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Identification of a Stanniocalcin Paralog, Stanniocalcin-2, in Fish and the Paracrine Actions of Stanniocalcin-2 in the Mammalian Ovary

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Stanniocalcin is a glycoprotein hormone important in the maintenance of calcium and phosphate homeostasis in fish. Two related mammalian stanniocalcin genes, STC1 and STC2, were found to be expressed in various tissues as paracrine regulators. We have demonstrated the existence of a second stanniocalcin gene in fish, designated fish STC2, with only 30% identity to fish STC1. However, phylogenetic analysis and comparison of the genomic structure of STC genes in vertebrates indicated that STC1 and STC2 genes were probably derived from a common ancestor gene. Based on the prominent expression of mammalian STC1 in the ovary, we tested STC2 expression in rat ovary and the regulation of STC2 expression by gonadotropins. Treatment of immature rats with pregnant mare serum gonadotropin increased STC2 transcripts, whereas subsequent treatment with human chorionic gonadotropin suppressed STC2 expression. Real-time PCR analyses also demonstrated that STC2 is expressed mainly in thecal layers. *In situ* hybridization studies also revealed that STC2 is expressed in thecal cell layers of antral and preovulatory follicles after gonadotropin stimulation. To elucidate the physiological functions of STC2, recombinant human and fish STC2 proteins were generated and found to be *N*-glycosylated homodimers. In cultured granulosa cells, treatment with human or fish STC2 suppressed FSH-induced progesterone, but not estradiol or cAMP, production. The STC2 suppression of progesterone production was associated with the inhibition of FSH-induced CYP11A and 3β-hydroxysteroid dehydrogenase expression. Thus, STC2 is a functional homodimeric glycoprotein, and thecal cell-derived STC2 could play a paracrine role during follicular development. (*Endocrinology* 146: 469–476, 2005)

STANNIOCALCIN IS A glycoprotein hormone first discovered in bony fish. In fish, stanniocalcin is mainly secreted from the corpuscles of Stannius and acts on gills, gut, and kidneys to maintain calcium and phosphate homeostasis (1–3). The potential role of stanniocalcin in terrestrial animals was determined after injections of fish stanniocalcin into frogs, birds, and rats led to the induction of hypocalcemia (4–6). The first mammalian stanniocalcin gene, human STC1, was isolated based on its sequence homology to fish stanniocalcin (7). STC1 was also found to be conserved in other mammalian species (7, 8). Unlike fish stanniocalcin, which regulates calcium metabolism by systematic actions, mammalian STC1 is probably a paracrine hormone. We have demonstrated that thecal cell-derived STC1 acts locally to regulate granulosa cell differentiation induced by gonadotropins in rat ovary. In cultured granulosa cells, STC1 suppressed gonadotropin-induced progesterone production and the FSH induction of functional LH receptors (9). Of interest, a recent study showed that theca/interstitial-derived big STC1 would probably interact with lipid storage droplets of ovarian steroidogenic cells and inhibit human chorionic gonadotropin (hCG) stimulation of progesterone biosynthesis by cultured luteal cells. However, the protein structure of this nonglycosylated big STC1 is unclear (10). In addition to ovarian actions, STC1 has been shown to protect the human cerebral neurons against hypoxic/ischemic damage (11). In transgenic mice, overexpression of STC1 led to a reduction of body weight, and STC1 was proposed to participate in mineral absorption and bone formation (12, 13).

Although human STC2, a paralog of STC1, was found by GenBank searches (14–16), no physiological actions for STC2 have been demonstrated. In addition, no second stanniocalcin gene was found in fish. The primary sequence of human STC2 showed less than 30% identity to fish stanniocalcin and human STC1. However, the cysteine residues found in fish stanniocalcin and human STC1 are conserved in human STC2. In this study, based on sequence homology to human STC2, we have isolated the STC2 ortholog in fish by searching the *Fugu rubripes* genome, followed by PCR amplification from a zebrafish cDNA library. Interestingly, like STC1, mammalian STC2 was also found to be expressed in the ovary (17). Hence, we analyzed the expression pattern of STC2 in rat ovary and generated recombinant human and fish STC2 proteins to demonstrate the physiological roles of STC2 in the ovary.

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Abbreviations: hCG, Human chorionic gonadotropin; 3βHSD, 3β-hydroxysteroid dehydrogenase; PMSG, pregnant mare serum gonadotropin; PNGase F, peptide *N*-glycosidase F; StAR, steroidogenic acute regulatory protein; STC, stanniocalcin.

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Materials and Methods

Animals

Immature female rats (Sprague Dawley) were obtained from Simonsen Laboratories (Gilroy, CA). Animals (22 d old; body weight, 53–68 g)

were anesthetized using CO_2 and were killed 72 h after insertion of diethylstilbestrol implants (18). Granulosa cells were obtained by puncturing the ovaries with fine needles. For analysis of STC2 mRNA expression using the superovulation model, immature female rats (26 d old) were treated with 15 IU pregnant mare serum gonadotropin (PMSG; Calbiochem, La Jolla, CA) at 0900–1000 h and received an ip injection of 10 IU hCG (Schein Pharmaceuticals, Florham Park, NJ) 48 h later. Rats were killed at different time points, and ovaries were collected for total RNA extraction or were fixed for *in situ* hybridization studies. All animals were housed under controlled humidity, temperature, and light regimen and were fed standard rat chow *ad libitum*. Animal care was consistent with institutional and NIH guidelines.

