exact function of Calpain5 in these tissues is not known. Calpain5 is highly conserved between human and mice—87% at the mRNA and 95% at the protein level. In murine models, Calpain5 expression is low in the uterus before pregnancy, but increases during the ongoing gestation, particularly in decidual cells (Nie *et al.*, 2003). The decidual cells are the only cells that express Calpain5 in mice during early and late pregnancy. These observations are consistent with our results that showed decidual Calpain5 expression in humans during the first trimester of pregnancy.

Here, we showed that Calpain5 was expressed in human endometrial stromal cells during the menstrual cycle. HOXA10 regulated the expression of Calpain5 in both stroma and decidua. Epithelial cell expression was present but not as highly regulated; this is likely due to the limited role of these cells in later pregnancy, including their lack of contribution to decidua. A cell type specific cofactor likely interferes with epithelial Calpain5 regulation in the secretory phase (Taylor, 2002). Similarly, the lack of a dramatic increase in Calpain5 in the secretory phase, a time when HOXA10 expression is increased, is likely due to the fact that increased HOXA10 expression at that time is primarily a glandular increase; HOXA10 is newly expressed in the glands at that time, whereas Calpain5 expression is not significant in the epithelial glandular cells. These results differ slightly from those of Nie *et al.* (2003) who found increased Calpain5 expression during the later phases of the estrous cycle in the mouse; however, decidualization occurs earlier in mouse than in human (Taylor, 2002). The earlier increase in mouse may reflect the relative earlier decidualization in this species. Similar to the mouse, human Calpain5 showed a significant increase in expression in first trimester decidua when compared with phases of the menstrual cycles (Nie *et al.*, 2003). In both species, the

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**Figure 4:** (A) Calpain5 protein expression in stromal cells and glands in eutopic endometrium of controls (left) and ectopic endometrium of endometriosis patients (right). (B) H score was used to quantify expression. C indicates controls, and Eu and Ec represent the eutopic and ectopic endometrium from women with endometriosis, respectively. *P < 0.05. (C) Real-time RT–PCR was used to quantify Calpain5 mRNA expression in the eutopic endometrium of women with endometriosis (E) and controls (C). *P < 0.05. (D) Western analysis was used to compare expression of Calpain5, the apoptosis marker, activated caspase-3, in the endometrium of controls (C) and women with endometriosis (E). Beta actin was used as a control.
stromal/decidual cell increase in Calpain5 expression is temporally and cellularity correlated with increased HOXA10 expression.

HOXA10 is essential for embryonic uterine development and persists in the adult uterus where it is necessary for the implantation process (Satokata et al., 1995; Benson et al., 1996; Block et al., 2000). The function of HOXA10 in the uterus is well defined; however, the molecular mechanism underlying these actions is poorly understood. HOXA10 is a transcription factor that regulates a battery of target genes. These include genes that are important for decidualization including IGF-binding protein 1 (IGFBP-1), β3 integrin, EMX2 and prolactin (Daftary et al., 2002; Troy et al., 2003; Daftary and Taylor 2004; Fei et al., 2005; Kim et al., 2007). The molecular mechanism by which HOXA10 directs endometrial receptivity and decidualization includes the regulation of Calpain5 in human endometrial stromal cells. This in turn likely regulates apoptosis of the endometrium in response to decidualization and pregnancy.

Here, we also showed decreased levels of Calpain5 expression in endometrium from patients with endometriosis. Endometriosis is characterized by the presence of endometrium outside of the uterine cavity. The most widely accepted theory for the etiology of endometriosis is retrograde menstruation and perpetuation of the endometrial tissue in ectopic locations (Sampson, 1924). The pathogenesis of the disease is associated with increased proliferation and decreased cell death. An increase in cell proliferation without an equivalent change in apoptosis has been described in the endometrium of women with endometriosis (Wingfield et al., 1995). One function of the Calpain family is to regulate apoptosis, activating both caspase-dependent and caspase-independent pathways. The action of Calpain5 in the uterus is likely also related to the regulation of apoptosis. We hypothesize that Calpain is a molecular mediator through which HOXA10 controls apoptosis in the normal endometrium. HOXA10 expression is decreased in eutopic and eutopic endometrium from women with endometriosis (Gui et al., 1999; Taylor et al., 1999; Browne and Taylor, 2006; Kim et al., 2007), which in turn leads to reduced expression of Calpain5. The decreased expression of Calpain5 in endometriosis limits apoptosis as demonstrated by the decrease in the apoptosis marker activated caspase-3. This in turn may promote the perpetuation of this tissue in ectopic locations (Lee et al., 2006; Kayisli et al., 2007). Moreover, the lower expression of Calpain5 in eutopic tissue and consequent decreased apoptosis may be related to the reduction in endometrial receptivity and fertility seen in these women.

In conclusion, we report the expression and molecular regulation of Calpain5 in human endometrium and decidua. Calpain5 is regulated by HOXA10 in the endometrial stromal cells and likely in decidua as well. Aberrant expression of Calpain5 is associated with endometriosis. The regulated expression of this protein is likely a mediator of apoptosis in both normal uterine physiology and disease.

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References


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