Stanniocalcin-1 expression in normal human endometrium and dysregulation in endometriosis

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Objective: To determine expression of stanniocalcin-1 (STC1) in human endometrium with and without endometriosis and its regulation by steroid hormones.
Design: Laboratory study.
Setting: University.
Patient(s): Nineteen women with endometriosis and 33 control women.
Intervention(s): Endometrial biopsy and fluid sampling.
Main Outcome Measure(s): Analysis of early secretory (ESE) and midsecretory (MSE) endometrial secretomes from fertile women with the use of nano–liquid chromatography–dual mass spectrometry; real-time quantitative polymerase chain reaction, and immunohistochemistry for STC1 and its receptor calcium-sensing receptor (CASR) mRNA and proteins in endometrium with and without endometriosis; evaluation of STC1 and CASR mRNA expression in endometrial stromal fibroblasts (eSF) from women with and without endometriosis decidualized with the use of E_2P or 8-bromo-cyclic adenosine monophosphate (cAMP).
Result(s): STC1 protein was strongly up-regulated in MSE versus ESE in endometrial fluid of fertile women. STC1 mRNA significantly increased in MSE from women with, but not from those without, endometriosis, compared with proliferative endometrium or ESE, with no significant difference throughout the menstrual cycle between groups. STC1 mRNA in eSF from control women increased >230-fold on decidualization with the use of cAMP versus 45-fold from women with endometriosis, which was not seen on decidualization with E_2/P. CASR mRNA did not exhibit significant differences in any condition and was not expressed in isolated eSF. STC1 protein immunoexpression in eSF was significantly lower in women with endometriosis compared with control women.
Conclusion(s): STC1 protein is significantly up-regulated in MSE endometrial fluid and is dysregulated in eutopic endometrial tissue from women with endometriosis. It is likely regulated by cAMP and may be involved in the pathogenesis of decidualization defects. (Fertil Steril® 2016;106:681–91. ©2016 by American Society for Reproductive Medicine.)
Key Words: Stanniocalcin-1, human endometrium, endometriosis, stromal fibroblasts, decidualization, proteomics

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Human endometrium is a dynamic tissue that undergoes cyclic morphologic and molecular changes under a changing hormonal milieu and plays a central role in human implantation. Endometrial tissue in humans and other mammals is a target of extensive research with the goal of increasing understanding of its physiology and pathophysiology and improving treatment of gynecologic pathologies such as endometriosis,

adenomyosis, recurrent pregnancy loss, as well as implantation failure and unexplained infertility.

High-throughput "omics" studies of global gene expression profiling in human endometrium have identified stanniocalcin-1 (STC1) as an important player in normal and diseased endometrial functions. STC is expressed in pre-pregnancy endometrium and early pregnancy decidua in rats and pigs, is regulated by estrogen and progesterone, and has been suggested as an implantation marker in pig endometrium (1, 2). In humans, consistent midsecretory endometrial (MSE) STC1 gene expression was demonstrated in patients who conceived with the help of assisted reproductive technologies (3). STC1 was significantly up-regulated in microarray analysis of MSE of the normal menstrual cycle compared with early secretory endometrium (ESE) (4), and was down-regulated in microarray analysis of MSE from women with unexplained infertility versus fertile control women (5), suggesting a possible role in human endometrial receptivity and implantation. Placental expression of STC1 was documented in a well designed microarray study of women with pregnancy complications, which demonstrated increased STC1 in placenta and serum at term in women with pregnancy complications, particularly preeclampsia and small-for-gestational-age babies (6).

STC1 is a glycoprotein phosphorylated by protein kinase C (7), initially described in a bony fish, and produced by unique endocrine glands, the corpuscles of Stannius, eliciting antihypercalcemic and antihypophosphatemic responses in various tissues (8, 9). In mammals, STC1 is expressed in a wide variety of tissues, and interestingly is not detected in the circulation under normal conditions except during pregnancy (9–11), suggesting an autocrine/paracrine rather than an endocrine function. STC1 in mammals is not necessarily directly linked to calcium/phosphorus pathways, but rather is regulated by multiple factors (9). Its roles in calcium homeostasis, bone, and muscle formation, angiogenesis, and reproduction were demonstrated in experiments with transgenic mice that experienced growth retardation and small litter size (11, 12).

The STC receptor, calcium-sensing receptor (CASR), is a G-protein–coupled receptor initially identified in bovine parathyroid cells and expressed in wide variety of tissues, including ovaries and uterus in human and rodents (13–15). Its major physiologic role is thought to be maintenance of calcium homeostasis, including regulation of secretion, gene expression, cell proliferation, differentiation, and apoptosis (13, 16). CASR was found to mediate STC1 secretion in response to extracellular calcium fluctuation in fish (17). It is expressed in first-trimester and term human placenta (18, 19) and is induced during implantation and decidualization in rat uterus (15).

Based on the above, there are limited data on human endometrial STC1 expression, regulation, and signaling through its receptor. We therefore aimed to investigate expression of STC1 and its receptor in human endometrium and regulation of STC1 in human endometrial stromal fibroblasts (eSF) in women without and with endometriosis in an effort to determine potential roles for this protein/receptor complex in normal and abnormal endometrial function.

MATERIALS AND METHODS

Study Subjects and Materials

Endometrial samples were obtained through the University of California–San Francisco (UCSF) National Institutes of Health Human Endometrial Tissue and DNA Bank and from healthy volunteers at the Department of Obstetrics and Gynecology, Karolinska University Hospital, Huddinge, Sweden, and Tartu University Women's Clinic, Tartu, Estonia, with appropriate Institutional Review Board (UCSF) and Ethics Committee (Karolinska Institute and University of Tartu) approvals. Written informed consents were obtained from all participating subjects. Table 1 summarizes characteristics of all participating women and the techniques applied for analyzing the samples.

