Progesterone Is a Sperm-Releasing Factor from the Sperm-Storage Tubules in Birds

Title: Progesterone is a sperm releasing factor from the sperm storage tubules in birds

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Author's names: Tomomi Ito[1], Norio Yoshizaki[2], Toshinobu Tokumoto[3], Hiroko Ono[4], Takashi Yoshimura[4], Akira Tsukada[4], Norio Kansaku[5] and Tomohiro Sasanami[1]

Institutions: [1]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan, [2]Department of Agricultural Science, Gifu University, Gifu 501-1193, Japan, [3]Department of Biology, Faculty of Science, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan, [4]Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan, [5]Laboratory of Animal Genetics and Breeding, Azabu University, Fuchinobe, Sagamihara 229-8501, Japan.

Address all correspondence and requests for reprints to: Dr. Tomohiro Sasanami, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan. Email address: atsasan@ipc.shizuoka.ac.jp.

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Abstract

Because of the presence of sperm storage tubules (SST) in the utero-vaginal junction (UVJ) in the oviduct, once ejaculated sperm have entered the female reproductive tract they can survive for a prolonged time in domestic birds, though the specific mechanisms involved in the sperm uptake into, maintenance within, and controlled release from the SST remain to be elucidated. In this report, we provide evidence that progesterone triggers the release of the resident sperm from the SST in the UVJ. The ultrastructural observation of the SST indicated that the resident sperm are released from the SST around 20 hr after oviposition. When laying birds were injected with progesterone, most of the sperm were released from the SST within 1 hr of injection. *In situ* hybridization analyses demonstrated the presence of the transcripts of membrane progestin receptor alpha (mPRα) in the UVJ, and the translated proteins were detected in the UVJ extracts by Western blotting. Moreover, the number of secretory granules in the SST epithelial cells fluctuates during the ovulatory cycle, and the progesterone administration mimics this phenomena. A binding assay using [^{3}H]-progesterone indicated the presence of a high affinity, limited capacity, saturable and single binding site for [^{3}H]-progesterone in the membrane fraction of the UVJ, and this receptor did not interact with the synthetic antiprogestin RU486. These results demonstrated for the first time that the progesterone stimulates the release of the resident sperm from the SST and that the release of the sperm might occur via mPRα-mediating signal transduction.

Introduction

Because of the presence of specialized simple tubular invaginations in the oviduct, once ejaculated sperm have entered the female reproductive tract they can survive up to 2-15 weeks in domestic birds, including chickens, turkeys, quail as well as ducks, for varying periods depending on the species (1, 2) in contrast to relatively short life span in mammalian spermatozoa. These specialized structures are generally referred to as sperm storage tubules (SST). SST are located in the utero-vaginal junction (UVJ) and in the infundibulum, though the primary storage site for sperm is the SST in the UVJ (3, 4). The spermatozoa are transported to the infundibulum, which is the site of fertilization and also serves as a secondary sperm storage site (5, 6). Bakst *et al.* (7) reported that the biological basis of sustained fertility in chicken and turkey hens is their capacity for sperm to reside in the SST of the UVJ, and the differences in the duration of fertility between domestic fowl (2 to 3 weeks) and turkeys (10 to 15 weeks) are, in part, related to their respective numbers of number of SST (the mean numbers of SST for chickens and turkeys are 4,893 and 30,566, respectively). Although extensive investigation concerning about the function of the SST in birds has been done since its discovery in the 1960s by means of ultrastructural analysis (8-10), the specific mechanisms involved in the sperm uptake into, maintenance within, and controlled release from the SST remain to be fully elucidated.

The ovarian and placental hormone progesterone is essential for almost all aspects of female reproductive function. Its involvement in the reproductive process is wide, including gonadotropin secretion, ovulation, implantation and maintenance of pregnancy as well as the control of sexual behavior (11). Until recently, these biological responses were believed to be transmitted via nuclear progesterone receptor (nPR), which functions as steroid/thyroid hormone receptor superfamily of transcription factors (12); however, in addition to their well-documented genomic effects, steroid hormones exert actions that are rapid, insensitive to inhibitors of transcription, and inducible by the steroids coupled covalently to cell-impermeable large molecules, like bovine serum albumin (13). These observations led endocrinologists to assume the presence of a novel steroid receptor localized on the cell surface and responsible for

the transmission of rapid non-genomic signals of steroid hormones.

In 2003, a novel gene and protein was identified in spotted seatrout ovaries that has the characteristics of the membrane progestin receptor (mPR) mediating progestin induction of oocyte meiotic maturation in the species (14), and the cDNAs of mPR identified in the 13 vertebrates were found to comprise three distinct groups (α, β and γ) (15). This novel mPR belongs to a larger, highly conserved family of proteins that are also present in eubacteria, and it possesses a core region containing 7 transmembrane domains (16). Later, this family was classified as Progestin and AdipoQ Receptor (PAQR), and according to this nomenclature, mPRα, -β and -γ were classified as PAQR 7, 8 and 5, respectively (16, 17). Since the distinct tissue localization of the three subtypes was reported (14, 15), the functions of each receptor were likely to be assigned. Indeed, the α subtype is mainly localized in the reproductive tissues and the β form expressed in neural tissues, whereas mPRγ is abundant in the kidney and colon in humans (15). Although knowledge about the mPR in a limited species, including fish and humans, has been accumulated in recent decades, the data of the mPRs in other species, particularly in avian species, is completely lacking.

