Expression of transforming growth factor- β isoforms and their receptors in utero-vaginal junction of hen oviduct in presence or absence of resident sperm with reference to sperm storage

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Abstract

Our goal was to determine whether transforming growth factor β (TGF β) isoforms were involved in the process of sperm survivability in the sperm-storage tubules (SST) in the utero-vaginal junction (UVJ) of hen oviduct. The birds were artificially inseminated. The mRNA expressions of three types of TGF β isoforms (TGF β 2, TGF β 3, and TGF β 4) and three types of receptors (T β R1, T β R2, and T β R3) were examined in the presence or in the absence of resident sperm in SST by semi-quantitative reverse transcriptase-PCR. The mRNA expression of TGF β s and T β Rs in sperm was also examined. Immunocytochemistry and western blot were performed for T β R2 to confirm its localization in UVJ. The sperm were observed at least 10 days after insemination by histology. The mRNA expressions of TGF β s and T β Rs were significantly increased in UVJ in the presence of resident sperm in SST. The mRNA expressions of TGF β s and T β Rs were also observed in sperm. Immunohistochemistry revealed that T β R2 were located in lymphocytes in UVJ and SST cells. The presence of T β R2 in UVJ was also confirmed by western blot. These results suggest that enhanced expressions of TGF β s and T β Rs in UVJ may protect sperm in SST, probably by suppressing anti-sperm immunoreactions.

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Introduction

In hen oviduct, sperm are stored for a prolonged period in the sperm-storage tubules (SST) in the utero-vaginal junction (UVJ) and infundibulum with the UVJ as the primary site for sperm storage (Fujii & Tamura 1963, Bobr *et al.* 1964, Bakst *et al.* 1994). The SST are formed by the invaginations of mucosal surface, where sperm are found to be close contact with the epithelium (Ashizawa & Nishiyama 1983). A number of studies have examined the features of sperm survivability in SST, and suggested that quiescence of spermatozoa during the storage (Holm *et al.* 2000), interaction of SST fluid outflow from SST cells and sperm mobility (Froman 2003, Zaniboni & Bakst 2004), and protection of sperm from immunoreaction (Das *et al.* 2005a, 2006) may be important events in this process. However, the precise mechanism of sperm survivability in the SST has not been established. There are reports that the leukocyte population was increased in UVJ after artificial insemination (AI) in infertile hens and the local immunity of vagina and UVJ may affect the sperm survivability (Higaki *et al.* 1995, Zheng *et al.* 2001, Das *et al.* 2005b). Thus, one of the requirements for prolonged sperm survivability in hen oviduct may be to suppress the

immune response to sperm stored in SST. Transforming growth factor β (TGF β) is one of the possible factors to suppress immunoreaction (Wahl *et al.* 1988, Mouri *et al.* 2002). Apart from the basic functions affecting growth, differentiation, and morphogenesis of cells (Sporn *et al.* 1986, 1987), TGF β isoforms mediate immunosuppression by suppressing the proliferation of T- and B-lymphocytes in mammals and birds (Kehrl *et al.* 1986a, 1986b, Quere & Thorbecke 1990, Huang & Huang 2005). TGF β in human seminal plasma may protect sperm from immune response in female reproductive tract (Nocera & Chu 1995, Srivastava *et al.* 1996). Thus, TGF β s may play an important role in the storage of sperm in SST by suppressing anti-sperm immune response. Chicken TGF β superfamily consists of TGF β 2, TGF β 3, and TGF β 4 (Chowdhury *et al.* 2003). Three types of TGF β receptors, namely types 1, 2, and 3 (T β R1, T β R2, and T β R3) were also identified in chicken (Chowdhury *et al.* 2004), and T β R2 binds with TGF β s directly and the complex is recognized by T β R1 (Massague 1996). However, the T β R3 does not participate in signal transduction but increases the receptor-binding affinity and cell responsiveness (Lopez *et al.* 1993).