Endometrial secretome samples for proteomics analysis were collected from early secretory (ESE, LH+2) and midsecretory (MSE, LH+8) phase endometria during the same menstrual cycle from fertile healthy volunteers ($n = 6$; age 31 ± 1.5 y). The day of the LH surge (LH+0) was determined with a urinary ovulation prediction test (Kaigert), which is an accepted and validated method for predicting ovulation in the clinical and translational research setting; it is also patient friendly, because it is a noninvasive test applicable for self-use at home. However, being aware of substantial interpatient variability, we supported our endometrial dating with endometrial histology. Histology samples were collected for confirmation of endometrial dating according to the criteria of Noyes et al. (20) and were found to correspond to days 19–24 of the 28-day cycle.

Endometrial biopsies for polymerase chain reaction (PCR), immunohistochemistry (IHC), and cell culture experiments were obtained from subjects with and without endometriosis ($n = 19$ and $n = 27$, respectively). Menstrual cycle phase was assigned by the day of LH surge and endometrial histology as above. In addition, all samples used for mRNA expression analysis were assigned cycle phase by means of bioinformatics methods (21). Only samples where all evaluation criteria were in agreement were used in the study.

Control subjects (no endometriosis) were healthy women 35.6 ± 0.86 years of age (range 23–49 y) undergoing gynecologic surgery for pelvic pain (with no endometriosis found during laparoscopy) or management of fibroids, healthy volunteers without uterine pathology, or women undergoing laparoscopic tubal ligation. Control subjects had regular menstrual cycles, were not pregnant, had no history of endometriosis, and had not been on hormonal treatment for at least 3 months before tissue sampling.

Women with endometriosis participating in the study were 35.9 ± 1.65 years of age (range 22–49 y), were not pregnant, and did not use any hormonal medication for at least 3 months before surgery. The diagnosis of endometriosis was based on visualization of lesions during laparoscopy and confirmed by histology. Staging of endometriosis was defined according to the revised American Fertility Society classification system (22).

Out of 19 samples from women with endometriosis and 27 samples from control women (without endometriosis), 14 and 10 whole tissue samples, respectively, were used for real-time quantitative reverse-transcription (RT) PCR ($n = 6$

TABLE 1

Characteristics of patients who donated endometrial biopsy or fluid samples for the study.

Patient	Cycle phase	Type of experiment	Diagnosis at laparoscopy	Age, y	Ethnicity
Endometriosis					
233	MSE	Cell culture and decidualization, qPCR	Mild endometriosis, pelvic pain	31	White
243	ESE	Cell culture and decidualization, qPCR	Minimal endometriosis, bilateral ovarian cyst, intramural myoma	46	White
267	LSE	Cell culture and decidualization, qPCR	Mild endometriosis, pelvic pain	32	White
268	Not Evaluated	Cell culture and decidualization, qPCR	Mild endometriosis, intramural myoma	38	Asian
288	PE	Cell culture and decidualization, qPCR	Severe endometriosis	22	White
270	PE	Cell culture and decidualization, qPCR	Mild endometriosis	49	White
651	PE	Tissue qPCR	Severe endometriosis/ endometrioma, chronic pelvic pain, fibroid uterus	37	White
575	PE	Tissue qPCR	Severe endometriosis/ endometrioma, chronic pelvic pain	26	Unknown
587	PE	Tissue qPCR	Severe peritoneal, rectovaginal endometriosis, chronic pelvic pain	36	White
ST90	PE	Tissue qPCR	Severe pelvic endometriosis, chronic pelvic pain	42	White
607	ESE	Tissue qPCR	Severe peritoneal and rectovaginal endometriosis, chronic pelvic pain	24	Asian
517	ESE	Tissue qPCR	Severe peritoneal endometriosis, chronic pelvic pain, fibroids, infertility	35	Unknown
ST112	ESE	Tissue qPCR	Severe peritoneal endometriosis, chronic pelvic pain	38	White
645	MSE	Tissue qPCR	Severe peritoneal endometriosis, chronic pelvic pain	39	Asian Indian
678	MSE	Tissue qPCR, immunohistochemistry	Severe endometriosis, fibroid uterus, chronic pelvic pain	44	White
ST96	MSE	Tissue qPCR	Severe peritoneal endometriosis, chronic pelvic pain	31	White
635	MSE	Immunohistochemistry	Mild endometriosis, bleeding, intramural myoma	42	White
516	MSE	Immunohistochemistry	Severe endometriosis, intramural myoma	34	Asian
540	MSE	Immunohistochemistry	Severe rectovaginal endometriosis	37	White
No endometriosis					
229	LSE	Cell culture and decidualization, qPCR	Pelvic pain (no endometriosis at laparoscopy)	47	White
236	PE	Cell culture and decidualization, qPCR	Symptomatic pelvic prolapse	47	White
237	ESE	Cell culture and decidualization, qPCR	Intramural myoma, left paratubal cyst	39	White
238	ESE	Cell culture and decidualization, qPCR	Endometrial polyp	41	Black
285	PE	Cell culture and decidualization, qPCR	Intramural myoma, pelvic adhesions	37	White
293	PE	Cell culture and decidualization, qPCR	Intramural myoma	49	White
310	PE	Cell culture and decidualization, qPCR	Intramural myoma, enterocele	41	Asian
ME 09	PE	Tissue qPCR	Undesired fertility	37	White
M182	PE	Tissue qPCR	Healthy volunteer	34	White
M169	PE	Tissue qPCR	Healthy volunteer	32	White
ETB038	PE	Tissue qPCR	Egg donor, natural cycle biopsy	23	White
ETB048	PE	Tissue qPCR	Egg donor, natural cycle biopsy	28	Black
ETB065	PE	Tissue qPCR	Egg donor, natural cycle biopsy	29	White
ME34	ESE	Tissue qPCR	Undesired fertility	33	White
ME13	ESE	Tissue qPCR	Pelvic pain (no endometriosis at laparoscopy)	33	Black

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TABLE 1

Continued.