In this report, we present the first evidence demonstrating that progesterone is a sperm releasing factor from the SST in birds. Moreover, we also provide the evidence that mPRα expressed in the SST might play an important role in the sperm release process.

Materials and Methods

Animals and tissue preparation

Male and Female Japanese quail, *Coturnix japonica*, 15-30 weeks of age (Kato-farm, Toyohashi, Japan), were maintained individually under a photoperiod of 14L: 10D (with the light on at 0500) and were provided with water and a commercial diet (Tokai-Hokuriku Nosan, Chita, Japan) *ad libitum*. Hens were inseminated via natural mating at 12 h after oviposition, and then used for the experiments. The birds were intravenously injected with 0.8 μg/ml of estradiol 17β, testosterone, or corticosterone, or with 0.08, 0.8 or 8 μg/ml of progesterone after 1 h of the mating. The stock solution of the steroids was dissolved in propylene glycol, and the

injection solution was prepared by diluting the stock solution with physiological saline. The circulating levels of progesterone after administration was estimated as 1, 10, or 100 ng/ml for 0.08, 0.8 or 8 μg/ml group, respectively, based on the facts that the blood volume of adult hens, and the average body weight of adult female quail is approximately 7.6% (18), and 150 g, respectively. For the evaluation of dose-response of progesterone treatment, we injected 0.56, or 0.64 μg/ml progesterone to the birds. The volume of injection was kept at 0.1 ml per 100 g of body weight. One hour after the administration, birds were decapitated, and the UVJ mucosa was isolated as described in the following section. For the injection of RU486, a synthetic anti-progestin, which is also referred to as mifepristone, 2 or 10 mg/ml of RU486 solution dissolved in DMSO was injected intraperitonealy to the bird 1 h after mating. Twelve hours after the injection, the UVJ mucosa was isolated for the SST observation.

All experimental procedures for the use and the care of animals in the present study were approved by the Animal Care Committee of Shizuoka University (approval number, 22-12).

Observation of SST

The animals were decapitated, and the UVJ were immediately dissected and placed in physiological saline. The adhering connective tissues were removed, and each UVJ was excised longitudinally. The mucosa was scraped with a scalpel, and the isolated mucosa containing the SST was incubated with 0.1% Triton X-100 in PBS for 10 min at room temperature. After washing with PBS, the specimens were fixed overnight at -20°C with a mixture of acetone and methanol (1:1). After again being washed with PBS, the samples were then stained with 1 μg /ml 4',6-diamidino-2-phenylindole (DAPI) for 10 min. The UVJ mucosa was cut into small pieces with scissors, mounted in glycerol and examined under a fluorescence microscope with a 20 x objective (BX 51, Olympus Optics, Tokyo, Japan). The total number of SST and the number of SST filled with sperm were counted, and the filling rate (%) of the SST was calculated. The length and the outer diameter of the SST were measured with LAS Interactive Measurement systems (Leica, Microsystems Japan, Tokyo, Japan).

cDNA cloning of quail mPRs

Total RNA was extracted from the brain (for mPRα and -β) or kidney (for mPRγ) with a

commercial kit, RNAiso (Takara Biomedicals, Otsu, Japan), according to the manufacturer's instructions. Messenger RNA was isolated using an oligotex-dT30 mRNA purification kit (Takara Biomedicals, Otsu, Japan), according to the manufacturer's instructions, and was reverse transcribed using a Super Script II First-Strand Synthesis System for RT-PCR kit with oligo (dT) primer (Invitrogen, Carlsbad, CA, USA).

Methods for Polymerase chain reaction (PCR), 3'-RACE (rapid amplification of 3' cDNA end) and 5'-RACE (rapid amplification of 5' cDNA end) for mPR cloning and Homology search were described in Supplemental Materials and Methods. The nucleotide sequences of the amplicons were verified by dye-terminator chemistry on an Applied Biosystem Model 310 sequencer by the dideoxy-mediated chain-termination method (19).

RT-PCR analysis

Total RNA was extracted from the brain, heart, lung, liver, kidney, spleen, ovary and UVJ of laying birds, and the cDNA was synthesized as described above. PCR amplification was performed as described in Supplemental Materials and Methods.

In situ hybridization

The birds were decapitated, the UVJ was removed and frozen sections were prepared. *In situ* hybridization was carried out as described previously (20). These procedures are briefly described in Supplemental Materials and Methods. For quantitative evaluation of the labeling density, the number of positive SSTs in randomly selected sections was counted manually, and each percentage of mPRα- or nPR-positive SSTs in the UVJ was calculated.