Reagents and hormones

McCoy's 5a medium (modified), L-15 Leibovitz medium, and DMEM-Ham's F-12 were obtained from Invitrogen Life Technologies, Inc. (Gaithersburg, MD). Recombinant human FSH was a gift from NV Organon (Oss, The Netherlands). Purified hCG (CR-129) and antibodies against cAMP were supplied by the National Hormone and Pituitary Program (NIDDK, NIH, Bethesda, MD). Peptide *N*-glycosidase F (PNGase F) was obtained from New England Biolabs, Inc. (Beverly, MA). Androstenedione, BSA, and 3-isobutyl-1-methylxanthine were obtained from Sigma-Aldrich Corp. (St. Louis, MO); L-glutamine, penicillin, and streptomycin were purchased from BioWhittaker (Walkersville, MD). Antisera against progesterone and estradiol were raised against progesterone-11-hemisuccinate-BSA and estradiol-17β-*O*-carboxymethyl-BSA, respectively (19).

Computational analysis

The human STC2 cDNA sequence was used to search the *F. rubripes* genome based on the BLAST server at National Center for Biotechnology Information (20). cDNA sequences in different species related to human STC1 and STC2 were collected after nucleotide to nucleotide BLAST. To compare the phylogenetic relationship of stanniocalcin genes from different species, full-length amino acid sequences from these genes were used. The sequence alignments and the phylogenetic tree were constructed by the neighbor-joining method using the Megalign program in the DNAStar software package (DNAStar, Madison, WI).

Construction of a zebrafish cDNA library and generation of zebrafish and human STC2 expression plasmids

Adult zebrafish, *Danio rerio*, were purchased from a local pet store. The whole fish total RNA was extracted from four animals using an RNeasy kit (Qiagen, Valencia, CA) following the suggested protocol. Five micrograms of total RNA were reverse transcribed to cDNA using the Ready-to-Go RT-PCR kit (Amersham Biosciences, Piscataway, NJ). Primers 5′-CTGCAGAACACAGCTGAGATCCAG-3′ and 5′-TGCCTCCTTCACTTCCTCTCCGCA-3′, deduced from conserved regions of the *F. rubripes* and human STC2 genes, were used to amplify an STC2 fragment from the zebrafish cDNA library. In addition, primers 5′-GATGTGGCTAGGTGTTTGAATGGC-3′ and 5′-GAGCAGAGTGCTGTAGTACCTGTT-3′, deduced from conserved regions of the *F. rubripes* and human STC1 cDNAs, were used to amplify a zebrafish STC1 cDNA fragment. The conserved fragments were cloned and sequenced to confirm them as STC1 and STC2 orthologs in fish. Flanking 3′ and 5′ end sequences were obtained using 3′ and 5′ rapid amplification of cDNA ends (BD Clontech, Palo Alto, CA).

Full-length human and zebrafish STC2 cDNAs were inserted into the pcDNA3.1/Zeo$^+$ expression vector. The constructed plasmids were purified and transfected into mammalian 293T cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). Forty-eight hours after transfection, transfectants were selected by the zeocin-containing medium. Selected cells were allowed to reach confluence and then were cultured for 72 h in a serum-free medium (DMEM/Ham's F-12 medium containing 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamate). After filtration of the conditioned medium, the recombinant proteins were purified at 4 C. The medium containing zebrafish STC2 was incubated with Con A-conjugated resin (Amersham Biosciences) that had been preequilibrated in the wash buffer (125 mM NaCl and 20 mM Tris-HCl, pH 7.4). The unbound protein was removed by washing with buffer. Recombinant proteins were eluted with the wash buffer containing 0.2 M methyl α-D-glucopyranoside. In addition, the medium containing recombinant human STC2 was adjusted to 20 mM imidazole and incubated with metal-chelating Sepharose (Amersham Biosciences). The bound proteins were eluted with a wash buffer containing 500 mM imidazole. Protein purity and biochemical characteristics were analyzed after electrophoresis using a 12% sodium dodecyl sulfate-polyacrylamide gel.

Quantitative real-time PCR

For assessing the gonadotropin regulation of STC2 mRNA expression, total RNA was extracted from rat ovaries after gonadotropin treatment. After isolation of individual follicles under the Hoffman modulation contrast microscopy (Nikon, Inc., Tokyo, Japan), total RNA also was extracted from follicles or isolated thecal or granulosa cells. For testing estrogen induction of STC2 expression, follicles were cultured in McCoy's 5a medium in the presence of 10^{-8} M estradiol. For detecting STC2 expressions in ovarian cell types, individual follicles were punctured to obtain granulosa cells, followed by cutting open the follicles and scrapping the remaining granulosa cells from the thecal shell. The purity of granulosa and thecal cells was confirmed based on the differential expression of FSH and LH receptor transcripts determined by real-time PCR (9).