Patient	Cycle phase	Type of experiment	Diagnosis at laparoscopy	Age, y	Ethnicity
ME42	ESE	Tissue qPCR	Undesired fertility	37	White
ME36	MSE	Tissue qPCR	Undesired fertility	35	Black
ME50	MSE	Tissue qPCR	Undesired fertility	35	White
16	MSE	Tissue qPCR	Healthy fertile volunteer	42	Unknown
22	MSE	Tissue qPCR	Healthy fertile volunteer	40	Unknown
30	MSE	Tissue qPCR	Healthy fertile volunteer	35	Unknown
MM1	MSE	Immunohistochemistry	Healthy fertile volunteer	36	White
MM2	MSE	Immunohistochemistry	Healthy fertile volunteer	41	White
MM5	MSE	Immunohistochemistry	Healthy fertile volunteer	30	White
MM16	MSE	Immunohistochemistry	Healthy fertile volunteer	35	White
ST18	MSE	Immunohistochemistry	Healthy fertile volunteer	37	White
ST21	MSE	Immunohistochemistry	Healthy fertile volunteer	35	White
Secr1	ESE, MSE	Mass spectrometry, histology	Healthy fertile volunteer	30	White
Secr2	ESE, MSE	Mass spectrometry, histology	Healthy fertile volunteer	32	White
Secr3	ESE, MSE	Mass spectrometry, histology	Healthy fertile volunteer	32	White
Secr4	ESE, MSE	Mass spectrometry, histology	Healthy fertile volunteer	33	White
Secr5	ESE, MSE	Mass spectrometry, histology	Healthy fertile volunteer	30	White
Secr6	ESE, MSE	Mass spectrometry, histology	Healthy fertile volunteer	29	White

Note: ESE = early secretory phase endometrium; LSE = late secretory phase endometrium; MSE = midsecretory phase endometrium; qPCR = quantitative polymerase chain reaction; PE = proliferative phase endometrium.

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and n = 4 in proliferative phase (PE) from women with and without endometriosis, respectively; n = 3 in ESE in each group; and n = 5 and n = 3 in MSE from women with and without endometriosis, respectively). IHC was performed on MSE samples: n = 4 and n = 6 for endometriosis and no endometriosis groups, respectively.

Isolated eSF from women with (n = 6) and without endometriosis (n = 7) were used in cell culture experiments (as shown previously, cycle phase does not confound response [23]).

Early Secretory and Midsecretory Phase Endometrial Fluid Proteomics Analysis

The secretomes were obtained by uterine flushing, i.e., injecting 0.5 mL of phosphate-buffered saline solution (PBS) into the uterine cavity, followed by aspiration of the fluid. Before protein extraction, the samples were precleared with centrifugation at 450*g* for 5 minutes. The collected secretomes were separated into six fractions according to molecular weight with the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen–Thermo Fisher Scientific). Proteins were reduced, alkylated, and in-gel digested with the use of dimethylated porcine trypsin (Sigma) followed by analysis with nano–liquid chromatography–dual mass spectrometry (Dionex Ultimate 3000 RSLC and Q Exactive MS/MS; Thermo Fisher Scientific). The label-free peptide elution profiles of different study subjects were identified and quantified with the use of the Maxquant software package (UniprotKB human reference proteome database, September 2014 version) (24). Label-free data were normalized with the use of the MaxLFQ (25) algorithm and compared by means of paired *t* test statistics.

Total RNA Isolation and Real Time RT-PCR

Total RNA from endometrial biopsies and cells was purified with the use of the Qiagen RNeasy Plus Mini kit according to the manufacturer's instructions. Samples were stored in RNase-free H_2O and quantified by spectroscopy, and the purity was analyzed with the use of the 260/280 absorbance ratio. RNA quality and integrity were analyzed with the use of the Agilent Bioanalyzer 2100 with all samples having high-quality RNA (RNA integrity number 9.7–10.0). For real-time quantitative RT-PCR analysis, 1 μg RNA was converted to cDNA with the use of the iScript cDNA Synthesis kit (Bio-Rad Laboratories). Duplicate mRNAs were pooled from each set of treatments. The real-time RT-PCR reaction was carried out for 40 cycles with primers to insulin growth factor–binding protein 1 (IGFBP1) (eSF only), PRL (eSF only), STC1 (eSF and tissue), and CASR (eSF and tissue). Relative gene expression was normalized with the use of RPL19 as the internal reference, which has been shown to have reproducibility and stable expression in endometrial tissue (23, 26). The following primer sequences were used: IGFBP1: forward, 5′-CTATGATGGCTCGAAGGCTC-3′, reverse, 5′-TTCTTGTTG CAGTTTGGCAG-3′; PRL: forward, 5′-CATCAACAGCTGCCA CACTT-3′, reverse, 5′-CGTTTGGTTTGCTCCTCAAT-3′; STC1: forward, 5′-GCAGGAAGAGTGCTACAGCAAG-3′, reverse, 5′-CATTCCAGCAGGCTTCGGACAA-3′; CASR: forward, 5′-CT CTTCACCAATGGCTCCTGT-3′, reverse, 5′-CCACACTCATCAA AGGTCACCTG-3′; and RPL19: forward, 5′-GCAGATAATGGG AGGAGCC-3′, reverse, 5′-GCCCATCTTTGATGAGCTTC-3′.

Immunohistochemistry

Paraffin-embedded sections (4 μm) of endometrial biopsies were deparaffinized and washed. Antigen retrieval was performed by submerging slides in citrate buffer (1× Citra Plus; Vector Laboratories) at 90°C for 10 minutes. Endogenous peroxidase was blocked with the use of 3% H_2O_2 in methanol for 10 minutes. Sections were then incubated with 10% blocking normal goat or horse serum in PBS (Vector Laboratories) for 30 minutes for anti-STC1 (rabbit anti–human

polyclonal; Santa Cruz Biotechnology) or anti-CASR (mouse anti–human monoclonal; Abcam) antibodies, respectively. Thereafter, STC1 and CASR primary antibodies were applied at dilutions of 1:150 and 1:200, respectively, at 4°C overnight. Nonimmune IgG of equivalent concentration from the same species was used as negative control.

The binding of primary antibodies was detected with the use of secondary antibodies: goat antirabbit and horse anti-mouse (Vector Laboratories) for 1 hour at room temperature, at a 1:300 dilution for both. After 30 minutes' incubation with ABC complex (Vectastain Elite ABC immunoperoxidase detection kit; Vector Laboratories), diaminobenzidine–hydrogen peroxide substrate (DAB kit; Vector Laboratories) was added to the slides; slides were rinsed with distilled water, counterstained with hematoxylin (Vector Laboratories), and mounted with the use of mounting medium. A Leica microscope was used to evaluate and photograph the slides.