Gel electrophoresis and Western blot analysis

The UVJ was homogenized in ice-cold PBS supplemented with 40 μg/ml bestatin, 0.5 μg/ml leupeptin and 10 μ g/ml soybean trypsin inhibitor. Debris was removed by centrifugation at 10, 000 x *g* for 5 min, and the supernatants were ultracentrifuged at 100, 000 x g for 30 min at 4°C. The precipitates were suspended in PBS containing 0.5% Nonidet p-40 and 250 μM digitonin supplemented with protease inhibitors as described above. The suspension was sonicated on ice for 6 sec for 3 times and served as the UVJ microsome fraction. The supernatant of the sample served as the cytosol fraction of the UVJ. The protein concentration in each sample was

determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).

SDS-PAGE under non-reducing conditions was carried out as described previously (21), using 12% and 5% polyacrylamide for resolving and stacking gels, respectively. For Western blotting, proteins separated on SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA) (22). The membrane reacted with anti-goldfish mPRα antiserum (1:1, 000), anti-goldfish mPRα antiserum preincubated with antigenprotein (1:1,000) (23) or anti-nPR monoclonal antibody (1 μg/ml) (24) was visualized by means of a chemiluminescent technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using horseradish peroxidase-conjugated anti-guinea pig IgG (Cappel, Durham, NC, USA) or horseradish peroxidase-conjugated anti-mouse IgG (Cappel), respectively. The anti-goldfish mPRα antiserum was produced by injecting bacterially-expressed full-length goldfish mPRα protein to guinea pig (23), and the amino acid similarity between goldfish mPRα (GenBank Accession number; AB122087) and quail counterpart is 55.6%.

Membrane preparation of UVJ and binding assay

Membrane fractions of the UVJ were prepared by the procedures as essentially described previously (23). Progesterone receptor binding in the membrane fractions was measured by the procedures established previously (14). These procedures are briefly described in Supplemental Materials and Methods.

The dissociation constant (Kd) and binding capacity (Bmax) were conducted using GraphPad Prism for Macintosh (ver. 4.0c; Graph Pad Software, San Diego, CA, USA). The results were visualized on Scatchard plots (25).

Immunofluorescence microscopy

To detect cuticle materials in the UVJ, we fixed the UVJ mucosa was fixed in Bouin's fixative and embedded samples in Paraplast (Oxford Labware, St. Louis, MO, USA). Immunohistochemical techniques were as described previously (26) using anti-cuticle materials antiserum (27) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel). The immunolabeled sections were examined under a fluorescence microscope, BX51 (Olympus).

Electron microscopy

For transmission electron microscopy, the UVJ mucosa were doubly fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and with 1% osmium tetroxide, dehydrated in acetone, embedded in epoxy resin and sectioned as described previously (28). The sections were stained with uranyl acetate and lead citrate. For scanning electron microscopy, the dehydrated specimens were dried in a critical point apparatus (HCP-2; Hitachi, Tokyo, Japan), coated with a layer of osmium and observed as described previously (28).

Statistical Analysis

All experiments were repeated two to seven times. Data were analyzed for significant differences using ANOVA, and means were compared using Duncan's Multiple Range test. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

Results

Sperm release regulation during the ovulatory cycle

To examine whether the sperm release from the SST is regulated during the ovulatory cycle, the female birds were mated 12 hr after oviposition, and we observed the SST in the UVJ 2 or 13 hr after the mating (corresponding to the time 14 or 25 hr after oviposition, respectively). The percentage of the SST containing sperm at 14 hr after oviposition was 55.5 ± 10.4% (n=7, mean ± SD) and was significantly decreased at 25 hr (41.3 ± 8.2%; n=8, mean ± SD). As shown by electron microscopy, the bundle of the sperm extruding into the lumen of the UVJ from the SST appeared 20 hr after the oviposition (Fig. 1B), while no such sperm was observed at 8, 14 or 25 hr (data not shown). At 23 hr, a free sperm was seen on the surface of the UVJ epithelial cells (Fig. 1C). The free sperm became visible on the surface of the uterus at 24 hr, upstream of the UVJ (Fig. 1D). These results indicated that the sperm release from the SST occurred around 20 hr after oviposition, and the free sperm moved upstream of the oviduct after the release.

To test whether the hormonal stimulation relates to the sperm release from the SST, we injected the birds with various steroid hormones and calculated the SST filling rate (Fig. 2). The percentage of the SST with sperm was only significantly decreased when the animals were