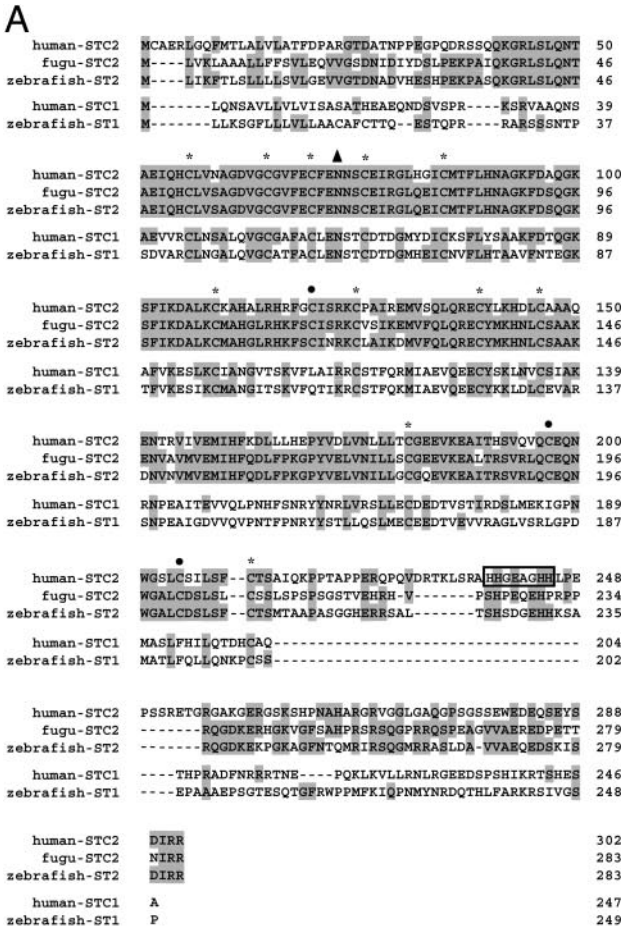
To study STC2 regulation of granulosa cell genes, cells (2×10^5 cells/ml) from diethylstilbestrol-treated animals were incubated with 10 ng/ml FSH in the presence or absence of 10 nM STC2 for various intervals. Cells were washed and collected for subsequent total RNA extraction. RNA was extracted using an RNeasy Mini Kit (Qiagen). Total RNA (2 μg) from each sample was reverse transcribed for subsequent PCR analysis (9). The primer pairs and fluorescent probes used for STC2 were as follows: rat STC2 forward primer, GTCGTGATTGTGGAGATGATTC; rat STC2 reverse primer, CTGTTCACACTGAGCCTGGA; and rat STC2 probe, TTCAAGGATCTCCTGCTGCATGAAC. Other primer pairs, probes, and the standard curves for STC2, LH receptor, CYP11A, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3βHSD), and β-actin were generated as described previously (9).

In situ hybridization studies

Rat ovaries were fixed at 4 C for 6 h in 4% paraformaldehyde in PBS, followed by immersion in 0.5 M sucrose in PBS overnight. Cryostat sections (8 mm thick) of fixed ovaries were mounted on microscope slides (Sigma-Aldrich Corp.), fixed in 4% paraformaldehyde in PBS, and stored at −80 C before the hybridization procedure. Sections were pretreated serially with 0.2 M HCl, 2× standard saline citrate, pronase E (0.125 mg/ml), 4% paraformaldehyde, and acetic anhydride in triethanolamine before dehydration in ascending grades of ethanol. A 300-bp PCR fragment of rat STC2 was amplified by primers TCGTCGTGATTGTGGAGATGAT and ACCCTTGGCACCTCTGTTGGCTGT and was subcloned for probe synthesis. The antisense and sense probes were generated and labeled with [^{35}S]UTP (1000 Ci/mmol; Amersham Biosciences). The sections were hybridized overnight at 45 C in 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, 1 μg/ml carrier transfer RNA, and 10 mM dithiothreitol. After ribonuclease A (20 μg/ml) treatment at 37 C for 30 min, posthybridization washing was performed to a final stringency of 0.1× SSC. Slides were dipped into NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) and exposed at 4 C for 3 wk before development. The slides were subsequently stained with hematoxylin and eosin and mounted with DPX Mountant (Electron Microscopy Sciences, Ft. Washington, PA). Photographs of the slides were taken using a MicroFire camera (Optronics, Goleta, CA) attached to a Zeiss microscope (Oberkochen, Germany) with bright- and darkfield illumination. To allow for direct comparison between the ovarian sections from different experimental groups, all slides were processed simultaneously and under identical conditions.

Preparation of granulosa cells and assessment of steroid and cAMP production

Granulosa cells were obtained from early antral follicles of diethylstilbestrol-treated rats. Ovaries were punctured in the L-15 Leibovitz



medium, and the medium containing granulosa cells was collected after low speed centrifugation at 500 × *g* for 10 min. Granulosa cells were dispersed by repeated washing and resuspended in the culture medium (McCoy's 5a supplemented with 10^{-7} M androstenedione, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Granulosa cells (2×10^5 viable cells/ml) were cultured in 24-well plates (Corning, Inc., Corning, NY) and treated with FSH in the presence or absence of increasing concentrations of STC2. After culture, medium progesterone, estradiol, and cAMP contents were determined using RIA as described previously (9).

Results

Identification of STC2 genes and cloning of STC2 cDNA from fish

By searching the genomic sequences of *F. rubripes*, we identified a stanniocalcin paralog, named STC2. Based on alignment of the conserved sequence between human and *F. rubripes* STC2, we designed primer pairs to amplify STC2 cDNA from a zebrafish (*Danio rerio*) cDNA library constructed by us. After sequence confirmation of the cDNA fragment, 5′ and 3′ rapid amplification of cDNA ends was performed to obtain a full-length zebrafish STC2 cDNA. Figure 1A shows the sequence alignment of STC2 and STC1 from human, *F. rubripes*, and zebrafish. Zebrafish STC2 shows 73% and 56% identity to *F. rubripes* and human STC2, respectively, whereas *F. rubripes* STC2 shows 56% identity to human STC2. In STC2 from these three species, all 14 cysteine residues and the putative *N*-linked glycosylation site (Asn-X-Ser/The) near the N terminus were conserved. Of interest, the C terminus of human STC2 had a cluster of histidines, HHxxxxHH, capable of interacting with divalent metal ions (21) that did not appear in the two fish STC2 sequences. We also aligned human STC1 with the zebrafish STC1 sequence deduced from a cDNA clone obtained by us. Of interest, 11 of the 14 cysteine residues and the amino-terminal glycosylation site found in two fish STC2 cDNAs were conserved in STC1. However, the overall identity between STC2 and STC1 sequences is less than 30%.