Semiquantitative evaluation of IHC was performed by two observers, blinded to the identity of the slides, using a grading system; each sample was analyzed twice. Staining intensity and the number of stained cells were graded on the following numeric scale: 0 = no staining; ± = few stained cells; + = faint staining; ++ = moderate staining; and +++ = strong staining. The average values from the two observers are presented.

Endometrial Stromal Cell Isolation and Culture

Fresh endometrial tissue was digested with the use of collagenase as described previously (27). Human eSF were separated from epithelium based on size and plated with the use of DMEM/MCDB-105 medium containing 10% charcoal-stripped fetal bovine serum (FBS), insulin (5 μg/mL), gentamicin, penicillin, and streptomycin, as described previously (23, 26). At passage 2, cells were cultured to near confluence in the same medium, followed by changing to low-serum medium (2% FBS) and cultured for 24 hours before the onset of treatment. All cell culture experiments were conducted with the use of 2nd to 4th cell passages; cell purity (99% stromal fibroblasts) was determined by means of immunostaining with cytokeratin, vimentin, and CD45 antibodies, as described previously (23).

Decidualization of Human Endometrial Stromal Fibroblasts

Human eSF were treated with 0.5 mmol/L 8-bromo-cyclic adenosine monophosphate (cAMP) for 96 hours or with E_2 (10 nmol/L) alone or E_2 (10 nmol/L) plus P (1 μmol/L) (E_2/P) for 14 days or vehicle. The duration and hormone concentrations were optimized previously (23). 8-Bromo-cAMP and steroid hormones were obtained from Sigma-Aldrich. All cultures were performed in duplicate. Culture media were changed every other day. Time "zero" (t = 0) control subjects were collected before initiation of treatment. Cells lysed in RLT lysis buffer (Qiagen) containing 0.1% β-mercaptoethanol and culture medium were collected after 96 hours of incubation (cAMP-treated samples) or 14 days (E_2/P-treated samples).

Enzyme-linked Immunosorbent Assay

IGFBP1 and PRL proteins in the culture media were measured to assess the decidualization status of eSF with the use of ELISA, according to the manufacturers' instructions (Alpha Diagnostic International and Diagnostic Systems Labs, respectively). All samples were assayed in duplicate. A standard curve was run in each experiment. Levels of IGFBP1 and PRL for each sample were normalized to total RNA. Inter- and intra-assay coefficients of variation for the IGFBP1 ELISA were 5%–7.4% and 2.4%–3.4%, respectively, and for PRL were 6.7%–10.4% and 7.8%–8.2%, respectively. Minimum detection limits for PRL and IGFBP1 assays were 0.14 ng/mL and 0.4 ng/mL, respectively.

Statistical Evaluation

Statistical analysis for the qunatitative RT-PCR and immunohistochemistry were performed with the use of the nonparametric one-way analysis of variance Kruskal-Wallis test. Results from the ELISA analysis demonstrated a normal distribution, and a two-tailed Student *t* test assuming unequal variances was applied. Significance was determined at $P \leq .05$.

RESULTS

Stanniocalcin-1 Protein is Up-regulated in Midsecretory Phase Endometrial Secretome

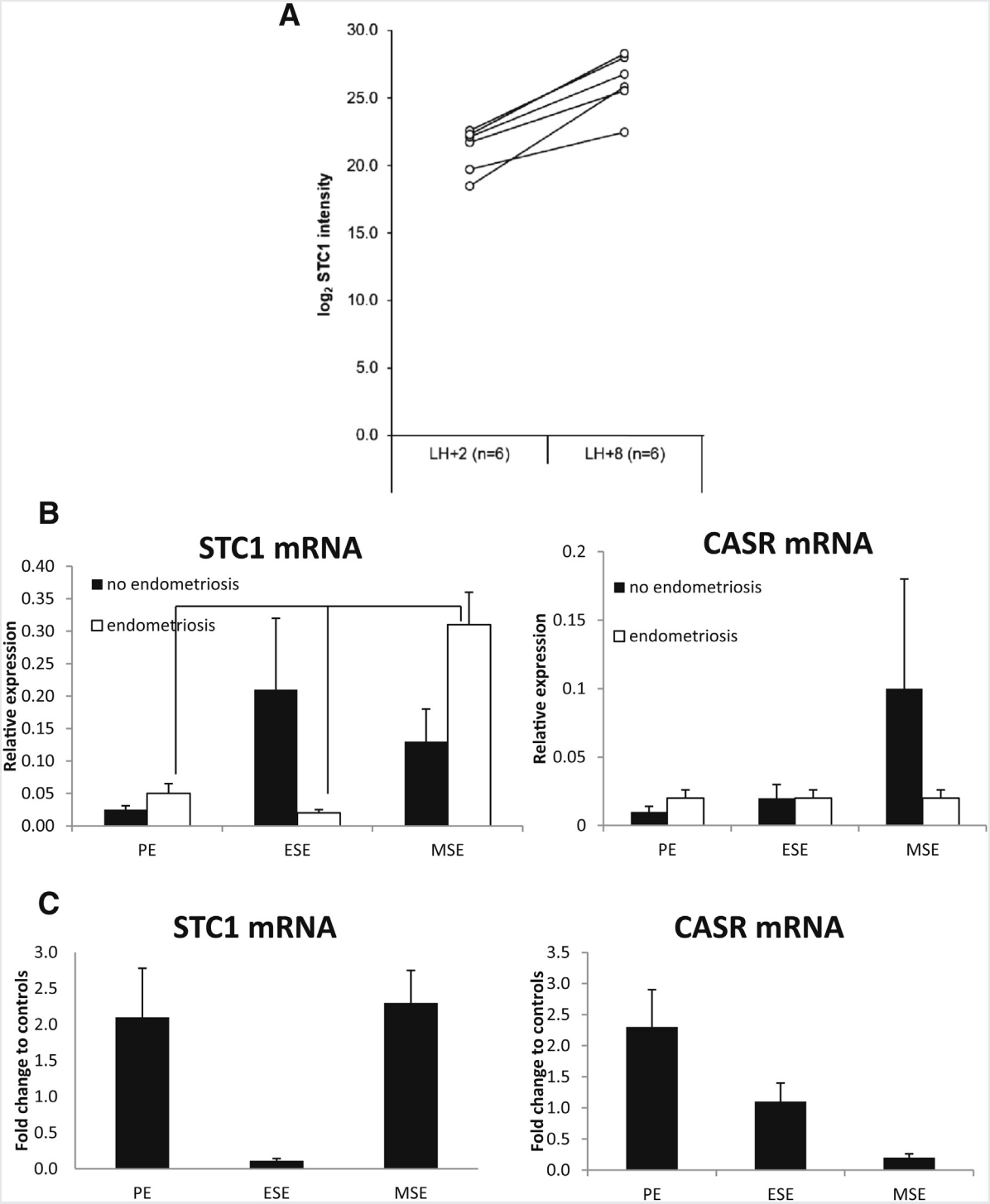
We identified STC1 protein with eight peptides (Supplemental Table 1, available online at www.fertstert.org) in endometrial aspirates from fertile women without endometriosis. With the use of label-free proteomics data, we observed STC1 protein to be significantly ($P=.0004$) up-regulated (mean fold change +39.6) in MSE (LH+8) versus ESE (LH+2) endometrial fluid or secretome (Fig. 1A).