treated with more than 0.8 μg/ml progesterone compared to that of the control (vehicle alone). Interestingly, the morphology of the progesterone-treated SST had been changed, that is the treated SST were shorter and wider than the control (Fig. 2B and 2C). The average of the width of progesterone-treated SST was approximately 1.4-fold times that of the control (24.6 ± 3.4μm *vs.* 30.5 ± 3.2μm for vehicle control and progesterone treatment, respectively, mean ± SD, n=100). On the other hand, the average length of progesterone-treated SST was only 72.2% of the control (340.9 ± 130.5μm *vs.* 246.2 ± 109.7μm for vehicle control and progesterone treatment, respectively, mean ± SD, n=100). To obtain EC_{50} of progesterone for the sperm release, we injected 0, 0.56 or 0.64 μg/ml of progesterone to the birds, and the filling rate was calculated. It showed 56.0 ± 13.4 % (n=3, mean ± SD), 51.3 ± 4.2 % (n=3, mean ± SD), or 32.2 ± 4.4% (n=4, mean ± SD) for 0, 0.56 or 0.64 μg/ml group, respectively, and the calculated EC_{50} of progesterone was 0.63μg/ml. Unexpectedly, when the birds after mating were injected with synthetic antiprogestin, RU486, which has a relatively high binding affinity for mammalian nPR (29), the sperm release was not blocked 13 hr after mating (Fig. 2B). Scanning electron microscopical observation revealed that the entrance of the SST became hollow due to the progesterone-treatment (Fig. 2E), whereas no such change was seen when the animals were injected with vehicle alone (Fig. 2D). Occasionally, the bundle of the sperm tail extruded from the SST was observed in the case of the progesterone treatment (Fig. 2F). These results demonstrated for the first time that the release of the sperm from the SST is stimulated by progesterone and that this phenomenon is not stopped by the administration of anti-progestin, RU486.

To determine whether the morphology of the SST epithelial cells changes during the ovulatory cycle, we observed the ultra-thin sections of the SST by electron microscopy (Fig. 3). At 8 hr after oviposition, there was no visible secretory granule in the cytosol of the cells (panel A). When the sample was prepared 14 and 20 hr after oviposition, the secretory granules were frequently found in the cytosol (arrow in panel B and C). However, these secretory granules had again disappeared at 25 hr after oviposition (panel D). These results indicated that the secretory activity of the SST epithelial cells had changed during the ovulatory cycle. To test

whether this secretory activity was also stimulated by progesterone treatment, we injected progesterone 14 hr after oviposition and observed the SST epithelium. Although secretory granules were observed in the cells of control animals (panel E), we failed to find secretory granules in the cells isolated from progesterone-treated animals (panel F). These results support the notion that progesterone triggers the secretion of the epithelial cells of the SST.

Expression of progesterone receptor in SST

The degenerate PCR and RACE procedures led to the isolation of the cDNA covering the open reading frame and untranslated regions of quail mPRα and partial sequences of mPRβ and γ. The sequences have been deposited in the GenBank database (mPRα, accession number: AB611832; mPRβ, accession number: AB611833; mPRγ, accession number: AB612146).

To investigate which type of progesterone receptor transcripts are expressed in the UVJ, we analyzed the mRNA isolated from various tissues of laying quail using a gene-specific RT-PCR analysis (Fig. 4A). We detected a single band in the UVJ sample when the cDNA was amplified with mPRα or nPR (lane 8). No such signal above the background was observed when the same sample was amplified with primer sets for mPRβ or -γ (lane 8). We also examined the changes in the abundance of mPRα (Fig. 4B) and nPR (Fig. 4C) transcripts during the ovulatory cycle. Although a band of the mPRα was detected in the sample isolated 8hr after oviposition, the intensity of the band dramatically increased when the RNA derived 14 hr after oviposition was detected, and it was maintained until 25 hr after oviposition (Fig. 4B). The expression level of nPR was always constant at any time point tested (Fig. 4C).

To further localize the expression of mPRα and nPR transcripts in the UVJ, we performed gene-specific *in situ* hybridization and observed the hybridized specimens under light microscopy (Fig. 4D and 4E). As shown in the Figure 4D, intense signals were observed in the epithelial cells of the SST when the specimen was detected with an mPRα probe (arrows in the figure), suggesting that the mPRα mRNA is transcribed in the SST. The specimens that had been hybridized with the sense probe did not contain these radiolabeled signals (data not shown). In the case of the nPR transcripts, these were localized evenly in the entire UVJ and the silver grains deposited in the edge of the SST ducts (Fig. 4E). The percentage of mPRα- positive

SSTs in the UVJ was greater than that of nPR-positive SSTs (mPRα: 85.8 ± 1.7 %, n= 397, mean ± SD; nPR: 10.5 ± 2.1%, n= 480, mean ± SD). These results clearly demonstrated that the mPRα gene is transcribed in the SST and that the expression fluctuates during the ovulatory cycle, whereas the mRNA for nPR ubiquitously localized in the UVJ is transcribed constantly.