Published information on the expression of TGF β isoforms and their receptors in UVJ tissues or infundibulum are not available. If TGF β s are involved in the protection of sperm from immunoreaction, the possibility of their synthesis by the sperm should also be examined. However, this possibility has also not been reported yet. Therefore, the goal of this study was to determine whether the cells in UVJ, and potentially the sperm themselves, express mRNAs of TGF β isoforms and their receptors (T β R) and whether their expressions are changed during the storage of sperm in SST. In Experiment 1, histological observations of UVJ tissue with or without AI were performed to confirm the changes in the population of SST-containing sperm and that of the lymphocytes after AI. In Experiment 2, mRNA expressions of TGF β s and T β R were investigated by reverse transcriptase (RT)-PCR. The expressions of TGF β s and T β R by chicken sperm were also examined in Experiment 3. Finally, immunocytochemistry and western blot for T β R2 were performed to localize it in UVJ in Experiment 4.

Materials and Methods

Birds and tissue collection

Healthy White Leghorn laying hens (aged approximately 50 weeks), regularly laying five or more eggs in a sequence were kept in individual cages under a regimen of 14 h light:10 h darkness and provided with commercial feed and water *ad libitum*. The hens were inseminated in Experiments 1, 2, and 4, and examined at two different terms, namely short- (trial 1) and long term after AI (trial 2). In trial 1, the birds were divided into four groups: 0, 1, 12, and 24 h after AI ($n=4$ each). In trial 2, birds were also divided into four groups: 0, 1, 10, and 20 days after AI ($n=4$ each). Semen used for AI and RT-PCR analysis of TGF β s and T β R expressions in sperm (Experiment 3) was collected from White Leghorn roosters ($n=3$) kept under the similar condition. For the insemination, 0.05 ml undiluted fresh semen containing approximately 2×10^8 sperm were intravaginally introduced from the cloaca using a plastic syringe. When the oviductal tissues were collected, the birds were euthanatized by decapitation under anesthesia with Nembutal (Abbott Laboratories, IL, USA) after approximately 5 h oviposition. The handling of birds was performed in accordance with the regulation by Animal Experiment Committee of Hiroshima University.

Experiment 1. Histological observation

A part of UVJ tissues of all female birds (trials 1 and 2) were fixed with Bouin's solution followed by embedding in paraffin in the usual manner. Paraffin sections (4 μ m in thickness) were prepared and stained with hematoxylin and eosin. The ratio of the SST-containing sperm and the

lymphocyte frequencies in the lamina propria were analyzed under a light microscope with a computer-assisted image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA) as described previously (Yoshimura *et al.* 2004). The lymphocytes were identified histologically with densely stained, small and round nuclei. Their frequencies in the lamina propria were analyzed by counting 15 different regions (approximately, $7-10 \times 10^4 \mu\text{m}^2$ area in each count) randomly selected from three UVJ sections. Then, the number of lymphocytes in $5 \times 10^4 \mu\text{m}^2$ areas was calculated.

Experiment 2. RT-PCR analysis for expression of TGF β s and T β R in UVJ

Changes in the expressions for TGF β s mRNA (TGF β 2, TGF β 3, and TGF β 4) and T β R mRNA (T β R1, T β R2, and T β R3) after AI were observed. In trial 1, four different oviductal segments, namely the infundibulum, uterus, UVJ, and vagina were collected. In trial 2, since significant changes in the expressions for TGF β s and T β R were observed only in UVJ in trial 1, only the UVJ tissues were collected.

Extraction of total RNA

Total RNA was extracted from the mucosal tissues of oviductal segments using Sepasol RNA I Super (Nacalai Tesque, Inc., Kyoto, Japan) as described previously by Barua & Yoshimura (2004). The pellet of RNA was suspended in TE buffer, incubated with DNase I (Roche) at a concentration of 10 U/ μ l, and the RNA concentration was measured with Gene Quant Pro (Amersham Pharmacia Biotech), and stored at -80°C until analysis.

