Expression and regulation of stanniocalcin 1 and 2 in rat uterus during embryo implantation and decidualization

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Abstract

Stanniocalcin-1 (STC-1) is a recently discovered polypeptide hormone, while stanniocalcin-2 (STC-2) is a subsequently identified homologue of stanniocalcin-1. Although previous studies have shown that both STC-1 and -2 are involved in various physiological processes, such as ion transport, reproduction and development, their expression in the uterus and roles in implantation and early pregnancy are unclear. Here we have investigated the expression and regulation of both STC-1 and STC-2 in rat uterus during early pregnancy under various physiological conditions. We show that only basal levels of STC-1 and STC-2 mRNA were detected in the uterus from day one (D1) to day five (D5) of pregnancy. STC-2 immunostaining was gradually increased in the glandular epithelium from day two (D2), with a peak occurring on D5. High levels of both STC-1 and STC-2 mRNA were observed in the stoma cells at the implantation site on day six (D6) of pregnancy, whereas their immunostaining signals were also significant in the luminal epithelium. Basal levels of both STC-1 and STC-2 mRNA and STC-1 immunostaining were detected in the uterus with delayed implantation. After the delayed implantation was terminated by estrogen treatment, both STC-1 and STC-2 mRNA signals were significantly induced in the stroma underlying the luminal epithelium at the implantation site, and STC-2 immunostaining was also observed in the luminal epithelium surrounding the implanting blastocyst. Embryo transfer experiments further confirmed that STC-1 and STC-2 expression at the implantation sites was induced by the implanting blastocyst. Both STC-1 mRNA and immunostaining were seen in the decidualized cells from day seven (D7) to day nine (D9) of pregnancy. STC-2 mRNA was also found in the whole decidua from D7 to D9 of pregnancy; STC-2 protein, however, was strictly localized to the primary deciduas on D7 and D8, with a weak expression in the whole deciduas on D9. Consistent with the normal pregnancy process, strong STC-1 and STC-2 mRNA signals were detected in the decidualized cells under artificial decidualization, whereas only basal levels of STC-1 mRNA and immunostaining were observed in the control horn. These data suggest, for the first time, that STC-1 together with STC-2 may play important roles in the processes of implantation and decidualization in the rat.
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Introduction

Stanniocalcin was initially identified as a disulfide-linked homodimeric hormone secreted from the bony fish endocrine gland corpuscle of *Stannius*. It was thought to be a regulator unique to fishes until the discovery of its mammalian counterpart in human (Olsen *et al.* 1996) and mouse (Chang *et al.* 1996). Subsequently, a related protein was cloned from human and mouse (Chang & Reddel 1998). The two mammalian stanniocalcins (STC) share 61% and 34% amino acid homology with their fish counterparts and were named

stanniocalcin-1 (STC-1) and stanniocalcin-2 (STC-2) respectively; the latter is also known as stanniocalcin-related peptide STCrP.

The STC-1 gene is widely expressed in various tissues. STC-1 is capable of promoting re-absorption of inorganic phosphate across renal and intestinal epithelia and reducing intestinal calcium transport (Wagner *et al.* 1997, Madsen *et al.* 1998). This hormone also has a possible role in maintaining the integrity of post-mitotic neurons (Zhang *et al.* 2000). The STC-1 gene has been found to be highly expressed in the ovary, in the androgen-producing thecal and interstitial cells (Deol

et al. 2000, Paciga *et al.* 2002). The ovarian STC-1 is structurally unique and its expression is stimulated by human chorionic gonadotropin. This gene is also highly expressed during embryogenesis, particularly in the urogenital system, suggesting that STC-1 probably acts as a signaling molecule during embryo development (Varghese *et al.* 1998, Stasko & Wagner *et al.* 2001*a*). Recently, it has been observed that STC-1 is expressed uniquely in mouse mesometrial stromal cells and sequestered by decidualizing anti-mesometrial cells, indicating a possible role in decidualization and uterine remodeling (Stasko *et al.* 2001). The expression and regulation of STC-1 in rat uterus during early pregnancy is still undefined.

STC-2 also appears to be widely expressed in mammalian tissues, including kidney (Wagner *et al.* 1995), pancreas (Moore *et al.* 1999), skeletal muscle (Ishibashi *et al.* 1998), small intestine (Chang & Reddel 1998) and ovary (Honda *et al.* 1999). Compared with STC-1, however, the potential function of STC-2 is even less understood. There are no reports about its expression and possible role in the mammalian uterus. In the kidney, STC-2 was reported to have an inhibitory effect on the Na^{+}/phosphate co-transporter activity while STC-1 is a stimulator of this transporter (Ishibashi *et al.* 1998). Honda *et al.* (1999) demonstrated that calcitriol, an active form of vitamin D_3, increased STC-1 mRNA levels more than threefold, but decreased STC-2 message to a trace level, suggesting that STC-1 and STC-2 may be counteracting molecules, STC-1 being anti-hypercalcemic and STC-2 anti-hypocalcemic.

This information prompted us to consider whether the STC-1 and STC-2 genes are temporarily and spatially expressed and whether they counteract each other in the uterus during the processes of implantation and decidualization. To assess this possibility, we examined expression of both STC-1 and STC-2 in rat uterus during early pregnancy by RT-PCR, *in situ* hybridization and immunohistochemistry. Furthermore, we established various physiological states, such as pseudopregnancy, delayed implantation, artificial decidualization and embryo transfer to confirm it.

Materials and Methods

Animals and treatments

Mature rats (Sprague–Dawley strain) were caged in a controlled environment with a 14 h light:10 h darkness cycle. The estrous cycle was determined by inspection of vaginal smears. Adult females were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (day 1 (D1) = day of vaginal sperm positive or day of vaginal plug). Pregnancy on D1–D5 was confirmed by flushing embryos from the reproductive tracts. The implantation sites on D6–D7 were identified by intravenous injection of 1% Chicago blue (Sigma) in 0.85% sodium chloride.

To induce delayed implantation, pregnant rats were ovariectomized on D4 at 0830–0900 h and treated with progesterone (5 mg/rat, s.c.) to maintain the delayed implantation condition on D4–D7. On D8, a random half of the progesterone-primed delayed-implantation rats were additionally given estrodiol (250 ng/rat) to terminate the delayed implantation condition. The remaining animals only received progesterone injection. The rats were sacrificed for collection of the uteri 24 h after the last hormonal treatment. The implantation sites were identified by tail intravenous injection of Chicago blue solution. To confirm that the rats receiving progesterone only were in a state of delayed implantation, uterine flushings were collected and examined for the presence of hatched blastocysts.

Artificial decidualization

Mature rats were ovariectomized. Artificial decidualization was induced according to the procedures described by Kennedy & Ross (1997). The uteri were collected at 24, 48 and 72 h after injection of the oil.

Embryo transfer

Embryo transfer was performed as previously described by Ertzeid & Storeng (2001). On D4.5, pseudopregnant rats were anesthetized by intraperitoneal injection of ketamine. One lateral uterine horn was exposed via a lateral incision. The embryos to be transferred were collected from the pregnant donors on D4.5. A fine-tipped glass pipette was inserted into a hole made previously by insertion of a 23-gauge needle through the uterine wall at the oviductal end. The contralateral uterine horn served as the control. Thereafter, the uterine horns were returned to the abdominal cavity and the skin incision was sutured. On D6, the recipients were sacrificed 5 min after tail intravenous injection of trypan blue solution.