Presence of mPRα and nPR proteins in the UVJ

To confirm the presence of the proteins of the progesterone receptors in the UVJ, we performed Western blot analysis using anti-goldfish mPRα antiserum or anti-nPR monoclonal antibody. The anti-goldfish mPRα antiserum reacted with the band with 45 kDa in the membrane extract under non-reducing conditions (Fig. 5A, lane 2). No band was detected when the sample was reacted with preimmunoserum (Fig. 5A, lane 1), or with anti-goldfish mPRα antiserum pre-incubated with antigen proteins (Fig. 5A, lane 4), which suggests that our anti-goldfish mPRα antiserum is specific to 45-kDa quail mPRα proteins. Furthermore, we compared the expression levels of mPRα protein during ovulatory cycle. A band of mPRα was detected in the sample before oviposition (14hr), whereas it was barely detected in the lysate prepared after egg-laying (25 hr), indicating that the mPRα protein might be down-regulated after oviposition. When the cytosol of the UVJ was detected, no such band was seen (Fig. 5A, lane 3). On the other hand, anti-nPR monoclonal antibody failed to detect an immunoreactive band in the membrane lysate (Fig. 5B, lane 3). Instead, it reacted well with 95-kDa bands in the cytosol (Fig. 5B, lane 2), and that the size of this band corresponds to the reported size of quail nPR. Although its nature is unknown, we also detected the immunoreactive ~210-kDa band in the cytosol (Fig. 5B, lane 2). This antibody is also specific to nPR protein, because no such band was seen when the same sample was reacted with control mouse IgG (Fig. 5B, lane 1). These results suggest that the immunoreactive materials that reacted with anti-goldfish mPRα antiserum exist in the membrane extract but not in the cytosol of the UVJ, whereas the protein that specifically reacted with anti-nPR monoclonal antibody was present in the cytosol and was barely detected in the membrane fractions of the UVJ.

The steroid-binding properties of the UVJ progesterone receptor localized on the membrane fraction were characterized by radio receptor assay and competitive binding assay (Fig. 5C). As

shown in the figure, a high affinity (Kd = 12.2 ± 4.4 nM, mean ± SD, n= 3), limited capacity (Bmax = 0.93 ± 0.52 nM, mean ± SD, n= 3), saturable, single binding site for [^{3}H]-progesterone was presented in the membrane fraction of the UVJ. Because there was no detectable nPR in the membrane fraction (Fig. 5), this binding appeared to be related to mPRα. The competitive binding assays indicated that this mPRα is highly specific to progesterone with an IC_{50} of 19.7 nM (Fig. 5D), and that this value is comparable that of human mPRα (87.3 nM, (30)). Most of the competitors do not displace [^{3}H]-progesterone (relative binding affinity: less than 0.1%), whereas testosterone was a weak competitor (relative binding affinity: less than 1%). More importantly, the synthetic anti-progesterone RU486 showed no binding to the membrane fraction of the UVJ. These results demonstrate that the progesterone receptor in the membrane fraction of the UVJ is mPRα, which is highly specific to progesterone, but it does not bind with RU486.

Coincidence of sperm release and the secretion of cuticle materials

In the next sets of the experiments, we stained the UVJ sections with anti-cuticle materials antibody, because the UVJ is reported to secret cuticle materials (27). In specimens derived 8 or 14 hr after oviposition, a weak immunoreactive signal was detected on the ciliated cells of the UVJ (arrowhead, Fig. 6 A and B). The signal was very strong at 20 hr after oviposition (Fig.6C) and disappeared at 25 hr after oviposition (Fig. 6D). Ultrastructural observations showed that the abundance of secretory granules localized in the cytosol of the cells also fluctuated during the ovulatory cycle (arrow in Fig. 6 I- K). Again, we failed to observe secretory granule at 25 hr after oviposition (Fig. 6L). To test whether the secretion of the cuticle materials was also stimulated with progesterone, we injected the birds with 0.8 μg/ml progesterone and exposed sections of the UVJ to anti-cuticle materials antiserum. As expected, although strong immunoreactions were detected in the specimens in the control animals (Fig. 7B), however, the UVJ sections derived from the progesterone-primed animals showed only limited signals (Fig. 7D). These results indicated that progesterone also triggers the secretory activity of the ciliated cells of the UVJ.

Discussion

The mechanism of sperm storage and release in the avian oviduct has been wrapped in obscurity since their discovery in the 1960s. There are several reports indicating that the sperm release from the SST is not regulated but occurs in response to the mechanical pressures of a passing ovum, because no contractile elements associated with the SST were found (10, 31). In addition, Burke and Ogasawara (3), who recovered the sperm from an inseminated hen oviduct, concluded that the sperm release from the SST is a slow and continuous event that occurs constitutively during the ovulatory cycle. In contrast, there is the conflicting evidence showing that egress of the spermatozoa is regulated. Indeed, Bobr *et al.* (8), who investigated the distribution of spermatozoa in the hen oviduct after insemination, reported that the spermatozoa are ordinarily found only in the UVJ, except about the time of oviposition or ovulation; however, resident spermatozoa were discharged from the SST close to the times of ovulations and/or ovipositions. In addition, Mero and Ogasawara (32) reported that the tubular enlargement of the SST is associated with sperm release in the chicken. Their observations indicated that the sperm release from the SST might be orchestrated with the ovulations and/or ovipositions. More recently, Freedman *et al.* demonstrated the presence of neurons, small ganglia and F-actin in the UVJ of the turkey oviduct and suggested that an unknown neural factor might play a role in the functions related to the sperm storage in and the release from the SST (33).