Semi-quantitative RT-PCR

The semi-quantitative RT-PCR was performed as described previously by Subedi & Yoshimura (2005). The RNA samples were reverse transcribed using ReverTra Ace (Toyobo Co. Ltd, Osaka, Japan) as described by the manufacturer's instructions. The primers used for TGF β 2 (Burt & Paton 1991; Accession no. NM001031045), TGF β 3 (Jakowlew *et al.* 1992; Accession no. S46000), and TGF β 4 (Jakowlew *et al.* 1988) and their receptors T β R1 (Accession no. U38622), T β R2 (Barnett *et al.* 1994; Accession no. NM205428), and T β R3 (Barnett *et al.* 1994; Accession no. NM204339) and also for chicken β -actin (Kost *et al.* 1983; Accession no. X00182) are shown in Table 1. An aliquot of cDNA corresponding to 1 μ g initial total RNA was used as a template in a volume of 25 μ l reaction mixture for PCR. The mixture was denatured at 95°C for 1 min followed by 30 cycles of 95°C for 1 min, 58°C for 1 min to anneal, 72°C for 1 min for extension and a final extension was done at 72°C for 10 min in a Programmable Thermal Controller PTC-100 (MJ Research, Inc., Waltham, MA,

Table 1 List of the PCR primers of TGFβ isoforms and their receptors.

Primers	Sequences
TGFβ2	F: 5'-AGGAATGTGCAGGATAATT-3' R: 5'-ATTTTGGGTGTTTGGCCAA-3'
TGFβ3	F: 5'-CAGATCCTGGCGCTCTACA-3' R: 5'-GAGGCCCTGGATCATGTCA-3'
TGFβ4	F: 5'-CACCGACTACTGCTTCGGC-3' R: 5'-GTCGGGCTCCAGATGTAC-3'
TβR1	F: 5'-GTTCCAAATGATCCAGTT-3' R: 5'-TGCTGATGGATTCTGATACC-3'
TβR2	F: 5'-CTATGCAAATCTGTGACGTT-3' R: 5'-ATCATCCTCTTTTCCTTCAT-3'
TβR3	F: 5'-TGATGTCTCAATCTCAGAA-3' R: 5'-GCTGACAAAGAGAAAATTTC-3'
β-actin	F: 5'-TTCCAGCCATCTTCTTG-3' R: 5'-TCCTTCTGCATCCTGTCA-3'

USA). In the preliminary experiments, different numbers of cycles (25, 30, 35, and 40 cycles) for TGFβs and TβRs were tested in each sample to optimize the amplification and it was confirmed that 30 cycles were optimal for the detection of quantitative differences between the samples. The PCR products were electrophoresed in a 3% (w/v) agarose gel with 0.4% ethidium bromide. The density of bands of TGFβs and TβRs were quantified with reference to that of β-actin using a Gel-Pro analyzer (Media Cybernetics, Inc., Silver Spring, MD, USA), and the ratio of TGFβs/β-actin was obtained. The sequence of PCR products of TGFβ2, TGFβ4, TβR2, and TβR3 had been confirmed by our previous reports that used same primers as the present study (Chowdhury *et al.* 2003, 2004). The PCR products of TGFβ3 and TβR1 were sequenced using the ExoSAP-IT (Amersham) and an ABI Prism BigDye Terminator Cycle Sequencing Kit (Amersham) as described by Nishibori *et al.* (2004). Sequences were analyzed with the GENETYX program package (version 7.04 Software Development, Tokyo, Japan). The sequence of PCR products corresponded to previous reports: TGFβ2 (Accession no. NM001031045), TGFβ3 (Accession no. S46000), TGFβ4 (Jakowlew *et al.* 1988), TβR1 (Accession no. U38622), TβR2 (Accession no. NM205428), and TβR3 (Accession no. NM204339).

Experiment 3. RT-PCR analysis for expression of TGFβs and TβRs by sperm

A possibility of the expressions of TGFβs and TβRs by sperm was examined. The sample of this experiment was fresh semen collected from roosters ($n=3$). The semen (0.5 ml) were added to 1 ml PBS (pH 7.4) and centrifuged at 1700 *g* for 10 min, and the precipitate containing sperm was washed three times with PBS by repeated centrifugation at 1700 *g* for 10 min. The mRNA was isolated and RT-PCR analysis for the expressions of TGFβs and TβRs was performed as described previously.