Stanniocalcin-1 and CASR Expression in Human Endometrial Tissue, mRNA, and Protein

In women without endometriosis, endometrial STC1 mRNA expression fluctuated throughout the menstrual cycle; however, the differences did not reach statistical significance ($P=.088$; Fig. 1B). In endometrium from women with endometriosis, STC1 mRNA expression significantly increased in MSE compared with PE or ESE ($P=.043$; Fig. 1B). However, when comparing STC1 mRNA expression in nonendometriosis and endometriosis samples, there was no statistically significant difference throughout the menstrual cycle ($P>.1$; Fig. 1C). On the other hand, STC1 receptor CASR mRNA did not change significantly throughout the menstrual cycle in both women with and without endometriosis ($P>.1$; Fig. 1B), and there was no significant difference when its expression was compared between subjects with versus without endometriosis ($P>.1$; Fig. 1C).

Immunohistochemistry was used to evaluate the specific cell-type expression of STC1 protein in midsecretory human endometrium. We observed cytoplasmic STC1 protein expression in both epithelial and stromal cells from women with and without endometriosis (Fig. 2A). STC1 immunoexpression in

FIGURE 1



(**A**) Stanniocalcin-1 (STC1) protein level is increased in receptive (LH+8) phase endometrial secretome. Paired data from six fertile women are presented. *X*-axis values were obtained by taking log2 from summed peptide peak areas of STC1 protein in different samples. Paired *t* test $P=.0004$, with significance accepted at $P \leq .05$. (**B**) Relative expression of STC1 and calcium-sensing receptor (CASR) mRNA in human endometrial tissue throughout the menstrual cycle in women with and without endometriosis. (**C**) Expression of STC1 and CASR mRNA in human endometrial tissue throughout the menstrual cycle in women with endometriosis normalized to samples from women without endometriosis and expressed as fold change. ESE = early secretory phase endometrium; MSE = midsecretory phase endometrium; PE = proliferative phase endometrium. Nonparametric one-way analysis of variance Kruskal-Wallis test was used for statistical analysis, and significance was accepted at $P \leq .05$.

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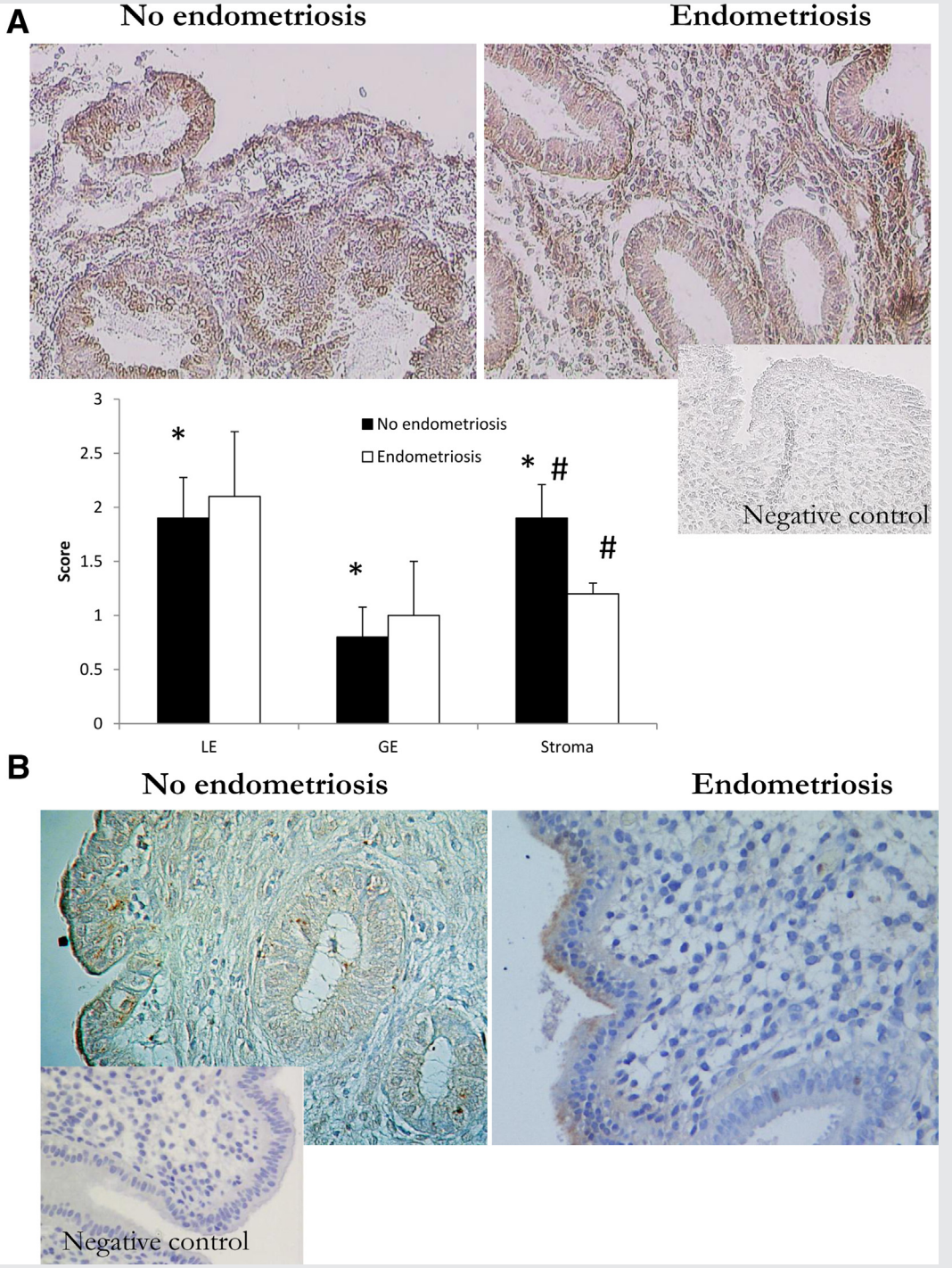
FIGURE 2



