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Stanniocalcin (STC) in the Endometrial Glands of the Ovine Uterus: Regulation by Progesterone and Placental Hormones[1]

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ABSTRACT

Stanniocalcin (STC) is a hormone in fish that regulates calcium levels. Mammals have two orthologs of STC with roles in calcium and phosphate metabolism and perhaps cell differentiation. In the kidney and gut, STC regulates calcium and phosphate homeostasis. In the mouse uterus, *Stc1* increases in the mesometrial decidua during implantation. These studies determined the effects of pregnancy and related hormones on STC expression in the ovine uterus. In Days 10–16 cyclic and pregnant ewes, *STC1* mRNA was not detected in the uterus. Intriguingly, *STC1* mRNA appeared on Day 18 of pregnancy, specifically in the endometrial glands, increased from Day 18 to Day 80, and remained abundant to Day 120 of gestation. *STC1* mRNA was not detected in the placenta, whereas *STC2* mRNA was detected at low abundance in conceptus trophectoderm and endometrial glands during later pregnancy. Immunoreactive STC1 protein was detected predominantly in the endometrial glands after Day 16 of pregnancy and in areolae that transport uterine gland secretions across the placenta. In ovariectomized ewes, long-term progesterone therapy induced *STC1* mRNA. Although interferon tau had no effect on endometrial STC1, intrauterine infusions of ovine placental lactogen (PL) increased endometrial gland STC1 mRNA abundance in progestinized ewes. These studies demonstrate that STC1 is induced by progesterone and increased by a placental hormone (PL) in endometrial glands of the ovine uterus during conceptus (embryo/fetus and extraembryonic membranes) implantation and placentation. Western blot analyses revealed the presence of a 25-kDa STC1 protein in the endometrium, uterine luminal fluid, and allantoic fluid. The data suggest that STC1 secreted by the endometrial glands is transported into the fetal circulation and allantoic fluid, where it is hypothesized to regulate growth and differentiation of the fetus and placenta, by placental areolae.

growth hormone, implantation, placental lactogen, pregnancy, progesterone, stanniocalcin, trophoblast, uterus

INTRODUCTION

Stanniocalcin (STC) was originally described as a hormone with calcitonin-like actions in fish [1–4]. The hormone was discovered in the corpuscles of Stannius, unique endocrine glands on the kidneys of bony fish [5]. Removal of the organ or stanniectomy causes hypercalcemia [6, 7]. Fish STC1 was subsequently purified from the corpuscles of Stannius and found to be a homodimeric phosphoglycoprotein that regulates calcium and phosphate homeostasis [8]. In fish, STC synthesis and secretion are controlled primarily by serum calcium levels [5], and STC acts to restore normocalcemia by acting on the gills to reduce further influx of calcium from the aquatic environment, on the kidneys to promote reabsorption of phosphate and chelate excess calcium, and on the gut to inhibit calcium uptake across the intestinal epithelium [1, 3, 5, 8, 9].

STC1, a mammalian ortholog of fish STC1, has relatively high amino acid sequence identity (approximately 50%) with fish STC and is expressed in a variety of tissues including brain, kidney, lung, and heart [10]. STC2 has lower identity (approximately 35%) with STC1 and fish STC1 [11]. Similar to STC1, STC2 is expressed in a variety of tissues. Research into the functions of STCs in mammals is at an early stage; therefore, its physiological roles have not been established (see for review [4, 12–14]). Similar to fish STC, mammalian STC1 regulates intracellular calcium and phosphate (Pi) levels in the kidney and intestine [5, 15], but the function of STC2 is unknown. Mammalian STC1 regulates renal transport of phosphate through stimulation of NaPi-2 cotransport activity [3, 16–18]. In rodents, *Stc1* expression increases in ovarian tissues during gestation and lactation [19], as well as in mesometrial decidua of the uterus during implantation [20]. In the rat ovary, STC1 and STC2 are expressed in ovarian theca/interstitial cells, and in vitro studies suggest that they act in a paracrine manner to dampen gonadotropin stimulation of granulosa cell differentiation [11, 21]. In mice, *Stc1* does not appear to be essential for reproduction or growth, because null mutants have no overt phenotype [22]; however, in that study, *Stc2* was found in all tissues that normally express *Stc1*, and may compensate for the lack of Stc1.

The STCs have not been investigated in the reproductive tract of mammals other than mice; therefore, these studies were conducted to determine whether the STC genes are expressed in the ovine uterus and to determine the effects of pregnancy, progesterone, and placental hormones on *STC1* and *STC2* expression in the endometrium. The results of these studies indicate that STC1 is expressed specifically by the endometrial glands of the pregnant uterus and suggest that it has a biological role(s) in regulating fetal and placental development and physiology.

MATERIALS AND METHODS

Animals

Crossbred Suffolk ewes (*Ovis aries*) bred and raised on the premises of Texas A&M University, were observed daily for estrus in the presence of vasectomized rams and used in the experiments after they exhibited at least two estrous cycles of normal duration (16–18 days). All experimental and surgical procedures were in compliance with the *Guide for the Care and Use of*

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Agriculture Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Designs

Experiment 1. At estrus (Day 0), ewes were mated to either an intact or a vasectomized ram as described previously [23] and then hysterectomized (n = 5 ewes/day) on either Day 10, 12, 14, or 16 of the estrous cycle or Day 10, 12, 14, 16, 18 or 20 of pregnancy. On Days 10–16, the uterine lumen was flushed with 20 ml of sterile saline. Presence of one or more morphologically normal conceptuses confirmed pregnancy in mated ewes. It was not possible to obtain uterine flushes on either Day 18 or Day 20 of pregnancy, because the conceptus had firmly adhered to the endometrial luminal epithelium (LE) and basal lamina. At hysterectomy, several sections (~0.5 cm) from the mid portion of each uterine horn ipsilateral to the corpus luteum (CL) were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the CL. No tissues from the contralateral uterine horn were used for this study. After 24 h, fixed tissues were changed to 70% ethanol for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware). Several sections (1–1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles), frozen in liquid nitrogen vapor, and stored at −80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80°C for subsequent RNA extraction. Uterine flushes were clarified by centrifugation (3000 × *g* for 30 min at 4°C) and frozen at −80°C for Western blot analysis.