If the sperm express TGFβs and TβRs, expressions of TGFβs and TβRs in the UVJ-containing sperm in

inseminated birds may be affected by sperm expression. It was examined whether the addition of sperm to isolated UVJ tissue causes the changes in their expression. Sperm were added to the isolated UVJ tissue at the ratio of 0, 1.5×10^3 , 3×10^3 , and 7.5×10^3 sperm/mg UVJ tissue, which is equivalent to 0, 0.5, 1, and 2% of the inseminated sperm (2×10^8 sperm) that calculated based on the total UVJ mucosal tissue weight (600 mg) respectively. However, Bakst *et al.* (1994) described the number of sperm that enter SST was <1% of the inseminated sperm. Total mRNA was collected from the mixed samples and their expressions of TGFβs and TβRs were observed in the same manner as described previously.

Experiment 4. Immunohistochemistry and western-blot analysis for TβR2

Imunostaining for TβR2 in the tissue of UVJ was performed using paraffin sections prepared in Experiment 1. After deparaffinization, sections were washed with PBS for 15 min (5 min $\times$ 3 times), and autoclaved for 1 min in 2 mM citric acid (pH 6.0) to enhance antigenicity. Then the sections were incubated overnight at 4 °C with sheep anti-chicken TβR2 polyclonal antibody (Abcam Ltd, Cambridge, UK) diluted to 1:100 in PBS containing 0.05% BSA (Nacalai Tesque, Inc., Kyoto, Japan). After washing with PBS (5 min $\times$ 3 times), the sections were incubated with biotinylated anti-sheep IgG (Abcam Ltd) for 1 h and with avidin–peroxidase complex (Nichirei Corporation, Tokyo, Japan) for 30 min. The sections were washed in PBS (5 min $\times$ 3 times) and immunoprecipitates were visualized by incubating with 0.02% (w/v) 3', 3'-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) and 0.001% (v/v) H₂O₂ in 0.05 M Tris–HCl buffer (pH 7.6). The slides were counterstained with hematoxylin, dehydrated, and covered.

For western-blot analysis, the UVJ tissue of non-inseminated birds and fresh sperm were homogenized in five times the volume of homogenization buffer consisting of 10 mM Tris–HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid, and 1 mM phenylmethylsulfonyl fluoride with a Polytron homogenizer (Kinematica AG, Littau, Lucerne, Switzerland). The samples were centrifuged at 12 000 *g* for 10 min, the supernatant was again centrifuged at 45 000 *g* for 1 h, and the supernatant was collected. The samples were separated by SDS-PAGE, 10% separating gel and 4% stacking gel, as described by Yoshimura *et al.* (1997). Briefly, the protein concentrations were measured using protein-assay reagent (Bio-Rad Lab.) as described by the manufacturer. Each sample of 62.5 μg in 50 μl was mixed with 20 μl sample buffer (35% (v/v) glycerol, 12% (v/v) mercaptoethanol, 7.2% (w/v) SDS, 0.15 M Tris–HCl (pH 6.8), 0.06% (v/v) bromophenol blue) and boiled for 2 min. The sample, 10 μl, was loaded onto gels and run at 80 V

in the stacking gel and at 120 V in the separating gel. After SDS-PAGE, the samples were electrophoretically transferred onto the nitrocellulose membrane (Hybond-C, Amersham Int.). The membrane was washed with western buffer (0.02 M Tris-HCl (pH 7.4), 0.15 M NaCl, 0.5% Tween-20, and 0.05% (w/v) BSA) for 30 min (10 min $\times$ 3 times) and incubated with 10% Block Ace (Dainihon Pharmaceutical Co., Osaka, Japan) in western buffer for 30 min. The membrane was then incubated with sheep anti-chicken T β R2 polyclonal antibody (Abcam Ltd) diluted to 1:1000 with western buffer for overnight. Following washing with western buffer for 45 min (15 min $\times$ 3 times), they were incubated with biotinylated anti-sheep IgG (Abcam Ltd) diluted to 1:10 000 for 2 h and with avidin-peroxidase complex (Nichirei Co., Tokyo, Japan) for 1 h. The membrane was washed with western buffer for 30 min (10 min $\times$ 3 times) and the immunoprecipitates on the membrane were visualized by incubating in a reaction mixture of 0.02% (w/v) 3', 3'-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Inc.) and 0.001% (v/v) H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.6).