(**A**) Immunohistochemical evaluation of STC1 protein expression in midsecretory human endometrium from women without and with endometriosis. LE = luminal epithelium; GE = glandular epithelium. *Statistically significant difference between LE and GE and between GE and stroma: $P<.02$. #Statistically significant difference in stromal staining between no endometriosis and endometriosis samples: $P=.04$. Magnification ×200. One-way Kruskal-Wallis test was used for statistical analysis, with significance accepted at $P\leq.05$. (**B**) Immunohistochemical evaluation of CASR protein expression in mid-secretory human endometrium from women without and with endometriosis. No stromal expression of CASR was observed, and no difference in epithelial (luminal and glandular) staining was noted between specimens from women with versus without endometriosis. Magnification ×200. Abbreviations as in Figure 1.

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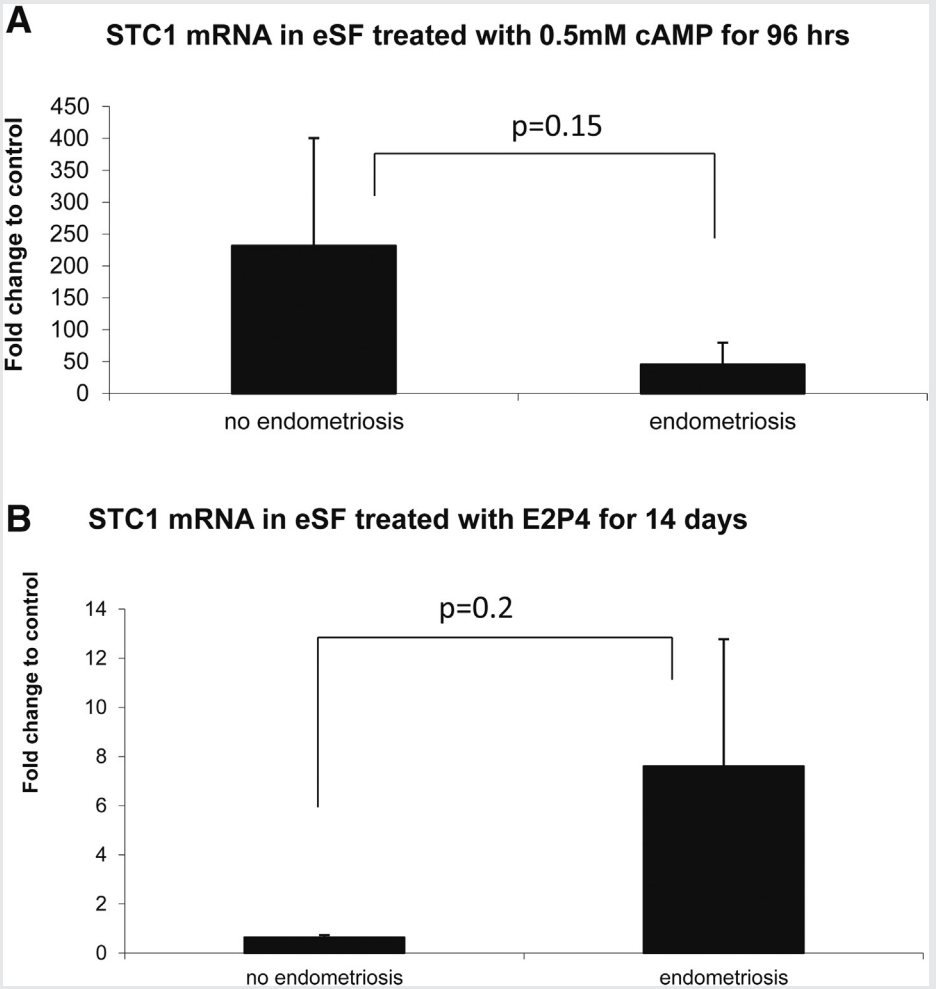
disease-free women was significantly higher in luminal epithelium and stroma compared with glandular epithelium ($P<.02$). In endometriosis, there was no significant difference in STC1 protein expression in epithelial cells; however, its expression in endometrial stromal cells was significantly decreased in women with endometriosis compared with control women ($P=.04$; Fig. 2A). When analyzing CASR protein immunoexpression, no stromal expression of CASR was observed, and it had faint cytoplasmic expression in epithelial cells, both luminal and glandular, which was not different between specimens from women with versus without endometriosis (Fig. 2B).