To analyze the evolutionary relationship of STC1 and STC2 genes, phylogenetic analyses were performed using the neighbor-joining and parsimony methods (Fig. 1B) (22). The results indicated that stanniocalcin genes could be divided into STC1 and STC2 subgroups, and the identity between STC1 and STC2 for a given species was less than 30%. In addition, genomic structures of *F. rubripes* and human STCs were compared (Fig. 1C). Both human and *F. rubripes* STC2 genes contained four exons, encoding similar numbers of

FIG. 1. Comparison of STC1 and STC2 genes from different species. A, Sequence alignment of human and fish stanniocalcin cDNAs. STC2 and STC1 protein sequences from human, *F. rubripes*, and zebrafish are aligned. Identical amino acids are *shaded*. *Dashed lines* indicate gaps introduced in the sequence for optimal alignment. The numbers on the *right* refer to the last amino acid on that line. The conserved cysteine residues (*) and a putative *N*-linked glycosylation site (▲) are indicated. Three additional cysteine residues found in STC2s are marked (●). A histidine cluster found in the C-terminal third of human STC2 is *boxed*. B, Phylogenetic analyses of fish and mammalian STC1 and STC2 genes. Full-length sequences of stanniocalcin genes from different species are used for comparison. C, Genomic structures of human and fish STC1 and STC2. Exons are represented by *boxes*; introns are shown by the *lines* between exons. The cysteine-rich region in the protein sequence is *shaded*. The sizes of introns are shown in kilobase pairs. The number of amino acid residues encoded by each exon is shown inside the exon box.

amino acid residues. In contrast, the human STC1 gene contained four exons with a sequence spanning 13 kb, whereas the *F. rubripes* STC1 gene contained five exons spanning about 3 kb. Based on comparison of STC1 gene structures, exons 3 and 4 of *F. rubripes* STC1 could be derived from a single exon with an intron insertion during evolution. Overall, the conservation of exon-intron boundaries among all STC2 and STC1 genes suggests their derivation from a common ancestor gene before the divergence of fish and mammals.

Generation and biochemical characteristics of recombinant human and zebrafish STC2 proteins

To study the structural characteristics and possible functions of STC2, an expression plasmid containing full-length cDNA of human or zebrafish STC2 was transfected into 293T cells and conditioned media were collected. Human STC2 was purified using metal chelate chromatography based on the presence of a histidine cluster near its C-terminus, whereas zebrafish STC2 was isolated using Con A affinity chromatography. Under reducing conditions, purified human STC2 migrated as a diffused band between 36–40 kDa based on Coomassie Blue staining (Fig. 2A, lane 2), whereas purified zebrafish STC2 migrated as a 34-kDa band (Fig. 2B, lane 2). In contrast, a prominent albumin band of 64 kDa was found in the conditioned medium before purification (Fig. 2, A and B, lane 1). Under nonreducing conditions, the purified human and zebrafish STC2 proteins migrated as bands of 80 and 70 kDa, respectively (Fig. 2, A and B, lane 3), suggesting that both recombinant human and zebrafish STC2 formed disulfide-linked homodimers. After treatment of recombinant STC2 with PNGase F to remove *N*-linked carbohydrate moieties, the human and zebrafish STC2 proteins migrated as bands of 36 and 29 kDa, respectively (Fig. 2C). These data demonstrated that human and fish STC2 proteins are probably *N*-linked homodimeric glycoproteins.

Gonadotropin and estrogen regulation of STC2 transcripts in thecal cells

Based on the prominent expression of STC1 in the ovary (7, 23), we tested ovarian STC2 expression and the possible regulation of STC2 transcript levels by gonadotropins during follicular development. Ovaries from immature rats treated with PMSG followed by hCG treatment were collected for total RNA extraction. Changes in STC2 gene expression were estimated using quantitative real-time PCR. As shown in Fig. 3A, STC2 mRNA levels increased 3-fold after 12 h of PMSG treatment ($P < 0.01$). STC2 transcript levels were suppressed in a time-dependent manner after subsequent hCG injection and returned to the baseline level 72 h after hCG treatment. We isolated early antral follicles and cultured them *in vitro*. In these follicles, treatment with estradiol (10^{-8} M) increased STC2 transcript levels in a time-dependent manner, with a 5.5-fold increase 24 h after treatment (Fig. 3B). STC2 expression was additionally analyzed in different ovarian compartments by isolating ovarian follicles and separating granulosa and thecal cells. As shown in Fig. 3C, STC2 transcripts were found mainly in thecal cells, but not granulosa cells. After treatment with PMSG for 24 h, thecal cell STC2 transcript levels were increased by 3.6-fold.