Statistical analysis

The significance of differences in TGF β s and T β Rs expressions (the ratio of TGF β s or T β Rs/ β -actin mRNAs) within each oviductal segment (infundibulum, uterus, UVJ, or vagina) was examined among different hours after AI (trial 1) or different days (trial 2) by one-way ANOVA, followed by Duncan's (1955) multiple range test. Differences were considered significant when *P* value was <0.05.

Results

Experiment 1. Histological observation

In the UVJ, the SST were distributed in the lamina propria of mucosal folds. The SST consisted of single layer of non-ciliated epithelial cells. No sperm was observed in the lumen of SST in non-inseminated birds (Fig. 1A), whereas there were SST filled with sperm in the inseminated birds of trials 1 and 2 (Fig. 1B and C) except for 20 days after AI (Fig. 1D). The ratio of SST structure containing sperm was approximately 35–50% at 1, 12, and 24 h after AI (trial 1; Fig. 2A), and showed a gradual decrease on 10 days (trial 2; Fig. 2B). The number of lymphocytes in the stroma surrounding the SST was not changed significantly during experimental periods in both trials 1 and 2 (Fig. 2C and D).

Experiment 2. RT-PCR analysis for expressions of TGF β s and T β Rs in UVJ

Trial 1

The expressions of TGF β 2, TGF β 3, and TGF β 4 were observed in the infundibulum, uterus, UVJ, and vagina. Figures 3–5 show the changes in the expressions of TGF β s in the oviduct until 24 h after AI. Differences in the expressions for TGF β 2 were not significant among 0, 1, 12, and 24 h after AI in the infundibulum, uterus, and vagina (Fig. 3A, B and D), however, that in the UVJ was significantly increased at 1 h after insemination and showed a gradual decreasing tendency (Fig. 3C). The expressions for TGF β 3 and TGF β 4 also did not show any significant changes within 24 h after AI in the infundibulum, uterus, and vagina (Figs 4A, B and D and 5A, B

Figure 1 Sections of sperm-storage tubules (SST) in utero-vaginal junction of hens with or without insemination. (A) Before insemination. (B) 1 day after insemination. (C) 10 days after insemination. (D) 20 days after insemination. Note that SST are filled with sperm 1 and 10 days after insemination (arrow heads; (B) and (C)), whereas no sperm is located in SST before and 20 days after insemination (arrows; (A) and (D)). (E) Surface epithelium; Lp, lamina propria; scale bars = 50 μ m. HE staining. The inserts of (B) and (C) (scale bar, 20 μ m) show the higher magnification of single SST with sperm in lumen (arrows).

Figure 2 Frequency of SST-containing sperm and lymphocyte population in UVJ of non-inseminated and inseminated birds. (A) Frequency of SST with spermatozoa within 24 h after insemination. (B) Frequency of SST with spermatozoa within 20 days after insemination. (C) Number of lymphocytes in 50 000 μm^2 areas of UVJ lamina propria within 24 h after insemination. (D) Number of lymphocytes in 50 000 μm^2 areas of UVJ lamina propria within 20 days after insemination. Values represent the mean $\pm$ S.E.M. ($n=4$). Values with different superscripts are significantly different ($P<0.01$).

and D). However, in UVJ, the expression of TGF β 3 was significantly increased during 1–12 h after AI and then decreased at 24 h after AI (Fig. 4C). The expression of TGF β 4 in UVJ was significantly greater during 1–24 h compared with the 0-h group (Fig. 5C).