Stanniocalcin-1 and CASR Expression in Cultured Human Endometrial Stromal Fibroblasts and Effect of Decidualization Stimuli

Next, we investigated the expression of STC1 and CASR mRNA in cultured eSF and their response to decidualization stimuli in vitro. We subjected eSF to short-term decidualization with the use of cAMP for 96 hours or long-term decidualization with P (with estrogen priming) for 14 days, as described in the Materials and Methods section. Decidualization of eSF was assessed by means of IGFBP1 and PRL mRNA and protein secretion (data not shown, but significance reported earlier [23]).

STC1 mRNA expression was dramatically up-regulated by >230-fold by cAMP in control women ($P=.02$ vs. vehicle control) compared with 45-fold in women with endometriosis ($P=.001$ vs. vehicle control). However, the difference in degree of decidualization between samples from subjects with versus without endometriosis did not reach statistical significance owing to large variability between samples ($P=.15$; Fig. 3A). In contrast, there was no significant change in STC1 mRNA in response to E_2/P stimulus by eSF from women without endometriosis, which did not differ significantly from the response of eSF from women with endometriosis ($P=.2$;

FIGURE 3



Expression of STC1 mRNA in decidualized human endometrial stromal fibroblasts (eSF) with (**A**) 0.5 mmol/L cyclic adenosine monophosphate (cAMP) for 96 hours, normalized to 96-hour control, or (**B**) 10 nmol/L E_2/1 μmol/L P for 14 days, normalized to 14-day control. One-way Kruskal-Wallis test was used for statistical analysis, with significance accepted at $P\leq.05$. Abbreviations as in Figure 1.

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Fig. 3B). CASR mRNA was not detected in any of the eSF samples analyzed in both groups.

DISCUSSION

Endometrial STC1 is Dysregulated in Endometriosis

We demonstrated that STC1 and CASR are expressed in human endometrial tissue. Despite STC1 mRNA detection during various microarray studies on human endometrium under different conditions, there were no attempts to characterize its expression in endometrial cell types and involvement in the decidualization process until now. Yet the very robust up-regulation of STC1 protein in endometrial fluid secretome in the midsecretory versus early secretory phase of healthy subjects reported here is potentially a clinically relevant finding. Unfortunately, we did not have endometrial fluid secretome available from subjects with endometriosis, which would have made the analysis more robust.

Interestingly, the present data showed a trend of up-regulation of STC1 mRNA in ESE versus PE or MSE in control samples, in contrast to microarray results from an earlier study (4) showing a trend of down-regulation of STC1 mRNA in ESE from endometriosis versus non-endometriosis samples, which correlated to modest down-regulation in another microarray study (28) but was different from the up-regulation of STC1 in MSE in women with endometriosis in the present study. It may be suggested that the increase in STC1 mRNA expression in women with endometriosis is an attempt to compensate the simultaneously low receptor expression during MSE in women with endometriosis. These differences could be explained by the fact that in the present study we used samples from an entirely different cohort of patients, and our control subjects for this part of the study were carefully selected to not have any uterine or endometrial pathology. In addition, the samples used for mRNA analyses in our study were entirely from subjects with severe endometriosis based on availability of tissue, which, while decreasing heterogeneity of the current results, may explain the discrepancy with earlier reports.

Correlation between mRNA and Protein

Significant abundance of STC1 protein in the secretome of receptive midsecretory endometrium from healthy fertile volunteers is a strong indicator of its involvement in the critical process of implantation. The changes in mRNA level from PE/ESE to MSE were not significant, but there was a trend toward up-regulation in control subjects. The discrepancy between transcript and protein expression is not surprising, because poor correlation between transcript level and the amount of corresponding protein has been shown previously, specifically in endometrial research (29–31). When analyzing proteomics data in the endometrium from women with versus without endometriosis, Stephens et al. (31) did not find a correlation between changes in protein abundance with the use of published transcriptome data, suggesting that extensive post-translation control of gene expression occurs in the tissue and is an important factor directly linked to the phenotype. Moreover, Fassbender et al. (29) did not find differences in mRNA level in the secretory endometrium from women with versus without endometriosis, although proteomic analysis of the same samples allowed the diagnosis of endometriosis with high sensitivity and specificity.

STC1 Regulation via cAMP-dependent Protein Kinase a Pathway

On the level of isolated pure human eSF, on the other hand, STC1 was significantly and strongly up-regulated by cAMP, but not by E_2/P, in control subjects, suggesting that regulation is likely mediated via the phosphokinase A (PKA) pathway. In vitro, cAMP was shown to up-regulate STC1 gene expression in human mesenchymal stem cells and rat neuroblastoma cells (32, 33) but to down-regulate it in rat Sertoli and Leydig cells (34). In the rodent male reproductive system, the effect of cAMP on STC expression was mediated by PKA pathway, because the phenotype could be rescued by H89, a PKA-pathway inhibitor (34). In rat cortical neurons, however, the cAMP effect was mediated through the ERK1/2, not the PKA, pathway (35). In equine endometrium, STC1 was highly up-regulated in pregnant endometrium and expressed exclusively by endometrial glands, although it was not significantly regulated by E_2/P treatment of cultured endometrial explants (36), the latter being in line with our findings.

STC1 as Possible New Marker of Decidualization

Some inconsistency between the MSE whole tissue STC1 quantitative RT-PCR and eSF decidualization results in the present study could derive from differences in tissue cell type composition. However, the PCR results in eSF culture experiments supported to some degree the IHC results, with higher STC1 eSF expression in control samples compared with endometriosis. Dysregulation of STC1 in eutopic endometrium and stromal cells from subjects with endometriosis suggests its involvement in the pathogenesis of decidualization defects. These observations lead us to suggest that STC1 is potentially a new marker of decidualization, with more studies needed to elaborate on this novel concept and the roles of P signaling and the PKA pathway and other pathways in this regard.

CASR in Human Endometrium

To the best of our knowledge, this is the first study reporting on the expression of CASR in human endometrium. Calcium homeostasis has been suggested to play a critical role in embryo implantation in rodents and humans, because calcium transporter genes are abundantly expressed in reproductive tissues, including endometrium, in a cyclic manner, they are regulated by ovarian steroid hormones, and their dysregulation affects the expression of some important markers of endometrial receptivity and decreases embryo implantation (37, 38).