RT-PCR

Total RNA was extracted from rat uteri with Trizol reagent (Gibco BRL Life Technologies Inc., Rockville, MD, USA) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg total RNA using Superscript II reverse transcriptase (Gibco) and oligo dT. The reaction was carried out at 42 °C for 50 min and 70 °C for 15 min. For PCR analysis, the cDNA was amplified for 26 cycles (denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, and elongating at 72 °C for 45 s) using STC-1 and STC-2 primers respectively: STC-1 primers were 5′-TCTCTTGGGAGGTGCGTT-3′ and 5′-GTCTTCCTTGCCATTCGG-3′ (428–844 bp, Genebank accession number U62667), and the expected

size of the amplified fragment was 417 bp. STC-2 primers were 5′-TTGTGAAATCCAGGGCTTA-3′ and 5′-TAGGTGGTGGTCGGTGTC-3′ (237–750 bp, Genebank accession number AB030707), with an expected product size of 514 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression remains fairly constant in the rodent uterus and was amplified for 23 cycles as an internal control. The primers were 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCAC-CACCCTGTTGCTGTA-3′ (566–1017 bp, Genebank accession number M32599), with an expected size of 452 bp (Sidhu & Kimber 1999). PCR products were cloned into T-easy vector, screened by restriction analysis and confirmed by sequencing. To confirm the specificity of RT-PCR, three controls were included to test for contamination by genomic DNA: (1) RNA samples were directly amplified without reverse transcription; (2) reverse transcription was performed without adding reverse transcriptase, followed by PCR amplification; and (3) RNA samples were replaced with diethylpyrocarbonate–distilled H_2O (DEPC–dH_2O) in the RT-PCR.

In situ *hybridization*

In situ hybridization was performed as described previously. In brief, the uteri were cut into 4–6 mm pieces and flash frozen in liquid nitrogen. The frozen sections (8 μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides and fixed in 4% paraformaldehyde solution in PBS. The sections were treated in 0.2 N HCl for 10 min after two washes in PBS, digested with 0.1 μg/ml proteinase K at room temperature for 10–15 min, and incubated twice in 0.2% (w/v) glycine in PBS for 5 min. Postfixation was performed in 4% paraformaldehyde for 2 min. After acetylation in freshly prepared 0.25% (w/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, the sections were washed twice in 2×SSC (0.3 M sodium chloride, 0.03 M sodium citrate) for 5 min, dehydrated in serial dilutions of ethanol, and air dried. Following the prehybridization in hybridization buffer (4×SSC, 50% (v/v) formamide, 5% (w/v) dextran sulfate, 1×Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, 0.25 mg/ml yeast tRNA) at 25 °C for 2–3 h, the sections were hybridized in hybridization buffer with 1–5 μg/ml DIG-labeled antisense or sense RNA probes at 55 °C for 16 h. After hybridization, the sections were washed in 4×SSC at room temperature for 10 min and digested in 10 μg/ml RNase A (Boehringer Mannheim) in 0.01 M Tris–HCl and 0.5 M NaCl (pH 8.0) at 37 °C for 30 min. The sections were washed twice in 4×SSC, 1×SSC and 0.5×SSC twice for 10 min at 42 °C. After non-specific binding was blocked in 0.5% (w/v) block mix (Boehringer Mannheim), the sections were incubated overnight in sheep anti-digoxigen (DIG) antibody conjugated to alkaline phosphatase at 4 °C (1:4000, Boehringer Mannheim). The signal was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma).

Immunohistochemistry

Excised rat uteri were immediately fixed in 10% neutral Buffered Formalin solution and embedded in paraffin. Sections (5 μm) were cut, deparaffinized and rehydrated. Antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0) at 95 °C for 15 min and cooling naturally to room temperature for 20 min. Nonspecific binding was blocked in 10% (v/v) normal horse serum in PBS for 1 h. The sections were incubated with goat anti human STC-1 or STC-2 antibody in 10% (v/v) horse serum (1:100) for 12 h at 4 °C (SC-14346 and SC-14350 for STC-1 and STC-2 respectively, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The sections were then incubated with biotinylated secondary antibody followed by an avidin–alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA, USA). Vector Red was visualized as a red color. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma). As a negative control STC-1 or STC-2 primary antibody was replaced with normal goat IgG. The sections were counterstained with hematoxylin and mounted.

Microscopic assessment

Uterine samples from three individual rats from each group were analyzed. Experiments were repeated at least three times, and one representative section from at least three similar results is presented. The mounted sections were examined using a Nikon microscope.

Statistical analysis

Samples from three individual animals at each tissue collection time point were analyzed. Statistical analysis was performed using Statistical Package for Social Science (SPSS for Windows package release 10.0, SPSS Inc., Chicago, IL, USA). Statistical significance was determined by one-way ANOVA. Post-Hoc comparisons between treatment group means were made using Fisher's protected least-significance-difference test. Differences were considered significant if $P < 0.05$. Values shown in all the figures were given as the mean±S.E.M.

Results

RT-PCR of STC mRNA expression during peri-implantation

As a first step towards examining a possible role of STC-1 and STC-2 in reproductive tissue, we determined the

levels of STC mRNA in the peri-implantation uterus by RT-PCR. Both STC-1 and STC-2 mRNAs were detected in all uteri examined. The mRNA levels were increased at the implantation site of pregnancy on D6, D8 and in the estrogen-activated delayed implantation uterus (Fig. 1A) compared with those in the uteri on D5 of pregnancy, D6 of pseudopregnancy and delayed implantation. The densitometry analysis showed that the increase in both mRNA levels at the implantation site of pregnancy on D6, D8 and in the estrogen-activated delayed implantation uterus was very significant ($P < 0.01$), while the mRNA levels in the uteri on D5 of pregnancy, D6 of pseudopregnancy and delayed implantation were not significant (Fig. 1B). These data suggest that STC expression in the uterus is temporarily and spatially regulated.

STC expression during early pregnancy

In order to study a possible role of STC in the uterus in more detail, we first characterized the developmental expression of the STC genes in normal pregnant rats. A basal level of STC-1 mRNA was seen in the luminal epithelium on D1 of pregnancy, and in the glandular epithelium from D1 to D5 of pregnancy (Fig. 2A, B and data not shown). Significant STC-1 immunostaining was detected in the luminal epithelium on D1 and in both luminal and glandular epithelium on D2 of pregnancy (Fig. 3A, B). The immunostaining was markedly reduced thereafter. On D4 and D5, STC-1 immunostaining was moderate in the glandular epithelium and sub-luminal stroma, but it was

weak in the luminal epithelium (Fig. 3C). While an elevated STC-1 signal was observed in the stromal cells near the lumen at the implantation site and embryo on D6 and D7 (Fig. 2C, F, Fig. 3D, E), no signal was observed at the implantation site on D6 of pregnancy when a DIG-labeled sense probe was used for hybridization (Fig. 2D). On D8, STC-1 signal in the primary decidual zone was still strong and gradually expanded to the secondary decidual zone (Fig. 2G). On D9, a high level of STC-1 was observed mainly in the secondary decidual zone (Fig. 2H), and high level of STC-1 immunostaining was observed in the whole decidua and the embryos on D8 and D9 of pregnancy (Fig. 3G and H). No immunostaining was detected when the goat anti-STC-1 was replaced with the normal goat IgG (data not shown).