The expressions of T β R1, T β R2, and T β R3 were observed in the infundibulum, uterus, UVJ, and vagina.

Figure 3 Changes in the expression of TGF β 2 in the tissues of (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina within 24 h after insemination. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TGF β 2 mRNA to β -actin mRNA. Values with different letters are significantly different ($P<0.05$).

Figure 4 Changes in the expression of TGF β 3 in the tissues of (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina within 24 h after insemination. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TGF β 3 mRNA to β -actin mRNA. Values with different letters are significantly different ($P<0.05$).

Differences in the expressions for T β R1 and T β R2 were insignificant among 0, 1, 12, and 24 h after AI within the infundibulum, uterus, or vagina (Figs 6A, B and D and 7A, B and D). In contrast, expressions of T β R1 and T β R2 in the UVJ were significantly increased at 1 h after AI and kept higher up to 24 h (Figs 6C and 7C). However, the expressions for T β R3 did not show significant change within 24 h after AI in any of the oviductal segments observed in the present study (Fig. 8).

Figure 5 Changes in the expression of TGF β 4 in the tissues of (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina within 24 h after insemination. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TGF β 4 mRNA to β -actin mRNA. Values with different letters are significantly different ($P<0.01$).

Figure 6 Changes in the expression of TβR1 in the tissues of (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina within 24 h after insemination. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TβR1 mRNA to β -actin mRNA. Values with different letters are significantly different ($P<0.05$).

Trial 2

Figure 9 shows the changes in the expression of TGFβs and their receptors in UVJ until 20 days after AI. Difference in the expression of TGFβ2 in UVJ was not significant among 0, 1, 10, and 20 days after insemination (Fig. 9A). In contrast, the expression of TGFβ3 was significantly greater on 1 day after AI than 0 day and it returned to same level as 0-day group in 10 days (Fig. 9B). However, in case of TGFβ4, the expression was significantly increased on 1 day after AI compared with 0 day, and then gradually declined on 10 and 20 days (Fig. 9C).

Figure 7 Changes in the expression of TβR2 in the tissues of (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina within 24 h after insemination. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TβR2 mRNA to β -actin mRNA. Values with different letters are significantly different ($P<0.05$).

Figure 8 Changes in the expression of TβR3 in the tissues of (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina within 24 h after insemination. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TβR3 mRNA to β -actin mRNA.

Figure 9 Changes in the expression of TGFβ isoforms and their receptors in the tissue of utero-vaginal junction within 20 days after insemination. (A) TGFβ2, (B) TGFβ3, (C) TGFβ4, (D) TβR1, (E) TβR2, and (F) TβR3. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TGFβs or TβRs mRNA to β -actin mRNA. Values with different letters are significantly different ($P<0.05$).

The expressions of T β R1 and T β R2 in UVJ were significantly increased on 1 day after AI and kept higher until 10 days followed by decreasing on 20 days that showed a same level as 0 day (Fig. 9D and E). However, expression of T β R3 in the tissue of UVJ did not show any significant differences during 0–20 days (Fig. 9F).

Experiment 3. Expression of TGF β s and T β Rs by sperm

Clear PCR products showing the expression of the TGF β 2, TGF β 3, and TGF β 4 and T β R1, T β R2, and T β R3 were observed in sperm samples (Fig. 10A and B). The expressions of TGF β s and T β Rs were insignificant among the tissues of UVJ added with 0, 1.5×10^3 , 3×10^3 , and 7.5×10^3 sperm/mg UVJ tissue (Fig. 11).

Experiment 4. Immunocytochemistry and western-blot analysis for T β R2

Immunoreactive T β R2 were observed in the lymphocytes in the lamina propria (Fig. 12A). The cells in SST were also stained positive with a strong intensity as compared to those of the surface epithelium. Western-blot analysis confirmed the presence of T β R2 in UVJ tissue and in sperm, which was observed approximately at 75 kDa (Fig. 12B).