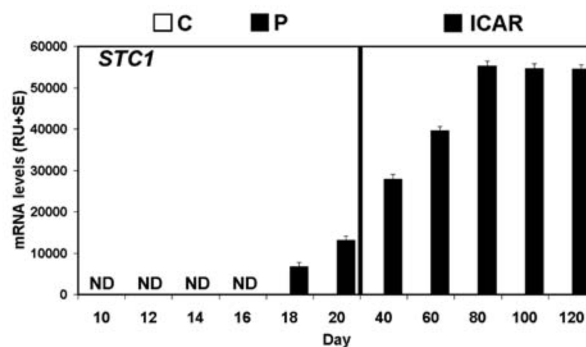
Experiment 2. At estrus (Day 0), ewes were mated to an intact ram as described previously [24]. Ewes were then hysterectomized (n = 5 ewes/day) on either Day 40, 60, 80, 100, 120, or 140 of pregnancy (gestation period is 147 days). Allantoic fluid samples were obtained and frozen at −80°C. At hysterectomy, the uterus was trimmed free of cervix and oviduct and opened along the mesometrial border. Several sections (~0.5 cm) of both intercaruncular and placentomal uterine wall regions from the mid portion of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). Placentomes were then removed by physical dissection, and remaining intercaruncular endometrium was dissected from the myometrium. Endometrial samples were frozen in liquid nitrogen and stored at −80°C for RNA extraction.

Experiment 3. Sixteen cyclic ewes were ovariectomized and fitted with intrauterine catheters on Day 5 postestrus as described previously [25]. Ewes were then assigned randomly (n = 4 ewes/treatment) to receive daily i.m. injections of progesterone (Sigma Chemical Co.) or progesterone and a progesterone receptor (PGR) antagonist (ZK 136,317; generously provided by Dr. Kristof Chwalisz, Schering AG, Berlin, Germany) and intrauterine infusions of either control serum proteins or recombinant ovine interferon tau (IFNT) protein as follows: 1) 50 mg progesterone (P4; Days 5–24) and 200 µg control (CX) serum proteins (Days 11–24; P4+CX); 2) P4 and 75 mg of ZK 136 317 (Days 11–24) and CX proteins (200 µg; P4+ZK+CX); 3) P4 and IFNT (2×10^7 antiviral units, Days 11–24; P4+IFN); or 4) P4 and ZK and IFNT (P4+ZK+IFN). All ewes were hysterectomized on Day 25 postestrus. Recombinant ovine IFNT was prepared in a yeast bacterial system and assayed for biological activity using an antiviral assay as described previously [26]. Control serum proteins and IFNT were prepared for intrauterine injections as described previously [27].

Experiment 4. Fifteen cyclic ewes were ovariectomized and fitted with intrauterine catheters on Day 5 postestrus as described previously [28]. All ewes received daily i.m. injections of 50 mg P4 (Days 5–25) and intrauterine injections of IFNT (2×10^7 antiviral units/day) from Day 11 to Day 20. Ewes (n = 5 per treatment group) also received daily intrauterine injections of either CX serum proteins (200 µg), recombinant ovine placental lactogen (PL; 200 µg), or recombinant ovine growth hormone (GH; 200 µg) from Day 16 to Day 25, when all ewes were hysterectomized. Recombinant ovine PL and ovine GH were prepared in bacteria and purified as described previously [29].

For both experiments 3 and 4, portions (~0.5 cm) from the middle region of the uterine horn were fixed at hysterectomy in fresh 4% paraformaldehyde in PBS (pH 7.2) for 24 h, washed in 70% ethanol for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware).

Experiment 5. Ewes (n = 4) were made unilaterally pregnant as described previously [30]. On Day 80 of pregnancy, uterine secretions, e.g., uterine milk, were collected from the nongravid uterine horn of unilaterally pregnant ewes (n = 4) on Day 80 of pregnancy by flushing the uterine horn with 100 ml of saline. In addition, samples of allantoic fluid and amniotic fluid (50 ml) were obtained using a syringe fitted with a 20-gauge needle from the gravid uterine horn.



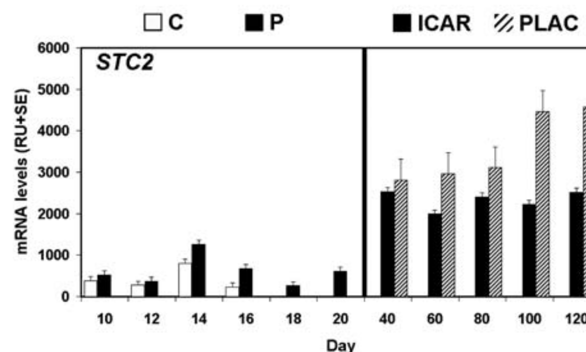


FIG. 1. Steady-state levels of *STC1* and *STC2* mRNAs in ovine uterine and placental tissues. **A**) *STC1* mRNA in the endometrium of cyclic (C) and early pregnant (P) ewes (Days 10–20) and in the intercaruncular (ICAR) endometria of later pregnant ewes (Days 40 −120). *STC1* mRNA was detected in the endometrium of pregnant ewes, but not in the endometrium of cyclic ewes (ND, not detectable) or in the placentomal tissues of pregnant ewes (data not shown). In pregnant ewes, *STC1* mRNA was first detected on Day 18 of pregnancy, increased ($P < 0.01$) ~6-fold to Day 80, and remained abundant thereafter in the intercaruncular (ICAR) endometrium. **B**) *STC2* mRNA in the endometrium of cyclic (C) and early pregnant (P) ewes (Days 10 to 20) and in the intercaruncular (ICAR) endometria and placentomes (PLAC) of later pregnant ewes (Days 40–120). *STC2* mRNA was detected in the endometria of both cyclic and pregnant ewes as well as in the placentomes. Overall, *STC2* mRNA levels were low in the endometrium and not different ($P > 0.10$) between cyclic and pregnant ewes on Days 10–16. *STC2* mRNA levels increased ($P < 0.05$) ~3-fold in the endometrium of pregnant ewes after Day 20. Low levels of *STC2* mRNA were observed throughout gestation in the placentomal tissues. Data are expressed as LSM relative units (RU) ± SEM.

Uterine milk and allantoic fluids were clarified by centrifugation and stored at −80°C.