In our tests, we observed that the release of the sperm from the SST in quail occurred only within the period 20 hr after oviposition (Fig. 1B). In accordance with our scanning electron microscopical observation, the sperm filing rate of the SST significantly decreased during the time between 14 and 25 hr after egg-laying (Fig. 1A). Based on these results, we think that the sperm release from the SST is the event that is controlled during the ovulatory cycle. In excellent agreement with this hypothesis, we observed that the intravenous injection of progesterone into the birds successively stimulated the sperm release from the SST (Fig. 2A). This observation clearly demonstrates for the first time that the progesterone is one of the factors that trigger the release of the resident sperm from the SST. Moreover, as a result of the progesterone injection, the change of the SST morphology that closely resembles contraction

was observed (Figs. 2C, 2E and 2F), which seemed to squeeze the resident sperm into the lumen. The peak of the circulating progesterone in poultry, including Japanese quail, is observed around 4 to 6 hr before the next ovulation (34-36) (corresponding to 19-21 hr after oviposition), and this is absolutely in concurrence with the timing of the sperm release we observed. Furthermore, Brillard and Bakst (37) reported that the SST of turkey hens inseminated within one week before the expected onset of egg production possessed more spermatozoa than that in the SST of turkey hens inseminated after the onset of laying. Similarly, the significantly lower number of empty SST in non-laying adult hens as compared to the laying hens was observed (38). These observations indicate that the increased numbers of resident spermatozoa in the SST of the hen without egg production might be due to the lack of a functional level of circulating progesterone in relation to the ovulatory cycle.

In the present study, we observed that the formation of the secretory granules in the SST epithelial cells fluctuated during the ovulatory cycle, and the progesterone treatment mimicking the phenomenon take place during the ovulatory cycle (Fig. 3). In the SST cells, there are well-developed tight junctions among the cells in the apical region, and the SST epithelial cells appear to secrete their content into the lumen of the tubules, where the resident sperm are located. Although we did not elucidate the nature of the secretory granules, it is very likely that the contents of the granules might affect the sperm physiology (*i.e*., sperm filling, storage and release) in the SST.

In the present study, we investigated the expression of progesterone receptor, which might transmit the progesterone signal in the SST for the sperm release. As shown by RT-PCR analysis and *in situ* hybridization, the SST epithelial cells evidently express mPRα (Fig. 4A and 4D). The mPRα protein is localized in the membrane fraction of the UVJ (approximately 45 kDa) but not in the cytosol (Fig. 5A), and it displayed high affinity, limited capacity and displaceable specific binding for [^{3}H]-progesterone (Fig. 5 C and D), similar to the previous reports (14, 23). To our knowledge, these results provide the first evidence showing that the avian oviduct expresses mPRα. Before the discovery of the mPR, Yoshimura *et al*. (39) demonstrated the presence of nPR protein in the SST cells by immunohistochemistry in laying

chicken, though the function of the nPR remains to be investigated. We also detected both the immunoreactive nPR protein (Fig.5B) and the mRNA of nPR (Fig.4A and 4E) in the UVJ; however, the injection of the synthetic anti-progesterone RU486 failed to block the sperm release from the SST of the birds that were previously inseminated. Since RU486 showed very low binding affinity to the mPRα in our competitive binding assay, we suggest that the mPRα, but not the nPR might transmit the progesterone signal for the sperm release from the SST of the quail oviduct. Because the release of the sperm after progesterone treatment occurs within 1 hr, it is not transmitted via the classical genomic action of the nPR, but might be conveyed with rapid, non-genomic action of the receptor. Although we are not able to specify the authentic receptor that is responsible for the sperm release due to the limitation of the technology (*i.e.,* lack of the gene knockout technique in birds), the evidence suggesting that the human nPR can exert its biological response by non-classical swift action was previously confirmed (40, 41).

If sperm are released from the SST without any regulation, most of the sperm ascending the oviduct may be trapped by the descending egg. This might be true for the excess sperm present just after insemination in the oviduct, because supernumerary sperm are lost as they are swept out of the lumen by successive descending egg had been suggested (42). However, Bobr *et al.* (8) found that the sperm after insemination had usually disappeared from the oviduct lumen within 24 hr of being deposited in a laying hen, in spite of their potency for producing an additional fertilized egg. Their observation might support our suggestion that the resident sperm in the SST are released for a restricted time under the control of progesterone. In our immunohistochemical observations, the secretory activity of the UVJ ciliated cells for the cuticle materials occurred in concurrence with the sperm release (Fig. 6). Moreover, the progesterone treatment stimulated the release of cuticle materials from the cells (Fig. 7). Rahman *et al.* (27), who investigated the role of cuticle for egg formation by electron microscopy demonstrated that the surface of the luminal epithelia in the uterus as well as the outside of the egg shell at 7 hr after oviposition showed a coarse appearance, whereas both became much smoother after cuticle secretion at 20 hr. From these observations, they suggested that the cuticle might function as a lubricant that facilitates egg rotation in the uterus.