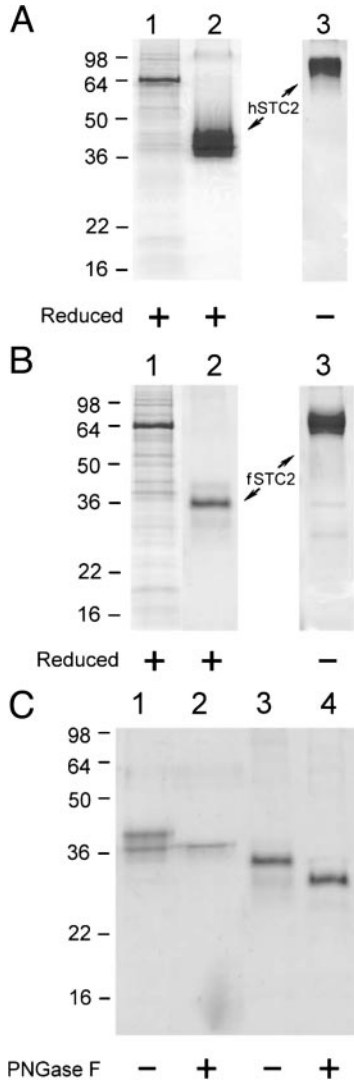


FIG. 2. Purification and biochemical properties of recombinant human and zebrafish STC2. A, Recombinant human STC2 was detected by Coomassie Blue staining after loading the conditioned medium from transfected 293T cells (lane 1, reducing conditions) and purified STC2 (lane 2, reducing conditions; lane 3, nonreducing conditions). B, Coomassie Blue staining of the recombinant zebrafish STC2 protein. Lane 1, Conditioned medium under reducing conditions; lane 2, purified zebrafish STC2 under reducing conditions; lane 3, purified zebrafish STC2 under reducing conditions. C, Purified human (lanes 1 and 2) and zebrafish STC2 (lanes 3 and 4) without or with PNGase F treatment were subjected to SDS-PAGE under reducing conditions. The gels were stained by Coomassie Blue.

In situ hybridization studies demonstrated negligible expression of STC2 mRNA before PMSG treatment (Fig. 4, A and B). After gonadotropin stimulation, STC2 expression was present in the thecal cell layer of antral follicles 12 h after PMSG stimulation (Fig. 4, C and D). Forty-eight hours after PMSG, STC2 expression was present in the thecal cell layer of large preovulatory follicles in addition to the thecal layer of antral follicles (Fig. 4, E and F).

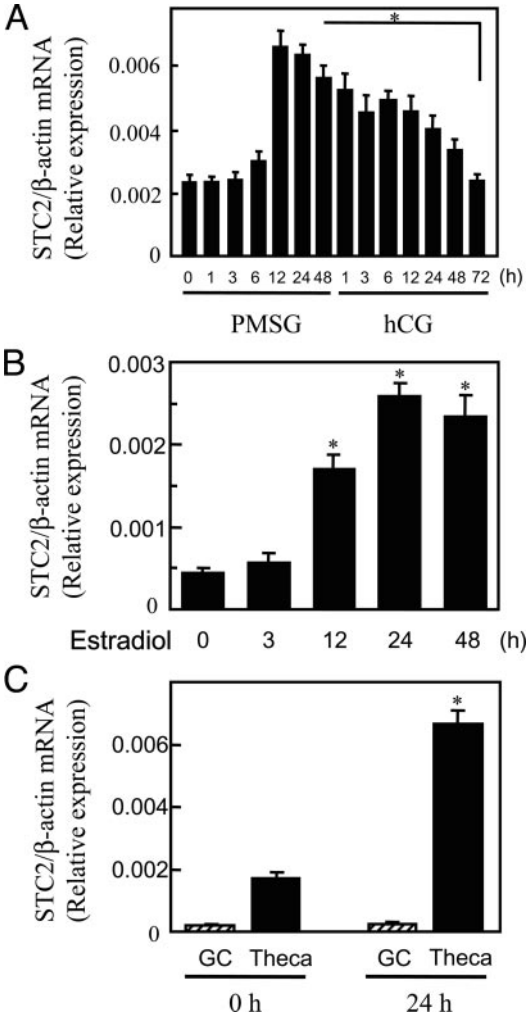


FIG. 3. Expression and regulation of STC2 in the ovary. A, Gonadotropin regulation of STC2 expression. Rats at 26 d of age were treated with PMSG, followed 48 h later by hCG for different intervals before extraction of ovarian RNA. B, STC2 as an estrogen response gene. Early antral follicles isolated from immature rats were incubated in the presence of 10^{-8} M estradiol for different intervals. C, Comparison of STC2 expression in different ovarian cell types. Granulosa and thecal cells were isolated from follicles before and 24 h after PMSG treatment. After RT, STC2 transcript levels were determined by real-time PCR and normalized using β-actin levels. Data are shown as the mean ± SE (n = 3). *, Significant difference between designated groups (by ANOVA, $P < 0.05$).

Effects of recombinant STC2 treatment on FSH-stimulated progesterone, estradiol, and cAMP production by cultured granulosa cells

Although the human STC2 gene was discovered in 1998 (14–16), its physiological function has not been clarified. Based on the expression of both STC paralogs in the ovary and the previous finding of a paracrine role for STC1 in the ovary (9), we tested the possible regulation of gonadotropin induction of granulosa cell steroidogenesis and cAMP production by STC2. Granulosa cells from early antral follicles were treated with FSH in the presence or absence of recombinant human STC2. FSH treatment dose-dependently increased progesterone, estradiol, and cAMP production, whereas cotreatment with human STC2 (10 nM) significantly