RNA Isolation

Total cellular RNA was isolated from frozen endometrium from the uterine horn ipsilateral to the CL (experiment 1) and intercaruncular endometrium or placentomes (experiment 2) using Trizol reagent (Gibco-BRL) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

Cloning of Partial cDNAs for Ovine STC1 and STC2

Partial cDNAs for ovine *STC1* and *STC2* mRNAs were amplified by RT-PCR using total RNA from endometrium from ewes on Day 18 of pregnancy. For *STC1*, the sense primer (5′-TGATCAGTGCTTCTGCAACC-3′) and antisense primer (5′-TCACAGTCCAGTAGGCTTCG-3′) were derived from the bovine *STC1* mRNA coding sequence (GenBank accession no. NM_176669). For *STC2*, the sense primer (5′-AACGCTGGAAAATTT-

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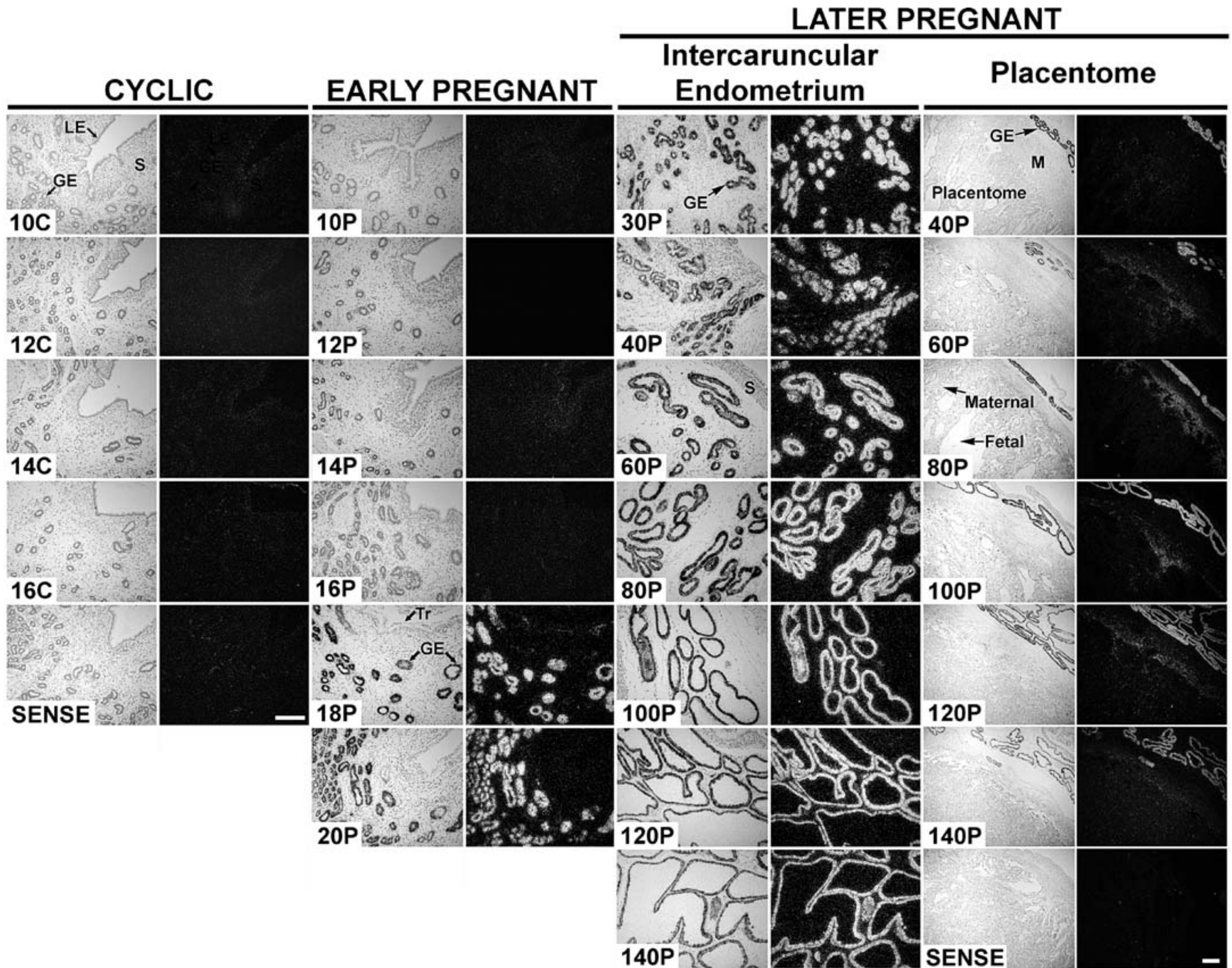

FIG. 2. In situ hybridization analysis of *STC1* mRNA in the uterus of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes and placentomes of pregnant ewes were hybridized with radiolabeled antisense or sense ovine *STC1* cRNAs. Note that *STC1* mRNA is expressed only in the glandular epithelia of the endometrium during pregnancy. GE, Glandular epithelium; LE, luminal epithelium; M, myometrium; S, stroma; Tr, trophectoderm. Bar = 10 μm.

GATGC-3′) and antisense primer (5′- CTCTTGCTACCTCGCTCACC −3′) were derived from the human *STC2* mRNA coding sequence (GenBank accession no. AF055460). PCR amplification was as follows: 1) 95°C for 5 min; 2) 95°C for 45 sec, 59.1°C for 1 min (for *STC1*), 61.8°C for 1 min (for *STC2*), and 72°C for 1 min for 35 cycles; and 3) 72°C for 10 min. Partial ovine *STC1* and *STC2* cDNAs were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and their sequences were verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems).

Slot Blot Hybridization Analyses

Steady-state levels of mRNA in ovine endometria from experiments 1 and 2 were assessed by slot blot hybridization as described previously [31, 32]. Antisense cRNA probes were generated by linearizing both pCRII-STC1 and pCRII-STC2 plasmids with *Xba*I and in vitro transcription with SP6 RNA polymerase, and sense cRNA probes were generated using *Bam*HI and T7 RNA polymerase. Radiolabeled antisense and sense cRNA probes were then generated by in vitro transcription with [α-^{32}P]-UTP. Denatured total endometrial RNA (20 μg) from each ewe was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics).

In Situ Hybridization Analyses

Location of STC mRNA expression in sections (5 μm) of the ovine uterus was determined by radioactive in situ hybridization analysis as described previously [31, 32]. Briefly, deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine *STC1* and *STC2* partial cDNAs using in vitro transcription with [α-^{35}S]-UTP. After hybridization, washing, and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak) and exposed at 4°C for 1 to 2 wk. Slides were developed in Kodak D-19 developer, counterstained with Gill hematoxylin (Fisher Scientific), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield and darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc.) fitted with a Nikon DXM1200 digital camera.

