Developing germ cells in mouse testis express pheromone receptors

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Abstract Pheromone receptors are expressed in the accessory olfactory system, which is vital for non-specific chemical communication and for sexual behavior. Under the hypothesis that some of the pheromone molecules released from female reproductive organs might regulate sperm chemotaxis or chemokinesis, we examined whether the V1R type pheromone receptor mRNAs are expressed in developing germ cells. By a reverse transcription-PCR method, we obtained nine kinds of cDNA fragments belonging to the receptor family. In situ hybridization analysis in testicular sections using probes of testicular pheromone receptors (TVRs) revealed that TVR mRNAs were expressed by spermatids. TVRs were also expressed in the accessory olfactory organ. In the testis, hybridization signals were localized in subsets of the seminiferous tubules, suggesting that TVRs were expressed by selective subsets of the spermatids. In situ hybridization study suggests also that each sperm expresses multiple pheromone receptors. The testicular pheromone receptors might have an important role in the maturation and/or migration of sperm.

Key words: Mouse; Pheromone receptor; Germ cell; Spermatid; In situ hybridization

1. Introduction

Sperm migration to egg is a critical step for ensuring fertilization, the direct interaction between these cells. While molecular and cellular mechanisms for the fertilization have been relatively well characterized [1,2], little is known about the mechanism of the sperm migration. There is an in vitro evidence for human sperm chemotaxis; human spermatozoa migrate to and accumulate in follicular fluid [3–6]. Ralt et al. recently demonstrated that this process involves both sperm chemotaxis, a response with movement to the direction of a chemical stimulus, and chemokinesis, a change in swimming speed in response to a chemical stimulus [4]. Sea urchin sperms respond to chemotactic peptides released from eggs with an increase in intracellular second messengers: cAMP and cGMP [7,8]. It has been postulated that chemotactic factors bind to their receptors expressed on the sperm membrane and cause an increase in intracellular second messengers and that this signal plays an important role in the sperm migration to egg [9,10]. Because of the coupling with the intracellular second messenger signals, members of the seven transmembrane G protein-coupled receptor gene family are candidates for the chemotactic factor receptors.

Odorant receptors (ORs) are seven transmembrane G protein-coupled receptors and form a large gene family with up to 1000 members in mice and rats [11]. They are expressed by sensory neurons in the olfactory epithelium (OE) of the nose. Recent studies demonstrated that a few members of the OR gene family are expressed in the male genital system by developing germ cells of the late stage and ejaculated sperm [12–14]. This observation led to the hypothesis that some odorants or odorant-like molecules function as chemoattractant to sperm and that ORs are involved in guiding sperm to egg by means of chemoattraction. In addition to odorants, mammalian olfactory system uses pheromone molecules as a means of chemical communication. Some of the pheromone molecules are thought to be present in the secretions of the genital tract. This raises a possibility that sperms are exposed to pheromone molecules in the genital tract while on their way to the egg and that the pheromone molecules might regulate the chemotaxis and chemokinesis of sperms.

Pheromone receptors are seven transmembrane G protein-coupled receptors [15–19]. They form a large gene family with about 100–200 members and are expressed by sensory neurons in the vomeronasal sensory epithelium of the vomeronasal organ (VNO) in rodents [15–18]. Pheromone receptors are classified into two subfamilies; one subfamily (V1Rs) is expressed by sensory neurons in the apical zone of the vomeronasal sensory epithelium while the other subfamily (V2Rs) is expressed by sensory neurons in the basal zone [18,19]. Signals received by the pheromone receptors are sent via axons of vomeronasal sensory neurons to the accessory olfactory bulb, and then to the higher centers of the accessory olfactory system. These signals provide information about gender, dominance or reproductive status, and elicit innate social and sexual behaviors along with profound neuroendocrine changes [20–22].

As a first step toward understanding possible roles of pheromone molecules in sperm migration, we examined in mice whether the pheromone receptors V1Rs are expressed in testis.

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Reverse transcription (RT-)PCR fragments were obtained from mouse testicular mRNA using degenerate primers directed against regions conserved among rat V1R pheromone receptors. Because all mRNAs are lost in mature sperm [14], we performed in situ hybridization study on developing germ cells in testis using probes for the testicular V1R pheromone receptors. The results demonstrate that pheromone receptor mRNAs expressed in the accessory olfactory system are present also in the developing germ cells. This suggests the possibility that pheromone molecules secreted in the genital tract can have two different functions: one as ligands for pheromone receptors expressed by migrating sperms and another as pheromones for sensory stimulation of the accessory olfactory system.