As seen for STC-1, STC-2 mRNA was at a basal level in the rat uterus during D1–D5 of pregnancy (Fig. 4A). No immunostaining was detected on D1 of pregnancy. From D2 on, however, a gradual increase in immunostaining in the glandular epithelium was observed, with maximal STC-2 immunostaining occurring on D5 (Fig. 5A, B). On D6 of pregnancy, when the blastocyst begins to implant, STC-2 mRNA levels were high and immunostaining was elevated in the stroma immediately surrounding the blastocyst (Fig. 4B, Fig. 5E). In the non-implantation site of the uterus, however, no mRNA was detected in the stroma, only a moderate immunostaining was observed in the glandular epithelium (Fig. 4C, Fig. 5G). From D7 to D9 of pregnancy, STC-2 mRNA was present in the decidual cells (Fig. 4E, G and H). Moderate levels of
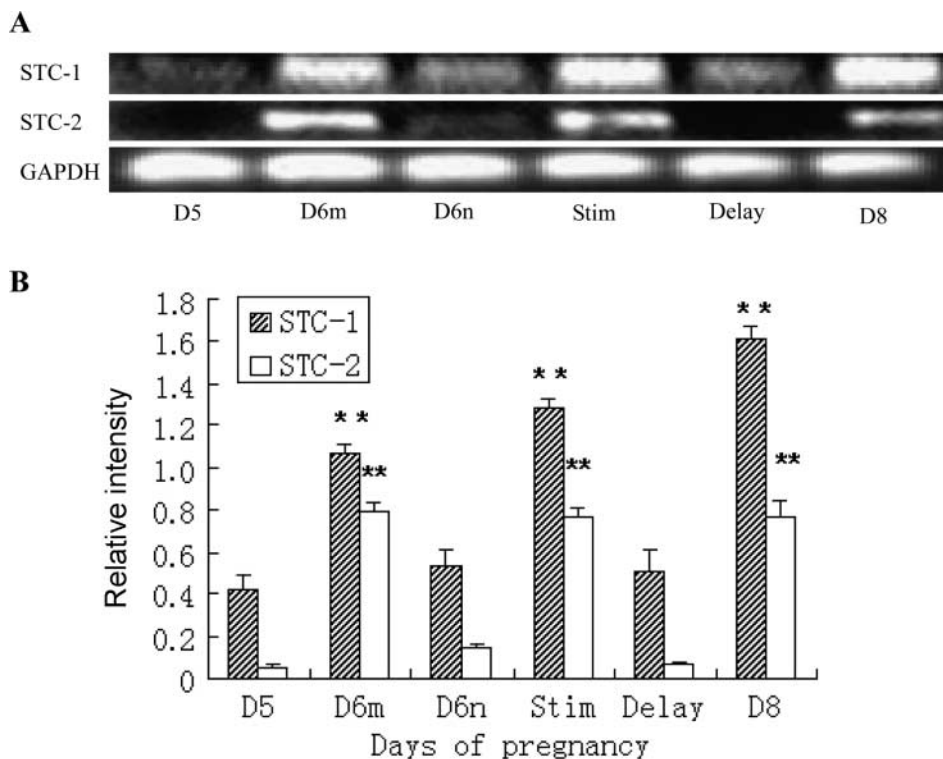

Figure 1 RT-PCR of STC mRNA in periimplantation uterus. D5: day 5 of early pregnancy; D6m: implantation site, day 6 of early pregnancy; D6n: inter-implantation site, day 6 of early pregnancy; Stim: implantation site of estrogen-activated delayed implantation uterus; Delay: delayed implantation uterus; D8: implantation site of day 8 of early pregnancy. GAPDH was used as a control for its constant expression. (A): RT-PCR. (B): Statistical analysis of RT-PCR results. The levels of mRNA were determined by densitometry. The relative intensity was the ratio of STC-1 and STC-2 mRNA to GAPDH. Data are presented as means±s.e.m. ($n=3$). Bar with ** is significantly different ($P<0.05$ or $P<0.01$).
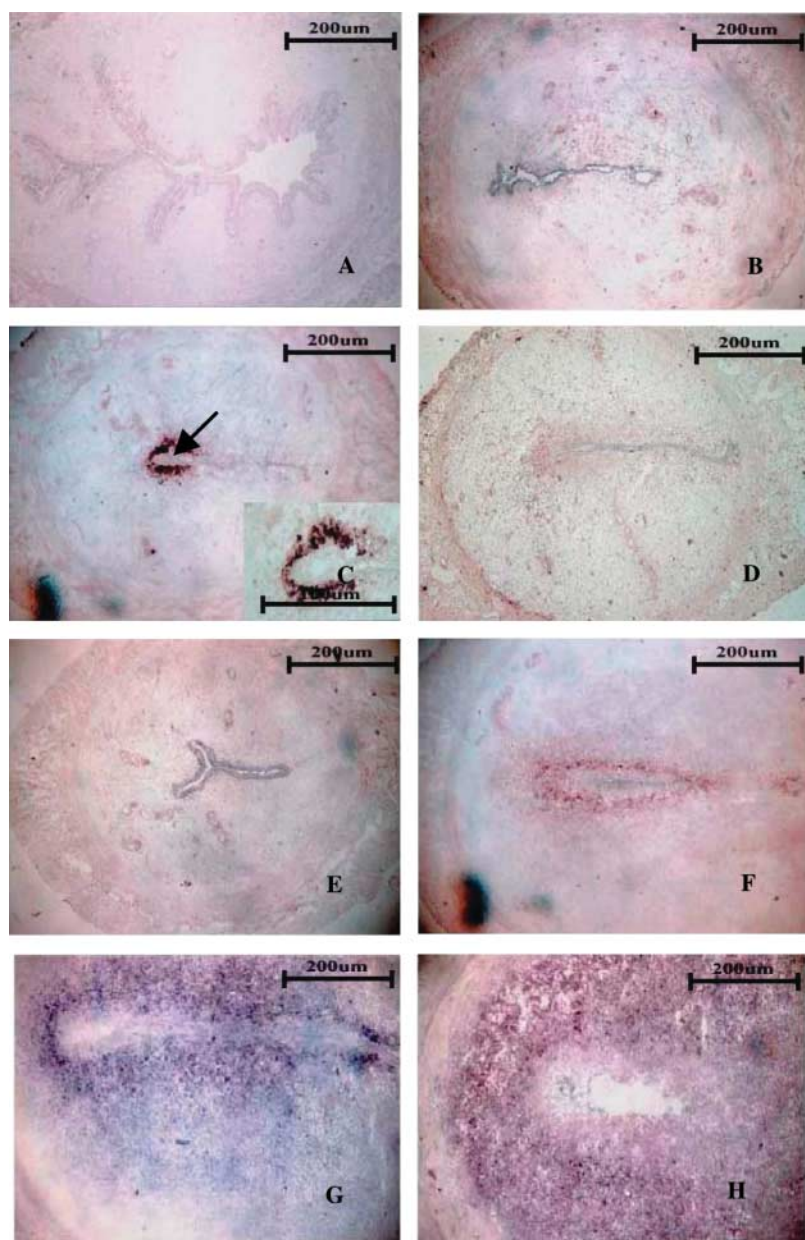
Figure 2 *In situ* hybridization of STC-1 gene in rat uterus. A basal level of STC-1 is seen in the luminal epithelium on day 1 and in the glandular epithelium on day 2 of pregnancy (A, B). On days 6 (C) and 7 (F) of pregnancy, an elevated STC-1 signal is seen in the stromal cells near the lumen at the implantation site, while no signal is seen at the implantation site on day 6 of pregnancy when the sense probe was used for hybridization (D). On day 6 of pseudopregnancy, only basal levels of STC-1 mRNA were seen (E). On day 8 of pregnancy, strong signal gradually expanded to the secondary decidual zone (G). On day 9 of pregnancy, a strong STC-1 signal was seen mainly in the secondary decidual zone (H). Arrow: implanting blastocyst.