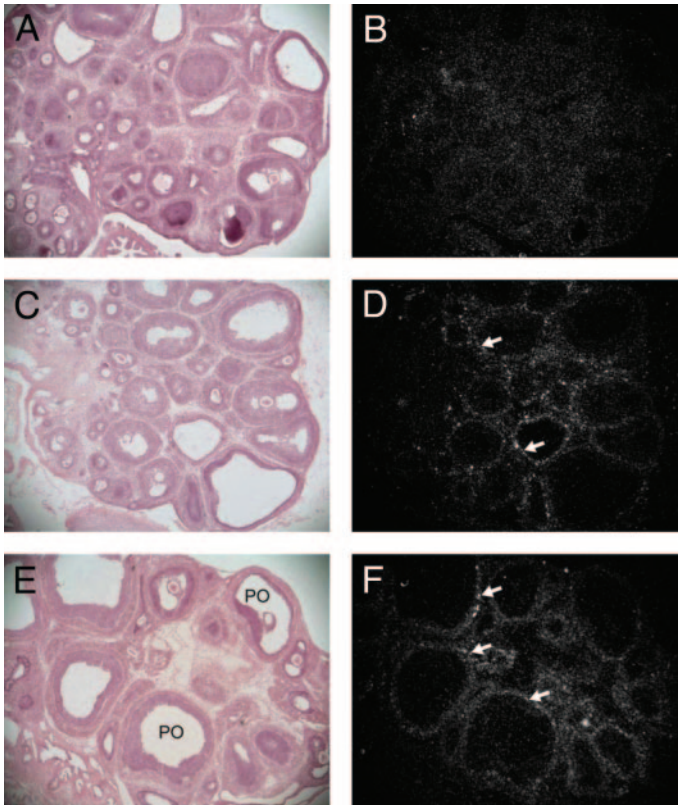


FIG. 4. *In situ* hybridization analyses of ovarian expression of STC2 in immature rats before and after gonadotropin stimulation. A and B, In ovaries from rats at d 26 of age, STC2 expression was negligible. C and D, At 12 h after PMSG treatment, STC2 was expressed in the thecal cell layer (*arrows*) of antral follicles. E and F, At 48 h after PMSG treatment, STC2 expression remained in the thecal layer of antral follicles and was also expressed in the thecal layer of the newly formed preovulatory follicles (PO). A, C, and E, Brightfield; B, D, and F, darkfield.

decreased FSH-stimulated progesterone, but not estradiol and cAMP, production (Fig. 5). In addition, treatment with human STC2 alone had no stimulatory effect on progesterone, estradiol, or cAMP production. Of interest, recombinant zebrafish STC2 had a similar suppressive effect on the FSH induction of granulosa cell progesterone production (Fig. 5A), but had negligible effects on estradiol or cAMP biosynthesis (data not shown), indicating the functional conservation of STC2 during evolution.

As shown in Fig. 6A, treatment of cultured granulosa cells with human STC2 led to a dose-dependent suppression of FSH-stimulated progesterone production, with 10 nM human STC2 producing maximum suppression. Time-course studies (Fig. 6B) also demonstrated that a significant inhibition of the FSH action on progesterone biosynthesis was seen after STC2 treatment for 24 h. At 48 h of treatment, FSH-induced progesterone production was suppressed by 55% in the presence of 10 nM STC2.

Regulation of the FSH induction of steroidogenic enzymes by STC2

To study the possible mechanisms underlying the STC2 suppression of progesterone production, we also quantified

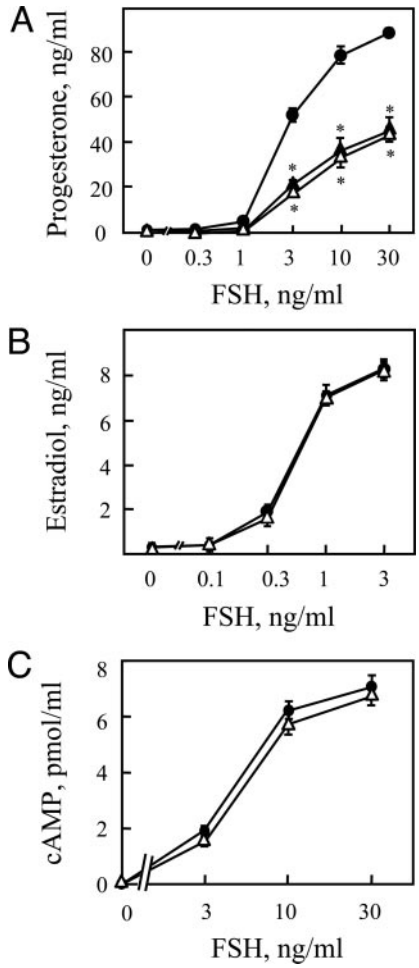


FIG. 5. Effect of treatment with STC2 on FSH-stimulated progesterone, estradiol, and cAMP biosynthesis by cultured granulosa cells from early antral follicles. Granulosa cells were cultured for 2 d in McCoy's 5a medium containing 100 nM androstenedione and various doses of FSH (0.1–30 ng/ml) without (●) or with 10 nM human STC2 (△) or zebrafish STC2 (▲). Medium concentrations of progesterone (A), estradiol (B), and cAMP (C) were determined by RIA. *Data points* represent the mean ± SE (n = 3). Data for estradiol (B) and cAMP (C) production by cells treated with zebrafish STC2 are not shown because they overlap those from cells treated with human STC2. *, Significant difference from control (by ANOVA, $P < 0.05$).

the transcript levels of different steroidogenic enzymes in cultured granulosa cells using quantitative real-time PCR. Human STC2 significantly inhibited FSH-stimulated CYP11A expression by 39% after 48 h of incubation (Fig. 7A). In addition, cotreatment with human STC2 suppressed FSH-induced StAR and 3βHSD mRNA levels in cultured granulosa cells by 21% and 28%, respectively (Fig. 7, B and C).