Discussion

We are reporting that the TGF β s (TGF β 2, TGF β 3, and TGF β 4) and T β Rs (T β R1, T β R2, and T β R3) are expressed in UVJ of hen oviduct and changed in association with the presence of sperm in the SST after AI. The significant findings of this study are: (i) among the four oviductal segments (infundibulum, uterus, UVJ, and vagina), the expressions for TGF β s and T β Rs were increased only in the tissue of UVJ during the storage of sperm in SST; (ii) the sperm also expressed TGF β s and T β Rs; and (iii) lymphocytes and SST cells were immunopositive for T β R2.

The expressions of TGF β 2, TGF β 3, and TGF β 4 in UVJ were significantly increased within 1 h after AI and that of TGF β 4 was kept higher even during 24 h–10 days after AI. In case of their receptors, expressions of T β R1 and

Figure 10 Expression of mRNAs of TGF β isoforms and receptors by sperm. (A) M, marker; lane 2, TGF β 2; lane 3, TGF β 3; and lane 4, TGF β 4. (B) M, marker; lane 2, T β R1; lane 3, T β R2; and lane 4, T β R3.

Figure 11 Changes in the expression of TGF β isoforms and their receptors in the tissue of isolated utero-vaginal junction added with sperm. Sperm were added to UVJ mucosal tissue at the ratio of 0×10^3 , 1.5×10^3 , 3×10^3 , and 7.5×10^3 sperm/mg of UVJ tissue. (A) TGF β 2, (B) TGF β 3, (C) TGF β 4, (D) T β R1, (E) T β R2, and (F) T β R3. Values show the mean $\pm$ S.E.M. ($n=4$ for each value) of the ratio of TGF β s or T β Rs mRNA to β -actin mRNA.

T β R2 but not T β R3 were increased at 1 h after AI and kept higher until 10 days. The UVJ is the primary site for sperm storage, where abundant SST distribute (Fujii & Tamura 1963, Bakst *et al.* 1994). The ratio of SST-containing sperm was high on 1 day after AI and gradually decreased by 10 days, followed by decreasing to a negligible level on 20 days. Thus, there seems a close association between sperm storage and expressions levels of TGF β s and their receptors. Among the three types of TGF β s, the TGF β 4 isoform may be the most noticeable molecule for this event as it maintained higher expression level for the longest term, which was similar to the term of sperm residence in SST. In contrast, the other three oviductal segments, namely infundibulum, uterus, and vagina did not show increase in expressions for any of the ligands or their receptors. We suggest that increased expressions of TGF β s and their receptors occur specifically in UVJ after insemination. The reason why the infundibulum, which is known as secondary sperm storage site, failed to show increase in expressions for any of the ligands or receptors in their SST is small.

Figure 12 (A) Sections of utero-vaginal junction of an inseminated bird immunostained for T β R2. Arrows indicate lymphocyte positive for T β R2 in lamina propria and surface epithelium. SST cells also show positive staining. *, SST; E, surface epithelium; Lp, lamina propria. Scale bar=40 μ m. Inset shows magnified view of lymphocyte in the lamina propria. Scale bar=10 μ m. (B) Western blot of T β R2 in the utero-vaginal junction of non-inseminated laying hen and in sperm. The bands of approximately 75 kDa are observed both in UVJ (lane 1) and sperm (lane 2).