In experiments 3 and 4, the relative abundance of *STC1* mRNA in the endometrial glands was determined with Scion Image software (release beta 4.03; Scion Corporation, NIH). Briefly, photomicrographs of at least 10 regions of the uterus from each animal were acquired under darkfield illumination and

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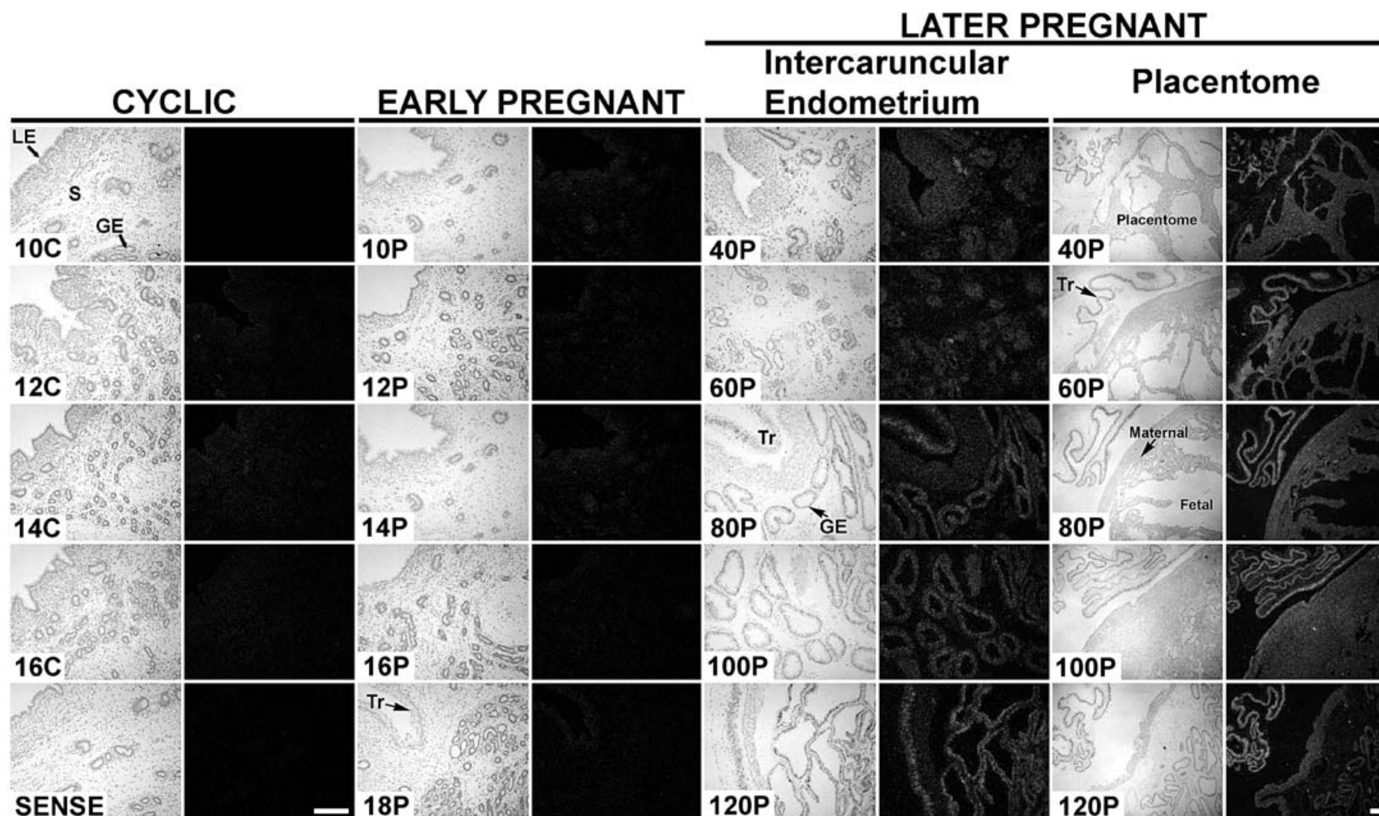

FIG. 3. In situ hybridization analyisis of *STC2* mRNA in the endometrium of cyclic and pregnant ewes and placentomal tissue of later pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *STC2* cRNAs. Very low levels of *STC2* mRNA were detected in the endometrial stroma and glands of the cyclic and early pregnant uterus. In later pregnant ewes, *STC2* mRNA was observed predominantly in the endometrial lumina epithelium (LE), glandular epithelium (GE) and conceptus trophectoderm (Tr) as well as in the maternal caruncular stroma (S) of the placentome. Bar = 10 μm.

converted to a TIFF file. Using Scion Image software, the optical intensity for the mRNA hybridization signals in the endometrial glands was determined. The inter- and intrasection variation in optical intensity value measurements was less than 5%.

Immunohistochemical Analyses

Immunocytochemical localization of STC1 protein in the ovine uterus was performed as described previously [28] in tissue sections from experiments 1 and 2 with rabbit anti-human STC1 antiserum [20] at a 1:25,000 dilution. Antigen retrieval was performed by using a boiling citrate buffer and negative controls included substitution of purified rabbit IgG for the primary antibody at the same final concentration.

Western Blot Analyses

Endometrial extracts of uteri from Days 12, 18, and 80 of pregnancy in experiments 1 and 2 were prepared by homogenizing the uterine tissues in extraction buffer (60 mM Tris pH 7.0, 1 mM Na3VO4, 10% glycerol, 2% SDS, and 1× protease inhibitor cocktail [Roche]). Uterine flushes from Day 16 pregnant ewes in experiment 1 were concentrated using Centricon-3 columns (Amicon). Protein concentrations of uterine flushes, uterine milk, and allantoic fluid were determined using the Bradford protein assay (Bio-Rad) with BSA as the standard. Proteins were denatured and separated by 15% SDS-PAGE, and Western blot analysis was performed as described previously [33] using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce) and X-OMAT AR X-ray film (Kodak). Immunoreactive STC1 protein was detected using the rabbit anti-human STC1 antiserum [20] at a 1:40,000 final dilution.

