

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

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1 **Abstract**

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

20

1 **Introduction**

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

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

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

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

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

19 20 **Materials and Methods**

21 *Animals and tissue preparation*

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

1 injection solution was prepared by diluting the stock solution with physiological saline. The
2 circulating levels of progesterone after administration was estimated as 1, 10, or 100 ng/ml for
3 0.08, 0.8 or 8 μ g/ml group, respectively, based on the facts that the blood volume of adult hens,
4 and the average body weight of adult female quail is approximately 7.6% (18), and 150 g,
5 respectively. For the evaluation of dose-response of progesterone treatment, we injected 0.56,
6 or 0.64 μ g/ml progesterone to the birds. The volume of injection was kept at 0.1 ml per 100 g
7 of body weight. One hour after the administration, birds were decapitated, and the UVJ mucosa
8 was isolated as described in the following section. For the injection of RU486, a synthetic anti-
9 progestin, which is also referred to as mifepristone, 2 or 10 mg/ml of RU486 solution dissolved
10 in DMSO was injected intraperitoneally to the bird 1 h after mating. Twelve hours after the
11 injection, the UVJ mucosa was isolated for the SST observation.

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

14 **Observation of SST**

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

27 **cDNA cloning of quail mPRs**

28 Total RNA was extracted from the brain (for mPR α and - β) or kidney (for mPR γ) with a

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

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

11 ***RT-PCR analysis***

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

15 ***In situ hybridization***

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

21 ***Gel electrophoresis and Western blot analysis***

22 The UVJ was homogenized in ice-cold PBS supplemented with 40 μ g/ml bestatin, 0.5 μ g/ml
23 leupeptin and 10 μ g/ml soybean trypsin inhibitor. Debris was removed by centrifugation at 10,
24 000 x g for 5 min, and the supernatants were ultracentrifuged at 100, 000 x g for 30 min at 4°C.
25 The precipitates were suspended in PBS containing 0.5% Nonidet p-40 and 250 μ M digitonin
26 supplemented with protease inhibitors as described above. The suspension was sonicated on ice
27 for 6 sec for 3 times and served as the UVJ microsome fraction. The supernatant of the sample
28 served as the cytosol fraction of the UVJ. The protein concentration in each sample was

1 determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA).
2 SDS-PAGE under non-reducing conditions was carried out as described previously (21), using
3 12% and 5% polyacrylamide for resolving and stacking gels, respectively. For Western blotting,
4 proteins separated on SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF)
5 membrane (Immobilon-P, Millipore, Bedford, MA, USA) (22). The membrane reacted with
6 anti-goldfish mPR α antiserum (1:1, 000), anti-goldfish mPR α antiserum preincubated with
7 antigenprotein (1:1,000) (23) or anti-nPR monoclonal antibody (1 μ g/ml) (24) was visualized by
8 means of a chemiluminescent technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA)
9 using horseradish peroxidase-conjugated anti-guinea pig IgG (Cappel, Durham, NC, USA) or
10 horseradish peroxidase-conjugated anti-mouse IgG (Cappel), respectively. The anti-goldfish
11 mPR α antiserum was produced by injecting bacterially-expressed full-length goldfish mPR α
12 protein to guinea pig (23), and the amino acid similarity between goldfish mPR α (GenBank
13 Accession number; AB122087) and quail counterpart is 55.6%.

14 ***Membrane preparation of UVJ and binding assay***

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

19 The dissociation constant (K_d) and binding capacity (B_{max}) were conducted using GraphPad
20 Prism for Macintosh (ver. 4.0c; Graph Pad Software, San Diego, CA, USA). The results were
21 visualized on Scatchard plots (25).

22 ***Immunofluorescence microscopy***

23 To detect cuticle materials in the UVJ, we fixed the UVJ mucosa was fixed in Bouin's fixative
24 and embedded samples in Paraplast (Oxford Labware, St. Louis, MO, USA).

25 Immunohistochemical techniques were as described previously (26) using anti-cuticle materials
26 antiserum (27) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel). The
27 immunolabeled sections were examined under a fluorescence microscope, BX51 (Olympus).