STC-2 immunostaining were strictly localized to the primary deciduas from D7 to D8 (Fig. 5H, I), while levels decreased in the whole deciduas on D9 (Fig. 5J). These data suggest that STC-1 and STC-2 may be induced by the process of blastocyst implantation, and that the molecules may be involved in the process of stromal cell decidualization.

STC expression during pseudopregnancy

To determine whether STC expression in the uterus was specifically induced by developing embryos and implanting blastocysts, we have examined the expression of both STC-1 and -2 in pseudopregnant animals. The STC-1 mRNA signal in uteri from D1 to D5 of pseudopregnancy was similar to that from D1 to D5 of early pregnancy. A basal level of immunostaining was observed in the luminal and the glandular epithelium from D3 to D5 of pseudopregnancy. However, there was no detectable signal of either mRNA or immunostaining observed in the uteri from D6 to D9 of pseudopregnancy (Fig. 2E and 3F).

STC-2 mRNA was at a basal level during D1–D8 of pseudopregnancy (Fig. 4D). At the protein level, however, we observed a similar expression pattern as that in the uteri from D1 to D5 of early pregnancy (Fig. 5C, D). From D6 on, STC-2 immunostaining decreased gradually, reaching an undetectable level on D8 of pseudopregnancy (Fig. 5F). These results,
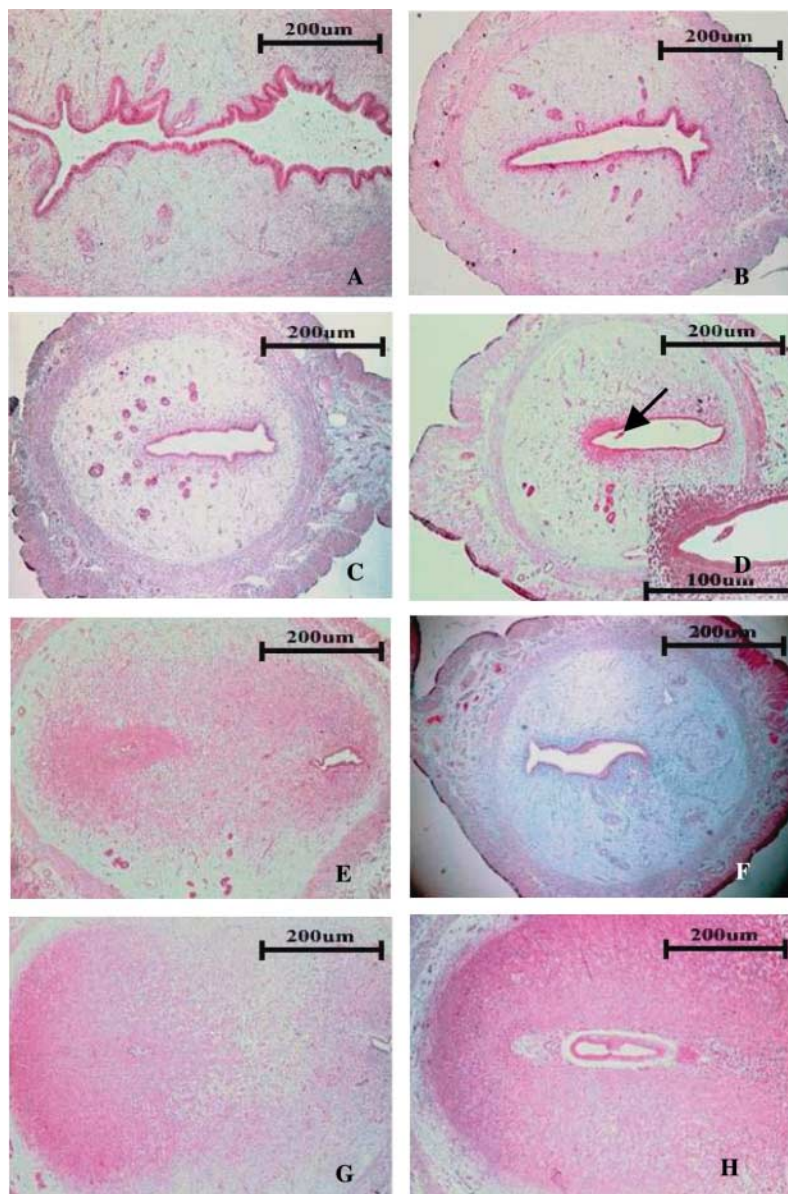
Figure 3 Immunohistochemistry of STC-1 expression in rat uterus. STC-1 protein detected in the luminal epithelium on day 1 (A) and in the glandular epithelium on day 2 (B). On day 5, levels of STC-1 protein were only moderate in glandular epithelium and sub-luminal stroma (C). A high level of STC-1 protein was observed in the primary deciduas and embryo on day 6 (D), while no obvious signal is seen on day 6 of pseudopregnancy (F). On day 7 of pregnancy, the STC-1 signal gradually expanded to the secondary decidua (E). STC-1 immunostaining was observed in the whole decidua and embryo on days 8 and 9 of pregnancy (G, H). Arrow: implanting blastocyst.

combined with those obtained from early pregnancy, suggest that STC expression in the stromal cells in the recipient uterus was induced by the implanting blastocysts, while expression in the epithelial cells in the preimplantation uterus may be induced by non-embryonic factors, such as ovarian hormones.

STC expression during delayed implantation

STC-1 and STC-2 mRNAs were at a basal level in the uterus under delayed implantation conditions (Fig. 6A, 7A). After the delayed implantation was terminated by estrogen treatment, elevated STC-1 and STC-2 mRNA signals were induced in the stroma surrounding the blastocyst (Fig. 6C, 7C).

Under delayed implantation conditions, no STC-1 immunostaining was detected in the uteri, while STC-2 protein was clearly present in the glandular epithelium with weak immunostaining in the luminal epithelium (Fig. 6B, 7B). Once the delayed implantation condition was terminated by estrogen treatment, both STC-1 and STC-2 expression was strongly induced in the embryo and the sub-luminal stroma immediately surrounding the embryo (Fig. 6D, 7D). This result supports the hypothesis that the activated blastocysts specifically induce STC expression in the adjacent stromal cells.