Statistical Analyses

All quantitative data were subjected to least-squares regression analyses (ANOVA) using the General Linear Models procedures of the Statistical Analysis System (SAS Institute). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from experiments 1 and 2 were analyzed for effects of day, pregnancy status (cyclic or pregnant), tissue (caruncular and intercaruncular endometrium), treatment, and their interactions. Within pregnancy status, least squares regression analyses were used to determine effects of day on endometrial mRNA levels. Optical intensity measurements of mRNA abundance in the endometrial glands as determined by in situ hybridization analyses of uteri from experiments 3 and 4 were analyzed for effects of treatment, animal, and slide. Preplanned orthogonal contrasts were used to determine main effects of treatment. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P value of 0.05 or less was considered significant, whereas a P value of 0.05 to 0.10 was considered a trend toward significance. Data are presented as least-square means (LSM) with SEM.

RESULTS

Steady-State Levels of STC1 and STC2 mRNA in the Endometrium of the Ovine Uterus

Steady-state levels of *STC1* and *STC2* mRNA in endometria of cyclic and pregnant ewes were determined by slot blot hybridization analysis (Fig. 1). *STC1* mRNA was detected in the endometrium of pregnant ewes, but not in the endometrium of cyclic ewes. In addition, *STC1* mRNA was not detected in the placentomal tissues of pregnant ewes (data not shown). In pregnant ewes, *STC1* mRNA first appeared on Day 18 of pregnancy, increased ($P < 0.01$) ~6-fold to Day 80, and remained abundant thereafter. *STC2* mRNA was found in the endometria of both cyclic and pregnant ewes as well as in the placentomes. Overall, *STC2* mRNA levels were low in the

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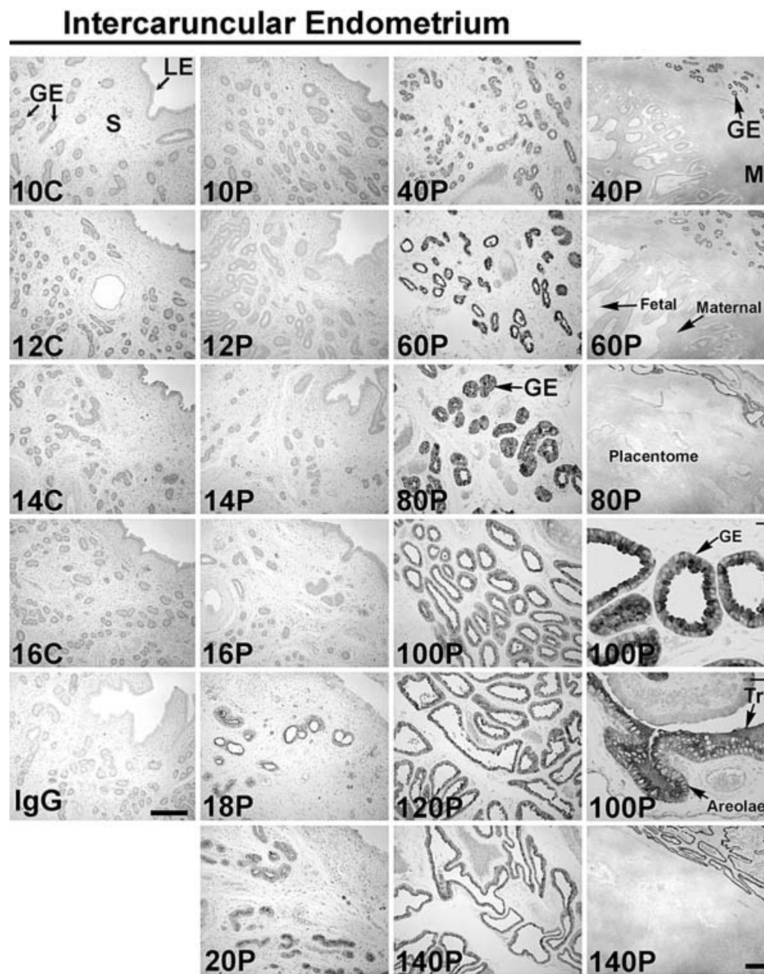

FIG. 4. Immunohistochemical localization of STC1 protein in uteri from cyclic (C) and pregnant (P) ewes. In the IgG control, normal rabbit IgG was substituted for rabbit polyclonal antibody to human STC1. Sections were not counterstained. Note the immunoreactive STC1 protein in the endometrial glandular epithelia and accumulation in the placental areolae as shown on Day 100 of pregnancy. LE, Luminal epithelium; GE, glandular epithelium; M, myometrium; S, stroma; Tr, trophectoderm. Bar = 10 μm, except in higher magnifications of the glands and areolae on Day 100 of pregnancy, for which bar = 100 μm.

endometrium and not different ($P > 0.10$) between cyclic and pregnant ewes on Days 10 to 16. *STC2* mRNA levels increased (linear, $P < 0.05$) ~3-fold in the endometrium of pregnant ewes after Day 20. Low levels of *STC2* mRNA were observed throughout gestation in the placentomal tissues.

Localization of STC1 *and* STC2 *mRNAs in the Ovine Uterus*

In situ hybridization analyses determined the location of *STC1* and *STC2* mRNAs in uteri of cyclic and pregnant ewes (Figs. 2 and 3). In cyclic ewes, *STC1* mRNA was not detected between Days 10 and 16 of the estrous cycle or pregnancy (Fig. 2). Similarly, *STC1* mRNA was not detected in the endometrium of Days 10 to 16 pregnant ewes. On Day 18 of pregnancy, *STC1* mRNA was detected in the endometrial glandular epithelium (GE), but not in any other uterine or placental cell types, including the LE, stroma, myometrium, blood vessels, immune cells, or conceptus trophectoderm. Throughout pregnancy, *STC1* mRNA was observed only in the endometrial GE. The photomicrographs of Day 60, 80, 100, and 120 placentomes contain red blood cells at the placentome-myometrium interface that diffract light under darkfield illumination, but are not positive for *STC1* mRNA.

In contrast to STC1, *STC2* mRNA was detected at very low levels in the endometrial LE, GE, and stroma of cyclic and early pregnant ewes (Fig. 3). In later pregnant ewes, *STC2* mRNA was detected predominantly in the endometrial LE and GE as well as in the conceptus trophectoderm, with lower levels in the stroma. Given the temporal and spatial alterations in the two *STC* genes in ovine uteroplacental tissues, we focused on STC1 in the remainder of the studies.