28 ***Electron microscopy***

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

7 ***Statistical Analysis***

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

11

12 **Results**

13 **Sperm release regulation during the ovulatory cycle**

14 To examine whether the sperm release from the SST is regulated during the ovulatory cycle,
15 the female birds were mated 12 hr after oviposition, and we observed the SST in the UVJ 2 or
16 13 hr after the mating (corresponding to the time 14 or 25 hr after oviposition, respectively).
17 The percentage of the SST containing sperm at 14 hr after oviposition was $55.5 \pm 10.4\%$ ($n=7$,
18 $\text{mean} \pm \text{SD}$) and was significantly decreased at 25 hr ($41.3 \pm 8.2\%$; $n=8$, $\text{mean} \pm \text{SD}$). As
19 shown by electron microscopy, the bundle of the sperm extruding into the lumen of the UVJ
20 from the SST appeared 20 hr after the oviposition (Fig. 1B), while no such sperm was observed
21 at 8, 14 or 25 hr (data not shown). At 23 hr, a free sperm was seen on the surface of the UVJ
22 epithelial cells (Fig. 1C). The free sperm became visible on the surface of the uterus at 24 hr,
23 upstream of the UVJ (Fig. 1D). These results indicated that the sperm release from the SST
24 occurred around 20 hr after oviposition, and the free sperm moved upstream of the oviduct after
25 the release.

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

1 treated with more than 0.8 $\mu\text{g/ml}$ progesterone compared to that of the control (vehicle alone).
2 Interestingly, the morphology of the progesterone-treated SST had been changed, that is the
3 treated SST were shorter and wider than the control (Fig. 2B and 2C). The average of the width
4 of progesterone-treated SST was approximately 1.4-fold times that of the control ($24.6 \pm 3.4\mu\text{m}$
5 vs. $30.5 \pm 3.2\mu\text{m}$ for vehicle control and progesterone treatment, respectively, mean \pm SD,
6 $n=100$). On the other hand, the average length of progesterone-treated SST was only 72.2% of
7 the control ($340.9 \pm 130.5\mu\text{m}$ vs. $246.2 \pm 109.7\mu\text{m}$ for vehicle control and progesterone
8 treatment, respectively, mean \pm SD, $n=100$). To obtain EC_{50} of progesterone for the sperm
9 release, we injected 0, 0.56 or 0.64 $\mu\text{g/ml}$ of progesterone to the birds, and the filling rate was
10 calculated. It showed $56.0 \pm 13.4\%$ ($n=3$, mean \pm SD), $51.3 \pm 4.2\%$ ($n=3$, mean \pm SD), or 32.2
11 $\pm 4.4\%$ ($n=4$, mean \pm SD) for 0, 0.56 or 0.64 $\mu\text{g/ml}$ group, respectively, and the calculated EC_{50}
12 of progesterone was 0.63 $\mu\text{g/ml}$. Unexpectedly, when the birds after mating were injected with
13 synthetic antiprogestin, RU486, which has a relatively high binding affinity for mammalian nPR
14 (29), the sperm release was not blocked 13 hr after mating (Fig. 2B). Scanning electron
15 microscopical observation revealed that the entrance of the SST became hollow due to the
16 progesterone-treatment (Fig. 2E), whereas no such change was seen when the animals were
17 injected with vehicle alone (Fig. 2D). Occasionally, the bundle of the sperm tail extruded from
18 the SST was observed in the case of the progesterone treatment (Fig. 2F). These results
19 demonstrated for the first time that the release of the sperm from the SST is stimulated by
20 progesterone and that this phenomenon is not stopped by the administration of anti-progestin,
21 RU486.

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

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

6 ***Expression of progesterone receptor in SST***

7 The degenerate PCR and RACE procedures led to the isolation of the cDNA covering the open
8 reading frame and untranslated regions of quail mPR α and partial sequences of mPR β and γ .

9 The sequences have been deposited in the GenBank database (mPR α , accession number:
10 AB611832; mPR β , accession number: AB611833; mPR γ , accession number: AB612146).