Discussion

The present study demonstrates the existence of fish STC2 and a potential paracrine role of STC2 in ovarian progesterone biosynthesis. PMSG treatment induced STC2 transcripts in the ovary, whereas subsequent hCG injection decreased STC2 mRNA levels. In antral follicles, STC2 transcripts were found in thecal, but not granulosa, cells. Treatment of granulosa cells with STC2 inhibited FSH stimulation of transcript

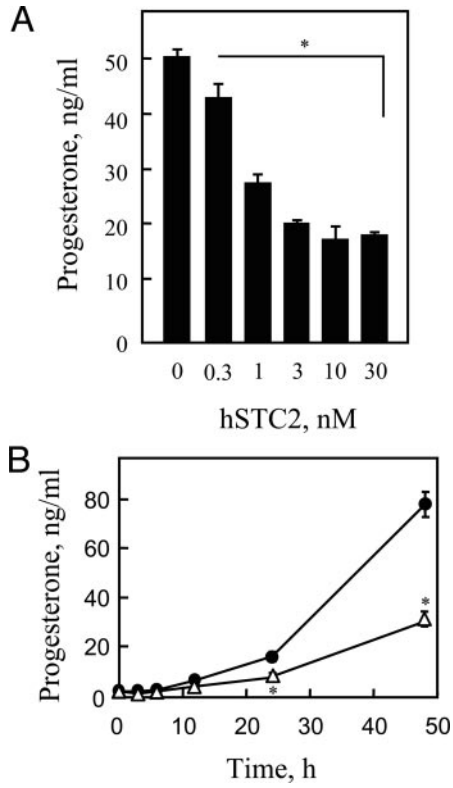


FIG. 6. Dose-dependent effect and time course of action of STC2 on progesterone production by cultured granulosa cells from early antral follicles. A, Granulosa cells from early antral follicles were cultured in medium containing 3 ng/ml FSH without or with increasing doses of purified STC2 (0.1–30 nM) for 2 d. Progesterone levels in the conditioned medium were assayed by RIA. B, Granulosa cells from early antral follicles were treated with 10 ng/ml FSH (●) or 10 ng/ml FSH together with 10 nM STC1 (△) for various intervals. Media were collected for progesterone determination. Data are shown as the mean ± SE (= 3). *, Significant difference from control (by ANOVA, $P < 0.05$).

levels for different steroidogenic enzymes and decreased FSH-stimulated progesterone production.

The existence of a second STC gene in fish had been a matter of speculation since the discovery of mammalian STC2 (14–16). However, no cDNA or protein sequence had been reported. In our study, a sequence similar to mammalian STC2 was found by searching the *F. rubripes* genome, and fish STC2 cDNA was generated by PCR amplification from a constructed zebrafish cDNA library. The discovery of the fish STC2 gene and the observed conservation of the genomic structure of different STC1 and STC2 genes demonstrated that STC1 and STC2 in mammals and fish arose from a common ancestor gene.

Salmon STC1 was purified and found as a secreted protein after cleavage of the propeptide (24). In contrast, processing of STC2 in vertebrates is unknown. The first 24 amino acids of human STC2 were predicted as a signal peptide (25), and the mature protein has a predicted molecular mass of 30,654 Da. Likewise, the mature zebrafish STC2 has a predicted molecular mass of 29,231 Da after cleavage at Gly^{20}-Thr^{21}. After PNGase F treatment, both human and zebrafish STC2 proteins migrated faster during electrophoresis. Unlike zebrafish STC2, which migrated to the predicted molecular

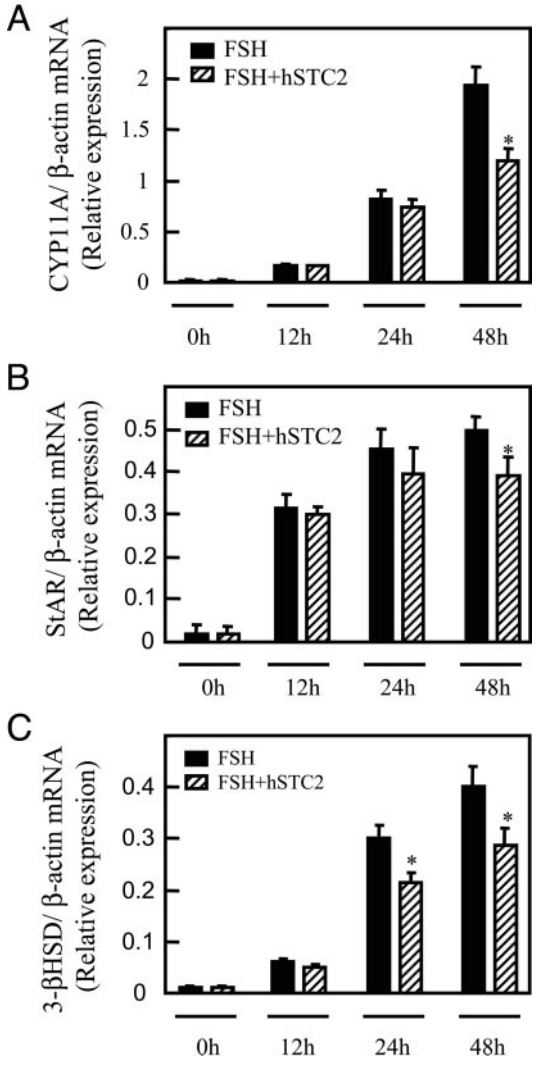


FIG. 7. Effects of STC2 treatment on FSH induction of steroidogenic enzyme transcripts in granulosa cells. Granulosa cells were cultured in McCoy's 5a medium containing FSH (10 ng/ml) only or FSH plus 10 nM STC2 for various intervals. Cells were collected for total RNA extractions. Transcript levels for CYP11A (A), StAR (B), and 3βHSD (C) were quantitated by real-time PCR and normalized using β-actin levels. Data are shown as the mean ± SE (n = 3). *, Significant difference from control (by ANOVA, $P < 0.05$).