STC expression during artificial decidualization

The initial attachment reaction between the uterine luminal epithelium and the blastocyst trophectoderm in
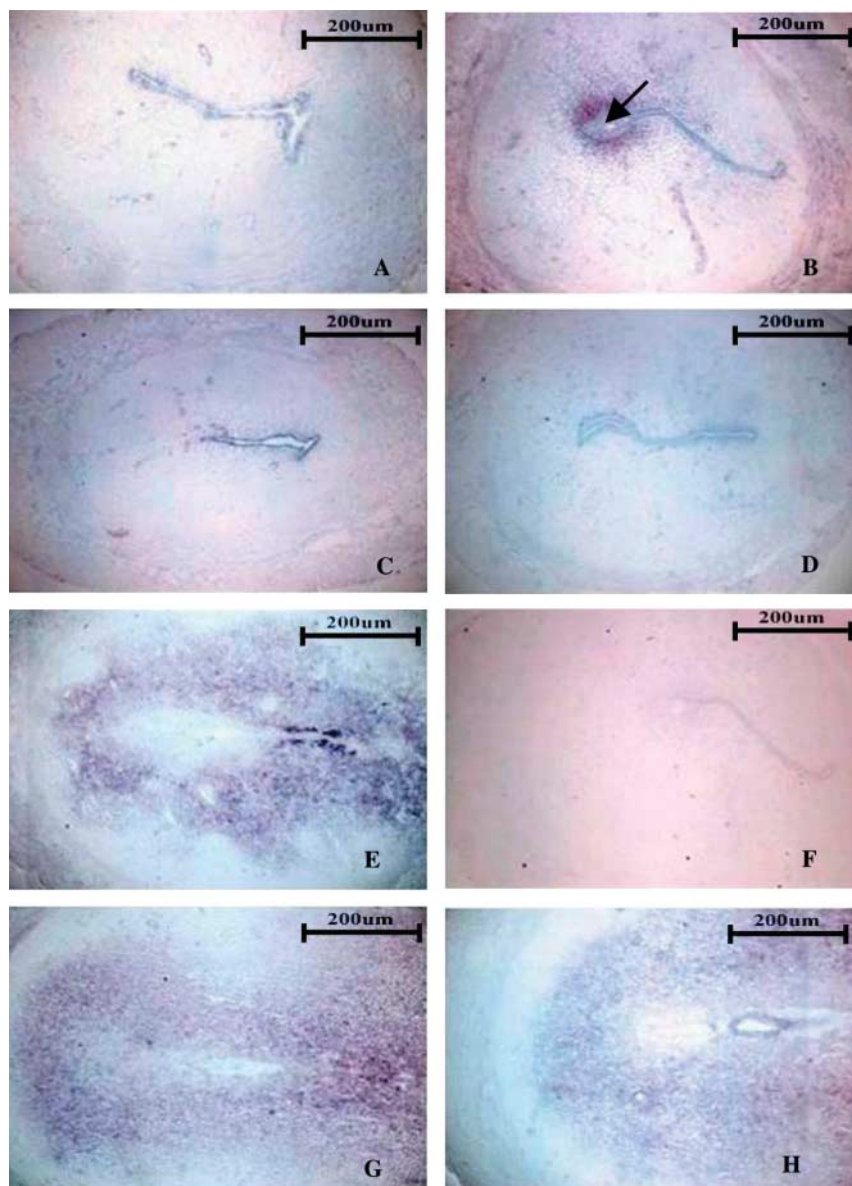
Figure 4 *In situ* hybridization of STC-2 gene in rat uterus. Only basal STC-2 mRNA is detected on day 5 of pregnancy (A), elevated STC-2 mRNA levels are seen in the stromal cells surrounding the blastocyst on day 6 (B), basal STC-2 mRNA levels are seen in the interimplantation site uterus (C) and on day 6 of pseudopregnancy (D). Elevated STC-2 mRNA is detected in the decidualized cells on days 7, 8 and 9 of pregnancy (E, G, H). No signal was seen when the antisense probe was replaced with the sense probe (F). Arrow: implanting blastocyst.

rat occurs at 1200–1600 h on D5 of pregnancy and is rapidly followed by proliferation and differentiation of stromal cells into decidual cells (decidualization) (Psychoyos 1973). The decidual reaction can also be induced experimentally by an intraluminal injection of a small amount of oil into the uteri of pseudopregnant rats. To confirm whether STC expression in the decidualizing stromal cells is specifically induced after embryo implantation, we examined the expression of STC in artificial decidualization uteri. Basal levels of STC-1 and STC-2 mRNA were detected in the luminal epithelium in the untreated control horn (Fig. 6G, 7F), whereas STC-1 and STC-2 mRNA were high in the decidualized cells under artificial decidualization (Fig. 6E, 7E). A moderate level of STC-1 immunostaining was detected in the luminal epithelium of the untreated control horn (Fig. 6H). After decidualization was artificially induced, STC-1 immunostaining was increased in the decidualized cells (Fig. 6F).