Localization of Immunoreactive STC1 Protein in the Ovine Uterus

Immunohistochemical analysis indicated that STC1 protein was localized predominantly on the apical surface of GE between Days 18 and 140 of gestation (Fig. 4). Consistent with results from in situ hybridization analyses, STC1 protein was predominantly detected in the endometrial glands near the apical surface. In caruncular areas, immunoreactive STC1 was detected only in GE adjacent to the placentome. Areolae are specialized areas of the intercotyledonary placenta that form over the opening of a gland duct on the endometrial luminal surface [34]. STC1 protein was consistently observed in the folded areolae of the intercotyledonary placenta.

Progesterone Induces STC1 *mRNA in the Endometrial Glands of the Ovine Uterus*

Osteopontin (secreted phosphoprotein 1 or SPP1 and uterine SERPIN, also known as uterine milk protein or UTMP) is also expressed only in the endometrial GE and induced by progesterone [25, 35–37]. Therefore, experiment 3 was conducted to determine if the induction of *STC1* mRNA in the endometrial glands of early pregnant ewes was caused by progesterone and/or IFNT from the conceptus (Fig. 5A). Continuous, long-term progesterone treatment for

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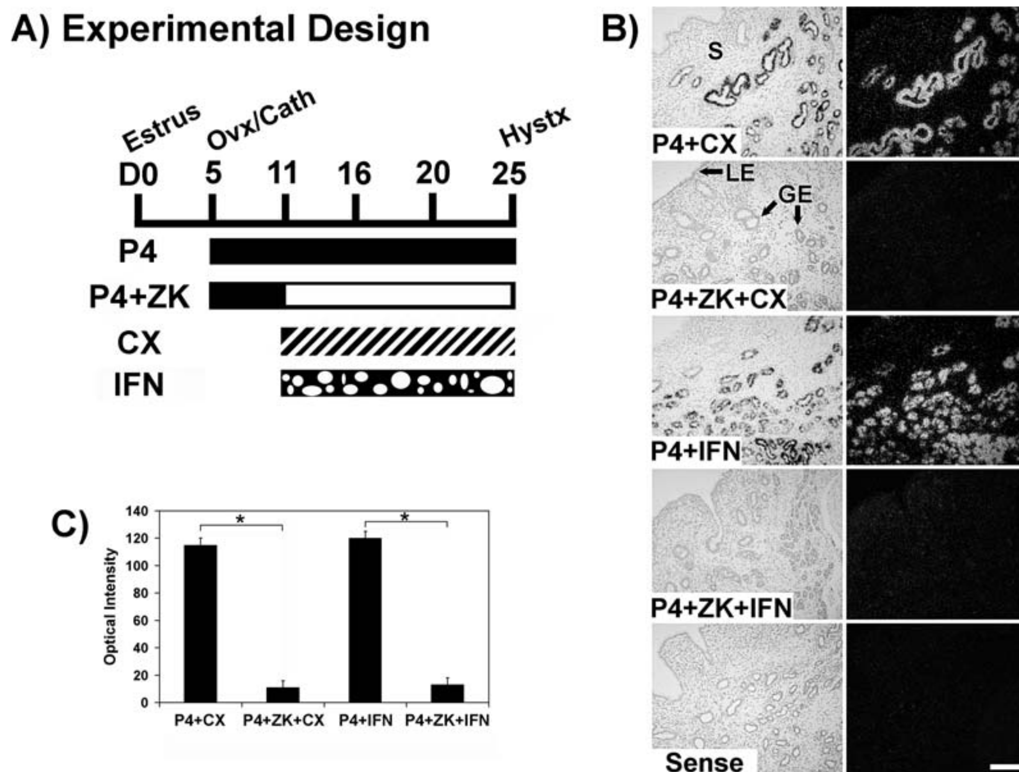

FIG. 5. Effects of progesterone and interferon tau on endometrial *STC1* mRNA. **A**) Experimental design (see *Materials and Methods* for complete description of experimental design). CX, Control serum proteins; Hystx, hysterectomy; IFN, recombinant ovine interferon tau; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; ZK, ZK137,316. **B**) In situ hybridization analysis of *STC1* mRNA in the uterus. *STC1* mRNA was not observed in the endometrial glands of uteri from any of the ewes receiving progesterone and the ZK antiprogestin. LE, Luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μm. **C**) Quantification of *STC1* mRNA in the endometrial glands of uteri. *STC1* mRNA was 11.6-fold higher ($P < 0.01$) in the endometrial glands of P4+CX-treated ewes as compared to P4+ZK+CX-treated ewes. However, intrauterine infusion of IFNT had no effect ($P > 0.10$) on *STC1* mRNA abundance in the endometrial glands when P4+IFN-treated ewes were compared to P4+CX-treated ewes. Data are expressed as LSM optical intensity ± SEM.

20 days alone induced *STC1* mRNA in the endometrium of the ovine uterus (Fig. 5B). As illustrated in Figure 5C, *STC1* mRNA was 11.6-fold higher ($P < 0.01$) in the endometrial glands of P4+CX-treated ewes as compared to P4+ZK+CX-treated ewes. Indeed, *STC1* mRNA was not observed in the endometrial glands of uteri from any of the ewes receiving progesterone and the ZK antiprogestin (Fig. 5B). Intrauterine infusion of IFNT had no effect ($P > 0.10$) on *STC1* mRNA abundance in the endometrial glands when P4+IFN-treated ewes were compared to P4+CX-treated ewes. *STC2* mRNA was not detected by in situ hybridization analysis in endometria of ewes in any treatment group (data not shown).

PL and GH Increase STC1 *mRNA in the Endometrium*

In addition to being progesterone-induced genes in the endometrial glands of the ovine uterus, SPP1 and SERPIN are also stimulated by intrauterine administration of ovine PL and ovine GH [28, 38]. Therefore, experiment 4 was conducted to determine whether ovine PL and/or ovine GH regulated STC expression in the endometrial glands of the ovine uterus (Fig. 6A). Intrauterine administration of recombinant ovine PL increased *STC1* mRNA in the endometrial glands of the ovine uterus by 1.8-fold ($P < 0.05$, CX vs. PL; Fig. 6, B and C). Similarly, intrauterine administration of recombinant ovine GH tended to increase *STC1* mRNA in the endometrial glands by 1.4-fold ($P < 0.10$, CX vs. GH). Similar to experiment 3, *STC2* mRNA was not detected by in situ hybridization analysis of the endometria from any of the ewes in the experiment (data not shown).