mass after removal of the *N*-linked carbohydrate moieties, the molecular size of the recombinant human STC2 after PNGase F treatment was still higher than its predicted size, suggesting additional posttranslational modifications. The predicted existence of two potential *O*-linked glycosylation sites at Thr^{28} and Thr^{220} of the human STC2 sequence (26) is consistent with our preliminary data indicating residual binding of human STC2 to Con A-Sepharose after PGNase F treatment (data not shown). Of interest, human STC2 has been found to be modified to a phosphorylated protein after modification by an ecto-protein kinase or casein kinase II (27).

A previous study indicated that STC1 forms a disulfide-linked homodimer through the 11th cysteine residue at the C terminus (28). In contrast, STC2 contains three additional cysteine residues not found in the STC1 sequence. Our analysis of recombinant STC2 proteins suggested that they are probably homodimeric proteins, and there could be at least two intermolecular disulfide bridges in the STC2 molecules. Due to the existence of additional disulfide linkages, STC2 proteins could have different tertiary structures compared with STC1.

The stanniocalcin gene was originally discovered in fish and was characterized as a hypocalcemic hormone to eliminate excess calcium through fish gills and intestines (1, 3). However, mammalian STC1 was found as a local paracrine regulator (9, 11). Like STC1, mammalian STC2 is also widely expressed (15, 21), but its physiological role is unclear. At present, only an indirect effect of mammalian STC2 on the phosphate transporter has been examined using a kidney cell line (16). Although STC2 was proposed to inhibit phosphate transport through the transcriptional control of the type II sodium-phosphate cotransporter in the kidney cell line, STC2 expression in normal kidney is negligible (21). In addition, Northern blotting and immunohistochemical analyses suggested that STC2 was expressed in the α-cells of pancreatic islets (21). However, possible roles for STC2 in glucose homeostasis have not been tested. Using cultured granulosa cells, we demonstrated that recombinant human and fish STC2 encode functional proteins, and granulosa cells provide a convenient bioassay for STC2.

In the present study we demonstrated the expression and regulation of STC2 in rat ovary. STC2 is regulated by gonadotropins and estrogen during follicular development. In addition to multiple paracrine factors found to be important during follicular development (29), we demonstrated that theca-derived STC2 could act as a paracrine factor on granulosa cells by suppressing gonadotropin induction of progesterone biosynthesis. In addition to regulating luteinization and pregnancy, progesterone plays a central role in the initiation of ovulation (30–32). Ovarian paracrine factors known to differentially inhibit progesterone biosynthesis without affecting estrogen production have been found in follicular fluid and postulated to be luteinization inhibitors (33, 34). In the present study we demonstrate that theca-derived STC2 functions as a paracrine factor to suppress FSH-induced progesterone, but not estrogen, production. Thus, ovarian STC2 may play a role as a follicular luteinization inhibitor. The observed increase in STC2 transcripts in thecal cells after PMSG treatment and the induction of STC2 expression by estrogen support the potential role of STC2 as a luteinization inhibitor in preovulatory follicles.

STC2 was found to inhibit the transcript levels of several FSH-induced steroidogenic enzymes, with CYP11A levels showing the greatest suppression. In steroidogenic pathways, the product of the CYP11A gene is the initial and rate-limiting enzyme to cleave the side-chain of cholesterol, leading to progesterone production (35, 36). The suppression of CYP11A transcripts by STC2 could result in the inhibition of progesterone production. Furthermore, in cultured follicles, we demonstrate that STC2 transcripts are regulated by estrogen in a time-dependent manner. These results imply that gonadotropin regulation of STC2 expression in the ovary may be related to the estrogen content. The importance of STC2 induction by estrogen during follicular development requires more study. Similar to our findings in cultured follicles, STC2 was found to be an estrogen-responsive gene in breast cancer cells and was suggested to be a breast cancer biomarker (37). The exact role of STC2 in tumorigenesis is unclear.

To date, limited information is available regarding STC2 function in mammals. Although overexpression of STC1 in transgenic mice could regulate mineral absorption resulting in decreased body weight (12, 13), similar studies of the STC2 gene have not been reported. Additional analyses of altered phenotypes in STC2-overexpressing or gene inactivation models are of interest.

In conclusion, we have demonstrated that the widely expressed STC2 gene is conserved in vertebrates during evolution. The present study provides evidence for functional STC2 proteins and for a potential paracrine role of STC2 in the regulation of granulosa cell function in the ovary. The ovarian STC2 system offers a model for functional testing of STC2 in diverse tissues to reveal its molecular mechanisms in different physiological processes.

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The nucleotide sequences reported in this paper have been submitted to GenBank with accession no. AY688947 for zebrafish STC2, AY688946 for zebrafish STC1, AY688945 for *Fugu rubripes* STC2, and AY688944 for *Fugu rubripes* STC1.

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