We observed moderate STC-2 immunostaining in the decidualized stromal cells immediately under the lumen 24 hours after injection of the stimulated oil (Fig. 7G); the immunostaining was significant and expanded outward 48 hours later (Fig. 8H), while only a low level of STC-2 protein was detected in all decidual cells 72 h later (Fig. 7I). In the untreated control horn, immunostaining was detected in the glandular epithelium (Fig. 7J). These results are consistent with those observed in the uteri of the early pregnant decidualization, showing that STC expression is temporarily and spatially involved in the
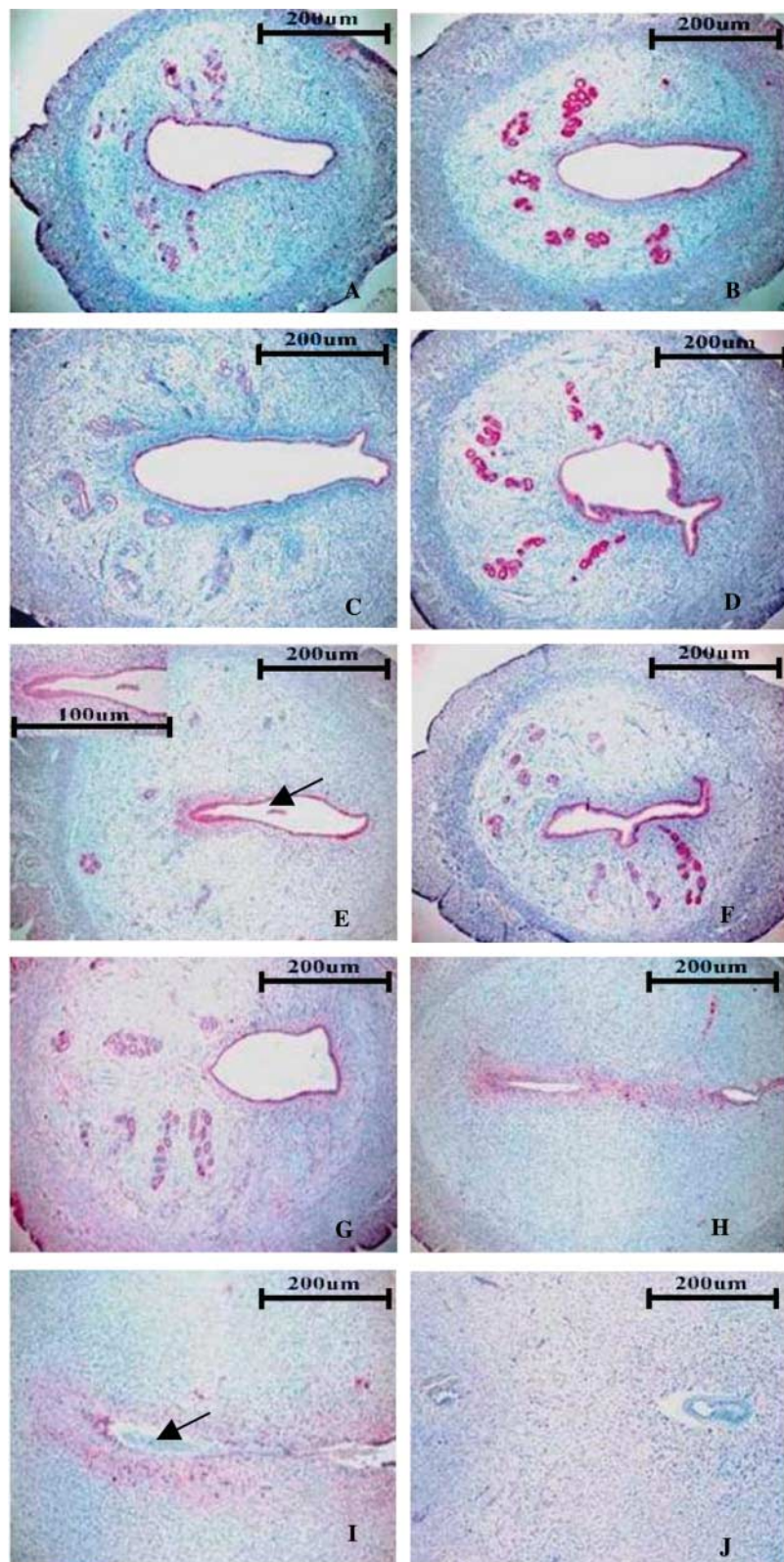
Figure 5 Immunohistochemistry of STC-2 protein in rat uterus. Low levels of STC-2 protein were detected on day 2 (A) in the luminal and glandular epithelium and elevated levels on day 5 (B) of pregnancy in the glandular epithelium. Similar expression patterns were seen on day 2 (C), 5 (D) and 6 (F) of pseudopregnancy. On day 6, elevated STC-2 immunostaining was seen in the luminal epithelium and moderate staining in the stromal cells near the lumen at the implantation site (E), and only moderate immunostaining in the epithelium at the interimplantation site (G). Moderate STC-2 immunostaining was strictly located to the primary deciduas on days 7 (H) and 8 (I), with only low STC-2 staining was seen in the whole deciduas on day 9 (J).
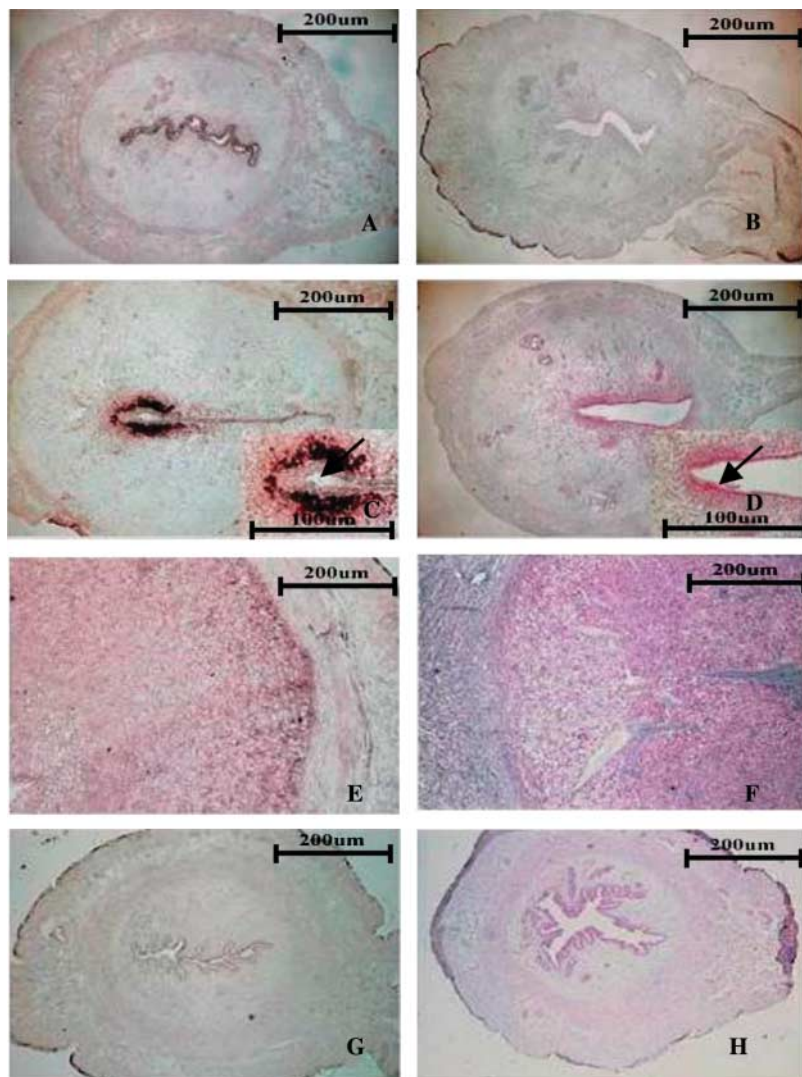
Figure 6 STC-1 mRNA (left panels) and immunostaining (right panels) expression under delayed implantation and artificial decidualization. Detection of basal level STC-1 mRNA under delayed implantation (A). After delayed implantation was terminated, strong STC-1 mRNA was induced in the stroma surrounding the blastocyst (C). No STC-1 immunostaining was detectable under delayed implantation conditions (B). Once delayed implantation was terminated, strong STC-1 immunostaining was induced in the embryo and the sub-luminal stroma immediately surrounding the embryo (D). Only basal STC-1 mRNA was seen in the uninjected control horn (G), while elevated STC-1 mRNA was seen in the decidualized cells 72 h after oil-induced artificial decidualization (E). A moderate level of STC-1 protein was detected in luminal epithelium of the uninjected horn (H), while significant STC-1 immunostaining was observed in artificially decidualized cells 72 h after oil injection (F). Arrow: implanting blastocyst.

decidualization during the implantation process, and suggesting that STC-1 is involved in the entire decidualizaion process, while STC-2 may mainly participate in the primary decidualization.

STC expression at the implantation site of transplanted uterus

Embryo transfer is another good model to study the interaction between the implanted embryo and the recipient uterus. For further confirmation of STC expression in the uterus, we transplanted early developing blastocysts into a synchronous pseudopregnant recipient uterus to induce embryo implantation. As shown in Fig. 8, both STC-1 and STC-2 mRNA and protein levels at the implantation site of the transplanted uteri were very similar to those in the uteri of early pregnancy (A, C, E, G), while in the non-transplanted control uteri, we observed the same expression pattern

as that in the uteri of D5 early pregnancy (B, D, F, H). The data obtained from the embryo transfer model show a high similarity to the results from the early pregnancy and the delayed implantation models, confirming that STC expression in the receptive uterus was specifically induced by the implanting blastocysts.