As mentioned, CASR, a known mediator of a wide range of calcium-dependent physiologic responses in various tissues, mediates STC1 transcription in response to extracellular

calcium in fish and is induced in rat endometrium during implantation and subsequent decidualization, particularly in luminal and glandular epithelial cells (15). In the present study, CASR did not exhibit variation in its mRNA expression under cyclic hormone influence or in endometriosis t the tissue level. In addition, we did not observe stromal expression of CASR in the samples analyzed, in contrast to its high expression in rat endometrium during implantation and decidualization (15). In our study, absence of amplification of the CASR transcript in eSF was confirmed by negative stromal immunoexpression of CASR in all samples, in line with an earlier study showing CASR expression in rat luminal epithelium but not stroma (39). In addition, the CASR transcript was not detected in any of the array studies reviewed. This pattern of expression suggests paracrine actions of stromal cell–derived STC1 on endometrial epithelial cells.

Strengths, Limitations, and Conclusion

The strengths of the current study include its novelty, obtaining endometrial flushings and biopsies from well characterized subjects. The limitations are the descriptive nature of the study and relatively small sample sizes.

Thus, we have reported on the pattern of STC1 and CASR mRNA and protein expression in endometrial tissue and endometrial stromal cells from women with and without endometriosis. Whether STC1 affects endometrial steroidogenesis, similarly to reports in ovarian tissue (40–42), is an appealing question for future investigation.

Further characterization of STC1 expression and function in human endometrium in pathologic conditions, such as unexplained infertility, is ongoing, as well as the analysis of secretome in women with unexplained infertility.

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SUPPLEMENTAL TABLE 1

Identified stanniocalcin-1 peptides.

Peptide sequence	Protein	Uniprot ID	Missed cleavages	Mass, Da	Observed peptide charges	Posterior error probability of identification	Andromeda identification score	Intensity, Secr1 LH+2	Intensity, Secr1 LH+8	Intensity ratio, LH+8/+2, Secr1	Intensity, Secr2 LH+2	Intensity, Secr2 LH+7
IGPNMASLFHILQ TDHCAQTHPR	STC1	P52823	0	2,643.280	3,4,5	3.33E−187	161.68	0.0E+00	1.4E+07	NA	0.0E+00	1.4E+07
MIAEVQEECYSK	STC1	P52823	0	1,485.648	2	7.47E−07	96.745	0.0E+00	1.5E+06	NA	0.0E+00	9.6E+05
NLRGEEDSPSHIK	STC1	P52823	1	1,480.727	3	1.26E−06	92.939	1.8E+05	7.3E+05	4.0	0.0E+00	5.6E+05
NLRGEEDSPSHIKR	STC1	P52823	2	1,636.828	3	2.60E−03	60.55	0.0E+00	3.4E+05	NA	0.0E+00	1.4E+05
NPEAITEVVQLPNHFSNR	STC1	P52823	0	2,064.039	2,3	4.86E−181	176.69	0.0E+00	3.9E+06	NA	0.0E+00	1.8E+06
RNPEAITEVVQLPNHFSNR	STC1	P52823	1	2,220.140	3,4	7.11E−168	177.08	0.0E+00	1.2E+07	NA	0.0E+00	1.1E+07
SLLECDEDTVSTIR	STC1	P52823	0	1,636.761	2,3	5.30E−24	108.58	0.0E+00	7.1E+06	NA	0.0E+00	5.4E+06
VAAQNSAEVVR	STC1	P52823	0	1,142.604	2	9.56E−04	89.266	9.1E+05	1.2E+07	13.7	7.0E+05	7.5E+06

Peptide sequence	Intensity ratio, LH+8/+2, Secr2	Intensity, Secr3 LH+2	Intensity, Secr3 LH+9	Intensity ratio, LH+8/+2, Secr3	Intensity, Secr4 LH+2	Intensity, Secr4 LH+8	Intensity ratio, LH+8/+2, Secr4	Intensity, Secr5 LH+2	Intensity, Secr5 LH+8	Intensity ratio, LH+8/+2, Secr5	Intensity, Secr6 LH+2	Intensity, Secr6 LH+8	Intensity ratio, LH+8/+2, Secr6
IGPNMASLFHILQ TDHCAQTHPR	NA	0.0E+00	5.1E+06	NA	6.3E+05	8.7E+07	137.1	0.0E+00	8.4E+06	NA	0.0E+00	3.1E+08	NA
MIAEVQEECYSK	NA	0.0E+00	6.5E+05	NA	0.0E+00	5.6E+06	NA	0.0E+00	1.6E+06	NA	0.0E+00	6.8E+07	NA
NLRGEEDSPSHIK	NA	0.0E+00	4.2E+05	NA	3.2E+05	2.9E+06	9.2	2.6E+05	5.3E+05	2.0	0.0E+00	3.6E+06	NA
NLRGEEDSPSHIKR	NA	0.0E+00	0.0E+00	NA	0.0E+00	1.4E+06	NA	0.0E+00	3.1E+05	NA	0.0E+00	2.6E+06	NA
NPEAITEVVQLPNHFSNR	NA	0.0E+00	0.0E+00	NA	0.0E+00	1.3E+07	NA	0.0E+00	1.3E+06	NA	0.0E+00	1.0E+08	NA
RNPEAITEVVQLPNHFSNR	NA	6.7E+05	6.4E+06	9.5	0.0E+00	7.5E+07	NA	1.1E+06	1.4E+07	12.3	1.8E+06	3.1E+08	169.2
SLLECDEDTVSTIR	NA	0.0E+00	3.8E+06	NA	0.0E+00	6.9E+07	NA	6.3E+05	3.4E+06	5.4	3.1E+06	2.2E+08	72.4
VAAQNSAEVVR	10.7	5.1E+05	5.2E+06	10.2	9.9E+05	5.7E+07	57.2	1.1E+06	1.3E+07	12.3	3.7E+06	2.1E+08	55.9

Note: Sequences along with peptide identification and quantification parameters are presented. Note that when the peptide signal had fallen below the limit of peak integration (limit of quantification), the intensity values are presented as zeros.

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