Western Blot Analysis of STC1 Protein in the Endometrium, Uterine Secretions, and Fetal Fluids

Western blot analysis (under reducing conditions) of endometrial extracts, uterine secretions and allantoic fluid from pregnant ewes with rabbit anti-human STC1 antibody detected a single protein of ~25 kDa in size. Immunoreactive STC1 was observed in the uterine luminal fluid, e.g., uterine milk, and allantoic fluid of Day 80 unilateral pregnant ewes, but not in the uterine luminal fluid obtained by flush of Day 16 pregnant ewes (Fig. 7). In addition, STC1 was not detected in the amniotic fluid from Day 80 pregnant ewes (data not shown). These results support the idea that STC1 is synthesized in GE, secreted by glands into the uterine lumen, transported by the areolae across the placenta into the fetal circulation, cleared by the kidney into the urachus, and then stored in the allantoic fluid during gestation.

DISCUSSION

The results of the present studies demonstrate that STC1 is exclusively expressed in the endometrial glands of the ovine uterus after Day 16 of pregnancy. In sheep, the blastocyst enters the uterus by Day 6, but only begins implantation on Day 16 [39]. In rodents, STC1 gene expression was found to shift from the uterine LE to the mesometrial decidua during implantation [20]. In contrast, in the endometrium of the ovine

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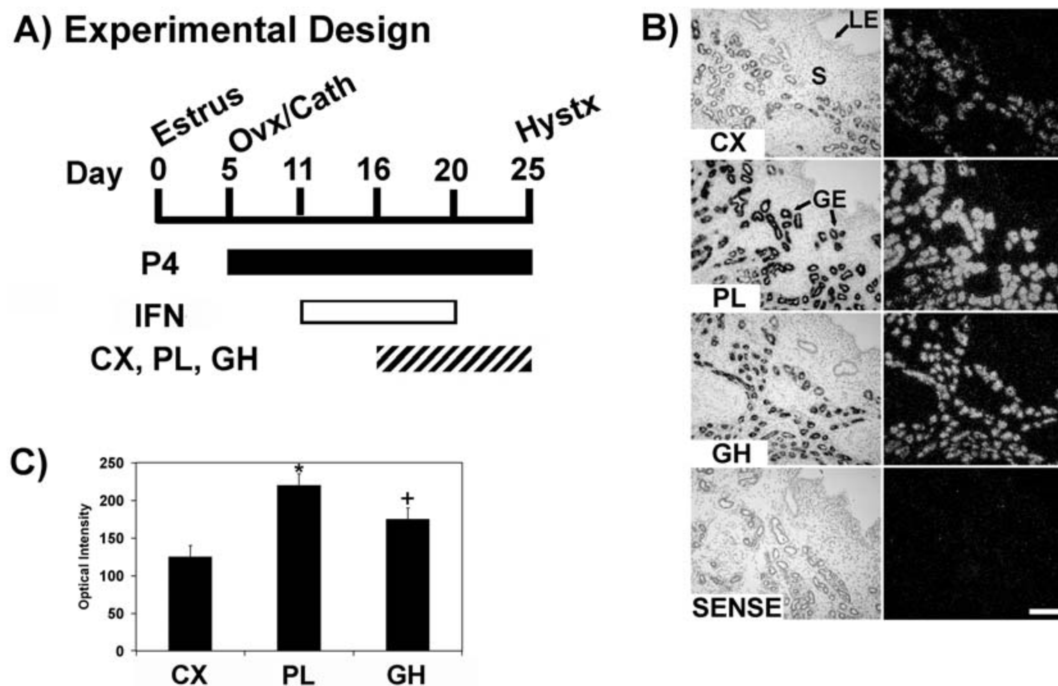

FIG. 6. Effects of intrauterine infusion of placental lactogen and growth hormone on endometrial *STC1* mRNA. **A**) Experimental design (see *Materials and Methods* for complete description of experimental design). CX, Control serum proteins; GH, growth hormone; Hystx, hysterectomy; IFN, recombinant ovine interferon tau; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; PL, placental lactogen. **B**) In situ hybridization analysis of *STC1* mRNA in the uterus. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μm. **C**) Quantification of *STC1* mRNA in the endometrial glands of uteri. Intrauterine administration of recombinant ovine PL increased *STC1* mRNA in the endometrial glands of the ovine uterus by 1.8-fold (*$P < 0.05$, CX vs. PL). Intrauterine administration of recombinant ovine GH tended to increase *STC1* mRNA in the endometrial glands by 1.4-fold (+$P < 0.10$, CX vs. GH). Data are expressed as LSM optical intensity ± SEM.

uterus, the STC1 gene was uniquely expressed in glandular epithelial cells. Likewise, STC1 protein was present near the apical surface of gland cells and secreted into the uterine lumen, as evidenced by the presence of immunoreactive STC1 protein in uterine secretions, placental areolae, and allantoic fluid. In this context, STC1 would appear to be secreted in an exocrine manner. If STC1 is also secreted in an endocrine direction by the endometrial glands, it may play an additional role in regulating maternal physiology, and perhaps be useful as an endocrine marker of pregnancy in sheep. STC2, a paralog of STC1, has been identified [11, 40, 41] and detected in various tissues, but its biological roles are not known. In the present study, low levels of STC2 mRNA were detected in the endometrial glands and placenta. Results of the present studies indicate that STC1 is the predominant form of the hormone produced in the ovine uterus and present in uteroplacental tissues of the sheep.

The gland-specific expression of the *STC1* gene in the endometrium of the ovine uterus is similar to that of *SPP1* and *SERPIN*, which also encode secreted proteins that are present in the uterine lumen and allantoic fluid during pregnancy [42–44]. All three genes are induced in the glands of the endometrium in response to progesterone. Available results indicate that continuous exposure of the uterus to progesterone specifically downregulates PGR in the endometrial epithelia [45, 46]. The disappearance of PGR from the endometrial GE after Day 13 of pregnancy is associated with subsequent induction of SPP1 after Day 13 followed by SERPIN and STC1 between Days 16 and 18 [25, 35, 47, 48]. Indeed, treatment of ewes with an antiprogestin inhibited progesterone-dependent downregulation of the PGR in the endometrial epithelia of the ovine uterus [25]. Furthermore, administration of estrogen with progesterone to ewes upregulated PGR in the endometrial GE, which, in turn, suppressed SPP1 and SERPIN [28]. Collectively, available evidence suggests that the STC1 gene is repressed by liganded PGR, and this repression is removed by progesterone downregulation of the PGR gene that occurs after Day 13 of pregnancy. Thus, progesterone induction of STC1, as well as SPP1 and SERPIN, is not a classical mechanism of gene regulation by progesterone and PGR. Indeed, downregulation of PGR by progesterone may be requisite for GE remodeling and differentiated function (see [46]). Given that STC1 gene expression was first observed in the endometrial glands on Day 18 of pregnancy, it is likely that another factor(s) besides progesterone regulates the STC1 gene, because PGR gene expression is lost between Days 13 and 15 of pregnancy [48].