Discussion

Both STC-1 and STC-2 mRNA were evident in the stromal cells surrounding the implanting blastocyst. No corresponding signal was observed in the uterus during pseudopregnancy. A high level of STC mRNA in the stromal cells surrounding the implanting blastocyst was also observed after delayed implantation was terminated by estrogen treatment and embryo implantation was initiated, whereas no signal was detected in the uterus during delayed implantation. Furthermore, in our study, both STC-1 and STC-2 mRNA levels and
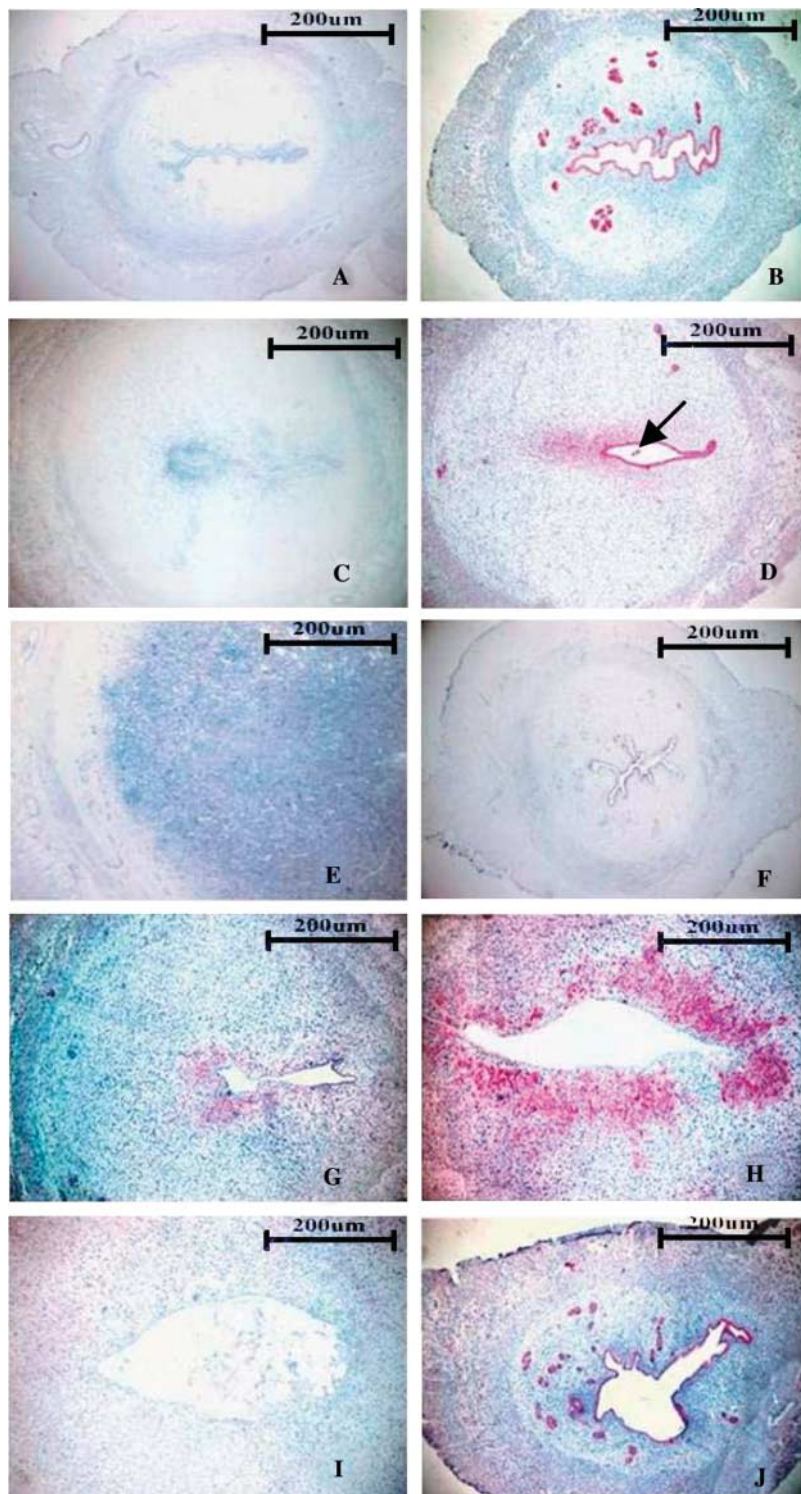
Figure 7 STC-2 expression under delayed implantation and artificial decidualization conditions. Basal STC-2 mRNA seen under delayed implantation (A). After delayed implantation was terminated, STC-2 mRNA was induced in the stroma immediately surrounding the blastocyst (C). STC-2 protein was located in glandular epithelium and weakly in luminal epithelium under delayed implantation conditions (B). Once delayed implantation was terminated, STC-2 immunostaining was induced in the stroma surrounding the blastocyst, the implanting embryos and the luminal epithelium (D). Only basal STC-2 mRNA was seen in the uninjected control horn (F), and elevated mRNA was detected in the artificial decidualized stromal cells (E). Twenty-four hours after injection of stimulating oil, only moderate immunostaining was induced in the decidualized stromal cells immediately under the lumen (G), immunostaining was elevated and expanded outward 48 h later (H), and only a low level of STC-2 protein was seen in the whole decidual cells 72 h later (I). In the uninjected control horn, significant immunostaining was detected in glandular epithelium (J). Arrow: implanting blastocyst.