We report the novel expressions of TGF β 2, TGF β 3, and TGF β 4, and T β R1, T β R2, and T β R3 by chicken sperm observed in the present study. In the previous reports, TGF β 1 was detected in human sperm by immunostaining (Chu *et al.* 1996) and the presence of TGF β 1, TGF β 2, and TGF β 3 were identified in human seminal plasma (Nocera & Chu 1995, Srivastava *et al.* 1996). It has also been reported that the majority of TGF β in seminal fluids is in a latent form, which becomes activated in female reproductive tract after insemination (Robertson 2005). These results suggest that sperm likely produce TGF β s in different species, including birds and mammals. However, the addition of 7.5×10^3 sperm to per milligram isolated mucosal UVJ tissue did not cause significant differences in the expression of TGF β s and T β Rs. Bakst *et al.* (1994) reported that the number of sperm that enter the SST is <1%, and approximately similar number of sperm were added to the UVJ tissue in this study. Therefore, the expressions of TGF β s and T β Rs in UVJ after AI might be increased by the interaction of SST cells and sperm, rather than the simple addition of sperm expression to the UVJ tissue expression. Sperm may influence some gene expressions and secretory proteomic profiles in the reproductive tract of mammals that may be related to sperm transport and selection (Fazeli *et al.* 2004, Georgiou *et al.* 2005). Long *et al.* (2003) reported the increase of gene expression for avidin in UVJ of turkey hens in response to insemination, and suggested that it might provide the nutrient sources of biotin or related vitamins for the resident sperm. These reports suggest that sperm may influence the gene expressions of some molecules in the oviduct of both mammals and birds. Thus, sperm could also induce the gene expression of TGF β s and their receptors in the UVJ. The TGF β s could be synthesized by sperm until ejaculation, however, it is not confirmed whether they are still synthesized even during the traveling in the oviduct. If sperm synthesize TGF β s even while traveling the oviduct, it may play roles in protecting themselves.

Immunohistochemical examination confirmed the presence of T β R2 in lymphocytes and SST cells,

suggesting a possibility of interaction between TGF β s and these cells. The specificity of the immunoreaction was confirmed by western-blot analysis. The TGF β s and its receptors exert a potent inhibitory effect on B-cell proliferation and differentiation (Kehrl *et al.* 1986a). The TGF β 1 in mammalian species, which is thought to be an ortholog of TGF β 4 in avian species (Pan & Halper 2003, Halper *et al.* 2004), suppressed immune response by maintaining development of suppressor T-cells in addition to the direct suppressive effect on the proliferation of B- and T-cells in chicken (Quere & Thorbecke 1990). In a recent study, Huang & Huang (2005) explained the involvement of T β R-V-signaling cascade along with T β R1/T β R2 for mediating the inhibitory function of TGF β s on various type of cell proliferation. Thus, these reports suggest that TGF β s exert suppressive effect on T- and B-cell proliferation or differentiation. Previous report described that lymphocyte population was increased in UVJ and sperm were not stored in SST in infertile hens after AI (Das *et al.* 2005b). Plasma cells were also shown in the UVJ of infertile hens (Van Krey *et al.* 1987). These results suggested that immune response to sperm may occur in infertile hens, resulting in the decline of sperm number stored in SST. Decline of fertility caused by immunoreponse to sperm has also been suggested in mammals (Mettler 1978). Our results showing the elevated levels of TGF β s and the presence of T β R2 in lymphocytes in UVJ suggest that TGF β s produced by UVJ tissue and sperm may suppress immune response to sperm to maintain the survivability of them in the SST. In the present study with healthy hens, the population of stromal lymphocytes was not significantly different between inseminated and non-inseminated birds. We assume that this is the normal dynamics of lymphocytes in UVJ stroma in healthy birds and TGF β might be involved in the suppression of increased lymphocyte population.

The SST cells showed the stronger immunoreactivity for T β R2 than the surface epithelium of UVJ. Sperm expressed T β R1, T β R2, and T β R3 with negligible immunoreactivity in SST on the immunostained sections,

which might be due to the fewer amount of receptor molecules. The interaction of TGF β s with the receptors in the SST cells and sperm may also be responsible for the survivability of sperm in SST.

In conclusion, we have provided evidence that the mRNA for TGF β 2, TGF β 3, and TGF β 4 and T β R1, T β R2, and T β R3 are expressed in the hen oviduct and in sperm, and their expressions in UVJ are increased with AI. The increase of expressions in UVJ might be caused by the stimuli of sperm stored in SST. The enhanced expressions of TGF β s and T β Rs in UVJ may be a mechanism responsible for the survivability of sperm during their storage in SST, probably via suppression of anti-sperm immunoreactions.

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