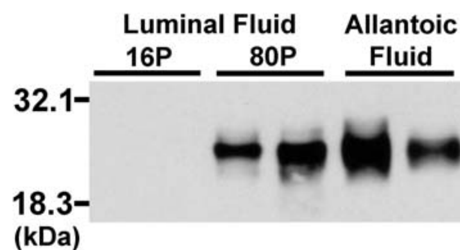

FIG. 7. Analysis of immunoreactive STC1 protein in the uterine luminal fluid and allantoic fluid of pregnant ewes. Proteins were separated by 15% SDS-PAGE under reducing conditions. Western blot analysis found a single immunoreactive protein of ~25 kDa in uterine luminal fluid and allantoic fluid samples from Day 80 unilaterally pregnant ewes, but not in uterine luminal fluid of Day 16 pregnant ewes or amniotic fluid of Day 80 ewes (data not shown). Positions of prestained molecular weight standards ($\times 10^{-3}$) are indicated.

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In the present study, ovine PL and GH were found to stimulate STC1 in the endometrial glands. During pregnancy, the uterus is sequentially exposed to progesterone from the ovary and then to IFNT, PL, and GH from the placenta. IFNT is produced by the mononuclear trophectoderm between Days 11 to 20 of early pregnancy and is the signal for maternal recognition of pregnancy [49]. IFNT acts in a paracrine manner on the endometrium to inhibit development of the luteolytic mechanism in the endometrial LE, thereby promoting continued production of progesterone by the corpus luteum. Although IFNT stimulates a large number of genes in the endometrial GE and stroma [49], *STC1* expression in the endometrial glands was not affected by IFNT in the present studies. PL is produced specifically by the trophoblast giant binucleate cells, which first differentiate between Days 14 and 15 in the conceptus [50]. Peak concentrations of PL in maternal serum closely parallel dynamic changes in total protein synthesized and secreted by the GE of the ovine endometrium during gestation [36, 51–54]. In the current studies, STC1 mRNA and protein was first observed on Day 18 of pregnancy and increased to maximal levels by Day 80 of pregnancy, which is associated with the onset of and increases in PL production by the trophoblast giant binucleate cells. Indeed, SPP1 and SERPIN are also stimulated in the endometrium of ovariectomized ewes treated with progesterone and IFNT [28, 38]. Lacroix et al. [55, 56] first described the expression of GH in the ovine placenta between Days 35 and 70. Similar to uterine SERPINs, STC1 tended to be stimulated in the endometrial glands by intrauterine infusions of ovine GH. Thus, somatolactogenic hormones from the conceptus act in a paracrine manner on the endometrium to increase *STC1* mRNA in the GE. Future studies will need to focus on the molecular mechanism of PL and GH modulation of *STC1* gene expression. The mechanism likely involves both prolactin receptors (PRLRs) and GH receptors (GHRs), because ovine PL can signal through a homodimer of PRLRs as well as through a heterodimer of PRLRs and GHRs, whereas GH signals only via a homodimer of GHRs [57]. Indeed, PRLRs are expressed exclusively in the endometrial glands of the ovine uterus, and PL binds to those receptors [35, 38, 58]. Furthermore, IFNT stimulates PRLRs in the endometrial glands of the ovine uterus [59]. Although likely more complicated, available evidence supports the idea that progesterone downregulates PGR, which is permissive for the onset of STC1, and then IFNT stimulates PRLRs, which in turn respond to PL from the new trophoblast giant binucleate cells, which further stimulate STC1 gene expression along with GH during later pregnancy.

Although mouse STC1 expression is highly upregulated in the ovary during lactation [19] and changes dynamically in the uterus during the preimplantation period [19, 20], its biological and molecular functions in the mammalian uterus during pregnancy are not known. In the present study, we found that STC1 is induced by progesterone and stimulated by PL and GH. The temporal alterations in endometrial STC1 mRNA and protein parallel fetal growth and development. Indeed, our results indicate that STC1 is secreted by the endometrial glands into the uterine lumen, where it is transported into the fetal circulation by the areolae of the intercotyledonary placenta. After implantation, the chorioallantois develops unique structures, termed areloae, that develop over the mouth of each uterine gland as specialized areas for absorption and transport of uterine histotroph into the conceptus [60]. These results support the idea that STC1 protein is synthesized by the endometrial glands and then secreted into the uterine lumen, where it is absorbed by the placenta, transported into the fetal circulation, and cleared by the kidney into the allantois via the urachus [60, 61]. Although the allantois was initially considered a reservoir for waste products of the fetus, it serves to store most secreted proteins from the endometrium, including SERPINs [36, 62]. In contrast, amniotic fluid is not in the path for protein clearance by the fetal kidney, and therefore does not function in this capacity. Alternatively, STC1 may originate from the fetus itself, given that *Stc1* is highly expressed by the mouse fetus, in particular by the kidneys, testes, bone and muscle [63].

Although the functions of uterine STC1 are not known, based on its biological properties in fish and mammals, it may be involved in the regulation of calcium and phosphate transport by placental membranes as well as their homeostasis in the fetus. All of the nutrients and minerals required to provide the anabolic requirements of the developing ovine fetus must pass from the maternal circulation through either the uterine glands (interplacentomal) or feto-maternal syncytiotrophoblast (placentomal) and then cross the placental trophoblast epithelium [64]. Calcium is essential for cellular homeostasis and function. During pregnancy, fetal calcium must cross the placenta, in exponentially increasing amounts during the second half of gestation to support fetal bone growth [65]. Calcium transport across the placenta is an active process, because the level of serum calcium in the fetus is higher than that in the mother [66]. Indeed, S100 calcium binding protein G (S100G, also known as calbindin-D9K) is present in the maternal endometrial glands and is higher in the trophoblasts of the interplacentomal placenta as compared to those of the placentomes [67, 68]. Given the importance of calcium in placental function and fetal growth, STC1 from the endometrial glands may regulate calcium and phosphate homeostasis in the placenta as well as perhaps the fetus.

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