immunostainning in the endometrium were much higher at the sites with the implanting embryo than that at other uterine segments without an embryo, suggesting that the expression of STC mRNAs and proteins in the luminal epithelium and sub-luminal stroma at the implantation site may be specifically induced by the implanting embryos. Again, in the embryo transfer model, high levels of STC expression were observed at the implantation site, while in the untransplanted control uterus, no obvious expression of
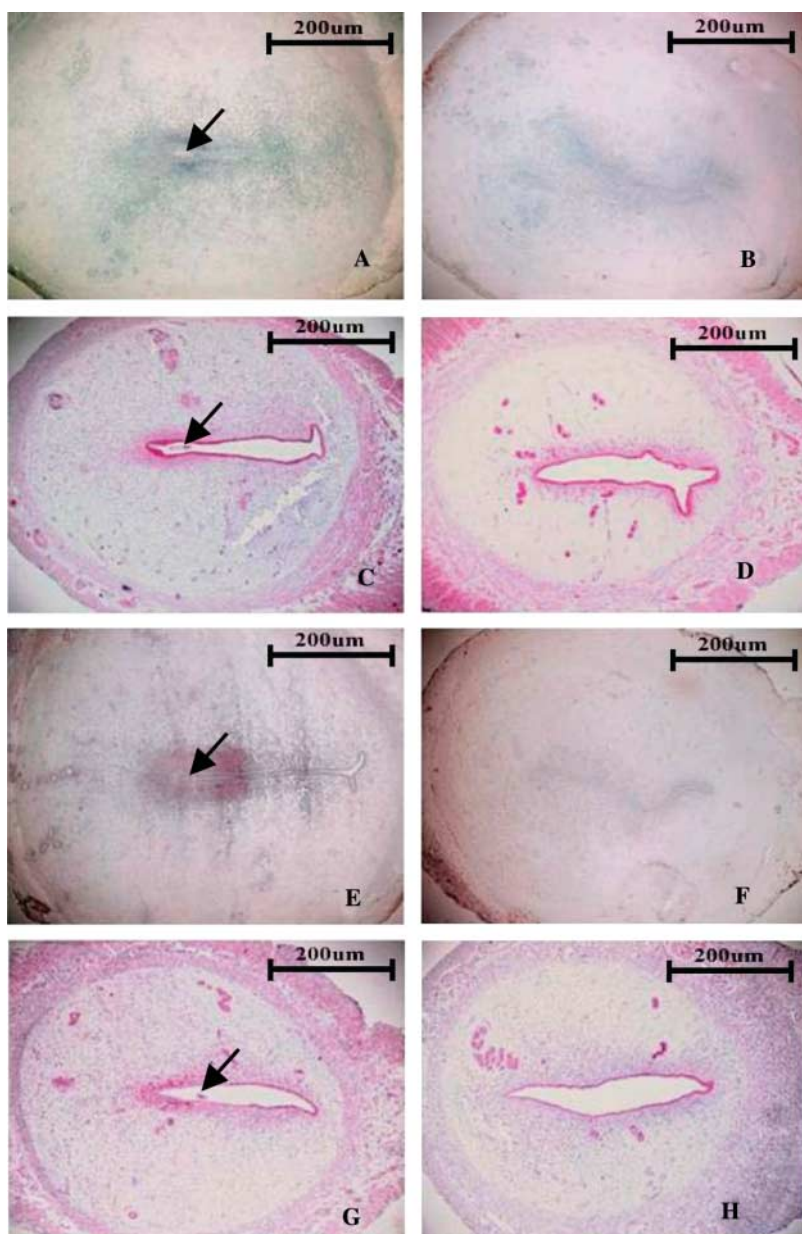
Figure 8 STC expression in transplanted uterus. STC mRNA and protein was seen in the stromal cells surrounding the blastocyst in the transplanted uterus (A, STC-1 mRNA; C, STC-1 protein; E, STC-2 mRNA; G, STC-2 protein). No obvious STC expression was seen in the control uterus (B, STC-1 mRNA; D, STC-1 protein; F, STC-2 mRNA; H, STC-2 protein). Arrow, implanting blastocyst.

these molecules was detected. These data suggest that expression of both STCs at the implantation site was specifically induced by either a blastocyst or an artificial decidualization stimulus under estrogen dominance.

It is interesting that a high level of STC-1 immunostaining was detected in the luminal epithelium at the implantation site on D6 of pregnancy. A similar expression pattern was also observed after the delayed implantation was terminated by estrogen treatment and embryo implantation was initiated. We also observed significant STC-1 immunostaining in the embryos of the pregnant uteri from D6 to D9, but no mRNA was detected in the post-implantation embryos. It is possible that STC-1 synthesized in the stromal cells at the

implantation site may be transferred to or sequestered by luminal epithelium and embryos. A different localization of STC-1 mRNA and protein was noticed in the mouse uterus, where the STC-1 mRNA was uniquely expressed in the mesometrial stromal cells, while STC-1 protein was also localized in the decidualizing antimesometrial cells and the adjacent luminal epithelial cells (Stasko *et al.* 2001). The same mRNA-protein disparity in the adult mouse ovary was also reported by Deol *et al.* (2000).

Disparities between STC-1 mRNA expression and protein distribution were previously reported in other mammalian organs in addition to the uterus and ovary. During mouse urogenital development, a

mesenchyme–epithelial signaling pathway was evident, whereby STC-1 mRNA was produced in the mesenchyme cells and the protein was much more evident in the adjacent epithelial cells (Stasko & Wagner *et al.* 2001*a*). During mouse skeletogenesis, the STC-1 gene was expressed in undifferentiated cells and targeted to cartilage cells in various stages of terminal differentiation (Stasko & Wagner *et al.* 2001*b*). It is evident that STC-1 plays a paracrine/autocrine signaling role during these processes.

In our study, STC-1 mRNA levels and immunostaining were both evident in the decidualized cells on D7–D9 of pregnancy. Similarly, high levels of STC-1 mRNA and protein could be induced in decidualized cells under artificial decidualization. STC-1 expression in the decidual cells may suggest a role in decidualization. However, the mechanism of STC-1 action during this process is still unclear.

STC-2 mRNA was also highly expressed in the decidual cells on D7–D9 of pregnancy and in the artificially induced decidual cells. STC-2 protein expression was interesting, with only a moderate level of STC-2 immunostaining in the primary deciduas on D7–D8, and very low immunostaining in the whole deciduas on D9. Similarly, only moderate immunostaining was seen in the decidualized stromal cells immediately under the lumen 24 h after injection of the stimulating oil, the immunostaining was elevated and expanded outward 48 h later, while only a low level of STC-2 protein was detected in the whole decidual cell 72 h later. These data show that STC-2 may mainly participate in the process of primary decidualization. However, the mechanism by which STC-2 fulfills its function during decidualization remains to be discovered.

Intriguingly, we could only detect a basal level of STC-2 mRNA in the uterus during the preimplantation period, while a strong STC-2 protein signal was localized in the glandular epithelium before embryo implantation on D4–D5 of pregnancy. There are three possible explanations for the disparities. One possibility is that STC-2 immunostaining detected in the uterus was derived from the maternal serum, originating from organs other than the uterus that may play a role as an endocrine regulator of glandular proliferation/differentiation in the uterus. The localization pattern of STC-2 in the uterus was highly reminiscent of the ovarian STC-1 distribution during pregnancy and lactation and of their counterpart in fish, where the stanniocalcin appears to act in a classical endocrine fashion as a regulator of calcium and phosphate homeostasis (Paciga *et al.* 2002). Another possibility is that our *in situ* hybridization protocol might not be sufficiently sensitive to detect the mRNA signal. We consider this unlikely as we could detect the mRNA signal in the uterus once embryo implantation occurred, using the same experimental protocol. The third possibility is an upregulation

mechanism of STC-2 protein by progesterone/estrogen at a translational level. No evidence is available in the literature, however, to support this hypothesis.

While the data from both ourselves and others (Deol *et al.* 2000, Stasko *et al.* 2001) show highly regulated STC-1 expression in the uterus and ovary, suggesting that STC-1 is involved in the regulation of uterine and ovarian function, the recently published STC-1 knockout paper presented us with unexpected results. The authors could not find any important change in fertility in STC-1 null mice, the histological examination of the uterus and ovary did not show any abnormalities compared with wild-type mice (Chang *et al.* 2005). The authors also ruled out the possibility of STC-2 compensating for the loss of STC-1, but checked STC-2 expression under normal status only. For figuring out the potential role of STC-1 in the uterus and the possibility of STC-1 function compensation by STC-2, the study should focus on the expression of STC-2 during different physiological conditions. It is also possible that compensation is fulfilled by other unknown candidates, the most efficient and accurate approach to figure this out would be to generate mice that are knockout both STC-1 and the candidate gene.

In summary, this is the first report to show expression and localization of both STC-1 and STC-2 in mammalian uteri under different physiological conditions. The high levels of STC expression at the implantation site and the decidual cells in rat uterus suggest that these hormones might be important for blastocyst implantation and uterine decidualization. STC-1 appears to be involved in the entire decidualization process, while STC-2 may participate mainly in the primary decidualization.

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