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

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

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

5 ***Presence of mPR α and nPR proteins in the UVJ***

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

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

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

13 ***Coincidence of sperm release and the secretion of cuticle materials***

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

28

1 **Discussion**

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

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

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

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

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

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

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

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

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

19

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25

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23

1 **Figure legends**

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

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

15 Fig. 2 Effects of various steroid hormones on the release of sperm from the SST. (A) The
16 animals were treated with vehicle alone (Cont), estradiol-17 β (E₂: 0.8 μ g/ml), testosterone (T:
17 0.8 μ g/ml), corticosterone (B: 0.8 μ g/ml) or progesterone (P₄: 0.08, 0.8 or 8 μ g/ml) 1h after
18 mating. The SST was isolated from the bird 1 h after the treatment, and the percentage of the
19 SST containing sperm was calculated as described in Methods. The data are expressed as the
20 mean \pm SD of 5 replicates. An asterisk indicates a significant difference, $P < 0.05$. (B) The
21 animals were treated with vehicle alone (Cont, n=6), 2 (n=4) or 10 μ g/ml (n=3) RU486 1h after
22 mating. The SST was isolated from the bird 12 h after the treatment, and the percentage of the
23 SST containing sperm was calculated. The data are expressed as the mean \pm SD. (C) Whole-
24 mount specimen of the UVJ isolated from the bird treated with vehicle alone. An arrow
25 indicates the resident spermatozoa in the SST. (D) The specimen of the UVJ isolated from the
26 bird treated with progesterone (0.8 μ g/ml). Scale bar, 50 μ m. (E-G) Ultrastructural observation
27 of the surface of the UVJ treated with progesterone. After mating, the animals were injected
28 with vehicle alone (E) or 0.8 μ g/ml progesterone (F and G). The UVJ was isolated 1 h after the

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

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

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

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

4 Fig. 5 Detection of membrane progesterin receptor in the UVJ. (A) and (B) Western blot analysis
5 of mPR α and nPR protein in the extract of the UVJ. (A) The membrane (lanes 1, 2 and 4) or
6 cytosol (lane 3) fractions prepared from the UVJ (10 μ g proteins/lane) were separated on SDS-
7 PAGE under non-reducing conditions, transblotted onto PVDF membrane and detected with
8 normal guinea pig serum (lane 1, 1:1 000) or anti-goldfish mPR α antiserum (lanes 2 and 3, 1:1
9 000) or anti-goldfish mPR α antiserum preincubated with the antigen protein (lane 4). (B) The
10 membrane (lane 3) or cytosol (lanes 1 and 2) fractions prepared from the UVJ (20 μ g /lane)
11 were detected with normal mouse IgG (lane 1, 1 μ g/ml) or anti-nPR monoclonal antibody (lanes
12 2 and 3, 1 μ g/ml). The results representative of 5 repeated experiments are shown. (C)
13 Representative Western blot of lysate of the UVJ membrane (20 μ g proteins/lane) prepared
14 from before (14) or after (25) oviposition.. The intensities of the bands were quantified, and
15 showed in right panel. Data is expressed as the mean \pm SD of 3 independent experiments. An
16 asterisk indicates a significant difference, $\underline{p} < 0.01$. (D) and (E) Specific [3 H]-progesterone
17 binding to membrane preparation from the UVJ of Japanese quail. (D) Representative saturation
18 curves (insert) and Scatchard plots of specific [3 H]-progesterone to the membrane fraction
19 prepared from the UVJ (representative of 3 different experiments). (E) Competition by natural
20 and synthetic steroids for binding to the membrane progesterin receptor. Samples were incubated
21 with 1 nM [3 H]-progesterone and various concentrations of the competitor. Competition curves
22 for steroid and progesterone binding are expressed as a percentage of maximum specific
23 binding. The data are expressed as the mean \pm SEM of 3 replicates. P4, progesterone; T,
24 testosterone; E2, estradiol-17 β ; B, corticosterone.

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

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

6 Fig. 7 Effect of progesterone administration on the release of cuticle materials. The animals
7 injected with vehicle alone (A and B) or 0.8 $\mu\text{g/ml}$ progesterone (C and D) were decapitated,
8 and the UVJ mucosa was processed for immunohistochemical observation using anti-cuticle
9 materials antiserum under fluorescence microscopy (B and D). The same field was observed
10 under light microscopy (A and C). The arrow indicates the SST. The representative results of
11 2 experiments are shown. Bar = 50 μm .

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

22