Considering these observations along with our results, we suggest that the cuticle materials secreted from the ciliated cells of the UVJ might support the sperm movement to ascend the oviduct by facilitating the rotation of the egg, as well as function as a fluid material for the sperm swimming after the release from the SST by the stimulation with progesterone (Fig. 8). Although identification of the receptor responsible for the cuticle release remains to be studied, cuticle release might be also promoted by nongenomic action via a membrane type receptor, since the secretion of this materials occurred within 1 hr after progesterone injection.

In conclusion, we demonstrated for the first time that progesterone stimulates the release of the resident sperm from the SST in the Japanese quail with a contraction-like morphological change of the SST, which is probably initiated via mPRα-mediating signal transduction. This process might be supported by the lubricant effect of cuticle materials secreted from the ciliated cells of the UVJ as well as the unknown materials supplied from the SST epithelial cells, in events coincidently triggered under the progesterone control (Fig. 8). In birds, it reasonable to suppose that two events, the sperm release from the SST and the cuticle secretion, are stimulated simultaneously by progesterone for efficient fertilization, because there is at least a 5-hr grace period before the next ovulation and the eluded sperm can reach the infundibulum, the site of fertilization, without hindrance of the descending egg. Our current findings might help to shed new light on the SST functions in birds.

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Figure legends

Fig. 1 Observation of the sperm release from the sperm storage tubules during the ovulatory cycle. (A) Filling rate of the sperm storage tubules before or after egg-laying. The female birds were inseminated via natural mating, and the UVJ was isolated before (approximately 14 h after oviposition) or after (approximately 25 h after oviposition) the laying. The percentage of the SST containing sperm was calculated as described in Methods. The data are expressed as the mean ± SD of 7 replicates. An asterisk indicates a significant difference, $P < 0.05$.

(B) Electron microscopy of the surface of the UVJ around the entrance of the SST 20 h after oviposition. The bundles of the sperm extruding into the lumen of the UVJ from the SST were seen. (C) Corresponding area of a different bird 23 h after egg-laying. Free sperm were seen on the surface of the lumen of the UVJ. (D) Observation of the surface of the uterus 24 h after oviposition. At this time, sperm appeared to ascend to the uterus (upstream of the UVJ). A representative photograph of three different observations is shown. Arrows in panel B-D indicate the sperm.

Fig. 2 Effects of various steroid hormones on the release of sperm from the SST. (A) The animals were treated with vehicle alone (Cont), estradiol-17β (E_2: 0.8μg/ml), testosterone (T: 0.8μg/ml), corticosterone (B: 0.8μg/ml) or progesterone (P_4: 0.08, 0.8 or 8μg/ml) 1h after mating. The SST was isolated from the bird 1 h after the treatment, and the percentage of the SST containing sperm was calculated as described in Methods. The data are expressed as the mean ± SD of 5 replicates. An asterisk indicates a significant difference, $\underline{P} < 0.05$. (B) The animals were treated with vehicle alone (Cont, n=6), 2 (n=4) or 10 μg/ml (n=3) RU486 1h after mating. The SST was isolated from the bird 12 h after the treatment, and the percentage of the SST containing sperm was calculated. The data are expressed as the mean ± SD. (C) Whole-mount specimen of the UVJ isolated from the bird treated with vehicle alone. An arrow indicates the resident spermatozoa in the SST. (D) The specimen of the UVJ isolated from the bird treated with progesterone (0.8μg/ml). Scale bar, 50 μm. (E-G) Ultrastructural observation of the surface of the UVJ treated with progesterone. After mating, the animals were injected with vehicle alone (E) or 0.8 μg/ml progesterone (F and G). The UVJ was isolated 1 h after the

injection, and the area of the entrance of the SST was observed by scanning electron microscopy. A representative photograph from those obtained from three different birds is shown. Arrows in (E) and (F) indicate entrance of SST.

Fig. 3 Ultrastructural analysis of the UVJ epithelial cells. Ultra-thin sections of the UVJ isolated 8 (A), 14 (B), 20 (C) or 25 hr (D) after oviposition were observed by transmission electron microscopy. Note that secretory granules in the SST epithelial cells are observed in specimens 14 and 20 hr after oviposition but are seldom seen in the cells 8 and 25 hr after oviposition. Shown are the results representative of three repeated experiments. The arrow in (B) and (C) indicate secretory granules. Well-developed tight junctions were observed between the cells in the apical region of the cells of all specimens. (E) and (F) Electron micrographs of progesterone-treated UVJ. Ultra-thin sections of the UVJ isolated from animals injected with vehicle alone (E) or 0.8 μg/ml of progesterone (F) were observed. Note that secretory granules in the SST epithelial cells can be seen (arrows in E), but are absent in the cells from hens with progesterone treatment (F). Shown are the results representative of three repeated experiments.

Fig. 4 Expression of mRNA for progesterone receptors. (A) mRNA extracted from the brain (lane 1), heart (lane 2), lung (lane 3), liver (lane 4), kidney (lane 5), spleen (lane 6), ovary (lane 7) or UVJ (lane 8) was reverse transcribed with oligo (dT) primers, and an aliquot was used in PCR using the primers set indicated on the right side of the figure (see Methods). For non-RT control, mRNA of UVJ (for mPRα, nPR and S17), brain (for mPRβ) or kidney (for mPRγ) was treated as described in Materials and Methods except for omitting the Super Script II reverse transcriptase (lane -). The panels of ethidium bromide gels reflect one representative experiment out of a total of three independent experiments. (B) The UVJ isolated from the female birds at 8, 14, 20 or 25 hr after egg-laying were processed for RT-PCR analysis using mPRα primers, and the intensities of the bands were quantified. Data was normalized with the intensity of the band of S17. (C) The same samples were analyzed with RT-PCR using an nPR primers set. Data in panels B and C is expressed as the mean ± SD of 3 independent experiments. An asterisk indicates a significant difference, $\underline{P} < 0.01$. (D-E) *In situ* hybridization of the UVJ. Autoradiograms of the UVJ sections after hybridization with ^{33}P-

labeled antisense probe specific to mPRα (D) or nPR (E) are shown. Arrows in A and B indicate the SST. Scale bar, 50 μm. The results are representative of repeated experiments (n=2).

Fig. 5 Detection of membrane progestin receptor in the UVJ. (A) and (B) Western blot analysis of mPRα and nPR protein in the extract of the UVJ. (A) The membrane (lanes 1, 2 and 4) or cytosol (lane 3) fractions prepared from the UVJ (10 μg proteins/lane) were separated on SDS-PAGE under non-reducing conditions, transblotted onto PVDF membrane and detected with normal guinea pig serum (lane 1, 1:1 000) or anti-goldfish mPRα antiserum (lanes 2 and 3, 1:1 000) or anti-goldfish mPRα antiserum preincubated with the antigen protein (lane 4). (B) The membrane (lane 3) or cytosol (lanes 1 and 2) fractions prepared from the UVJ (20 μg /lane) were detected with normal mouse IgG (lane 1, 1μg/ml) or anti-nPR monoclonal antibody (lanes 2 and 3, 1μg/ml). The results representative of 5 repeated experiments are shown. (C) Representative Western blot of lysate of the UVJ membrane (20 μg proteins/lane) prepared from before (14) or after (25) oviposition.. The intensities of the bands were quantified, and showed in right panel. Data is expressed as the mean ± SD of 3 independent experiments. An asterisk indicates a significant difference, $\underline{P} < 0.01$. (D) and (E) Specific [^{3}H]-progesterone binding to membrane preparation from the UVJ of Japanese quail. (D) Representative saturation curves (insert) and Scatchard plots of specific [^{3}H]-progesterone to the membrane fraction prepared from the UVJ (representative of 3 different experiments). (E) Competition by natural and synthetic steroids for binding to the membrane progestin receptor. Samples were incubated with 1 nM [^{3}H]-progesterone and various concentrations of the competitor. Competition curves for steroid and progesterone binding are expressed as a percentage of maximum specific binding. The data are expressed as the mean ± SEM of 3 replicates. P4, progesterone; T, testosterone; E2, estradiol-17β; B, corticosterone.

Fig. 6 Immunohistochemical analysis of cuticle materials in the UVJ. Sections of UVJ isolated from 8 (A), (E) and (I); 14 (B), (F) and (J); 20 (C), (G) and (K), or 25 (D), (H) and (L) hr after oviposition were processed for histochemical observations. (A)-(D): Immunohistochemical observation using anti-cuticle materials antiserum under fluorescence microscopy. The

arrowhead indicates the surface of the UVJ mucosa. (E)-(H): The same field of each specimen was observed under light microscopy. The arrowhead indicates the corresponding position in (A)-(D). The arrow indicates the SST. Note that no immunoreaction was observed in the SST cells. (I)-(L): Ultrastructural analysis of the ciliated cells in UVJ mucosa. The arrow indicates the secretory granules. The results representative of 2 repeated experiments are shown.

Fig. 7 Effect of progesterone administration on the release of cuticle materials. The animals injected with vehicle alone (A and B) or 0.8 μg/ml progesterone (C and D) were decapitated, and the UVJ mucosa was processed for immunohistochemical observation using anti-cuticle materials antiserum under fluorescence microscopy (B and D). The same field was observed under light microscopy (A and C). The arrow indicates the SST. The representative results of 2 experiments are shown. Bar = 50 μm.

Fig. 8 Schematic model of the proposed mechanism of the sperm release from the sperm storage tubules (SST) in the quail oviduct. Before the onset of the progesterone surge (panel A), the spermatozoa in the SST are quiescent, and the lumen of the SST is not extended. When ovulation is imminent (panel B), the blood concentration of progesterone increases, and it stimulates the release of the resident sperm from the SST with the contraction-like morphological change of the SST, which is probably initiated via mPRα-mediating signal transduction. This process might be supported by the lubricant effect of cuticle materials secreted from the ciliated cells of the UVJ (black dots) as well as the unknown materials supplied from the SST epithelial cells (red dots), the events coincidently triggered under the progesterone control.