2. Materials and methods

2.1. RT-PCR cloning

Total RNA from tissues of adult mice (ddY: 10 weeks old) was prepared according to the method of Chomczynski and Sacchi [23]. DNaseI-treated RNA samples were reverse-transcribed with Superscript II (Gibco-BRL, MD, USA) and subjected to PCR with AmpliTaq Gold PCR kit (Perkin Elmer, NJ, USA). Degenerate primers for amplifying the cDNAs encoding V1R subtype of pheromone receptors were designed to direct against the sequences conserved among the known rat V1Rs, corresponding to the transmembrane domains III and VI (V1R 5′ primer 5′-C(C/T)A (A/G)AA (A/G)(A/C)T CCT GTT TA(A/G) CAA-3′, V1R 3′ primer 5′-ATG G(A/T)(A/C) (C/T)(C/G/T)(G/T) GTG GCC CTT TG-3′). Degenerate primers for the ORs were similarly designed to the sequences conserved among the known mouse ORs (OR 5′ primer 5′-(A/G)(C/T)(C/G) ATG (G/T)C(C/T)T ATG ACC GIT (A/T)(C/T)(C/G) T(A/G)G-3′, OR 3′ primer 5′-CT(A/C/T) AG(A/G) (C/G)T(A/G) TAG AT(A/G) AA(A/C/G) GG(A/G) TTC A-3′). After the activation of the AmpliTaq Gold enzyme at 95°C for 11 min, 40 cycles of the PCR reaction were performed; each cycle consisted of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 1 min. To confirm the cDNA synthesis and the genomic DNA digestion, we also performed RT-PCR with β-actin primer set (Clontech, CA, USA) on the same samples used in the other RT-PCRs. The amplified products were analyzed by the electrophoresis on 1% agarose gel. The products obtained from testis cDNAs were cloned into pGEM-T-easy vector (Promega, WI, USA) and their nucleotide sequences were analyzed. To obtain the control probe for identifying the spermatid, we performed an RT-PCR cloning of protamine 2 cDNA fragment using specific primer set (5′ primer: 5′-TGG ACA AGC CAT GAA CGC-3′, 3′ primer: 5′-CTG CAT CTC CTC CTC CTT CG-3′).

2.2. In situ hybridization

Riboprobes were transcribed from the testicular V1R pheromone receptors, OR or protamine 2 cDNA fragments in the pGEM-T-easy vector, using [^{33}P]UTP (Amersham, UK) with RNA transcription kit (Stratagene, CA, USA). All steps of the in situ hybridization reactions were carried out essentially according to Simmons et al. [24]. The 15 μm sections of VNO and testis were prepared from 2 and 10 week old mice, respectively. Mice were deeply anesthetized with Nembutal and then perfused with 4% paraformaldehyde. Head and testis were emerged into OCT compound, frozen, and cut with a cryostat. The sections were then mounted on the slide glasses coated with poly-L-lysin (Matsunami, Japan), dried, fixed with 4% paraformaldehyde for 10 min at room temperature, digested with proteinase K (10 mg/ml, 25°C, 30 min), acetylated, dehydrated and air-dried. The sections were then hybridized with hybridization buffer containing labeled riboprobe (10^6 cpm/ml) for overnight at 56°C in a humidified chamber. Hybridization buffer consists of 50% formamide, 20 mM Tris–HCl (pH 7.6), 1 mM EDTA, 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 mg/ml yeast tRNA, 1× Denhardt's solution, and 10% dextransulfate. After hybridization, the sections were washed in 4× SSC, treated with RNase A (10 mg/ml, 37°C, 30 min), washed in 0.1× SSC (70°C, 30 min), dehydrated with ethanol, and dipped in NBT-2 emulsion (Kodak, NY, USA), exposed for 3–4 weeks, developed in Kodak D-19, fixed with Fuji Fix, and counterstained with cresyl violet. The sections were observed with a BX-60 microscope (Olympus, Japan).

3. Results

3.1. Identification of putative pheromone receptor gene fragment in mouse testis by RT-PCR

Pheromone receptors (both V1Rs and V2Rs) are expressed in the sensory epithelium of the VNO, but are absent from the OE. To examine whether the V1R subset of pheromone receptors are expressed by mouse testis, RT-PCR was performed using a degenerate primer set designed to direct

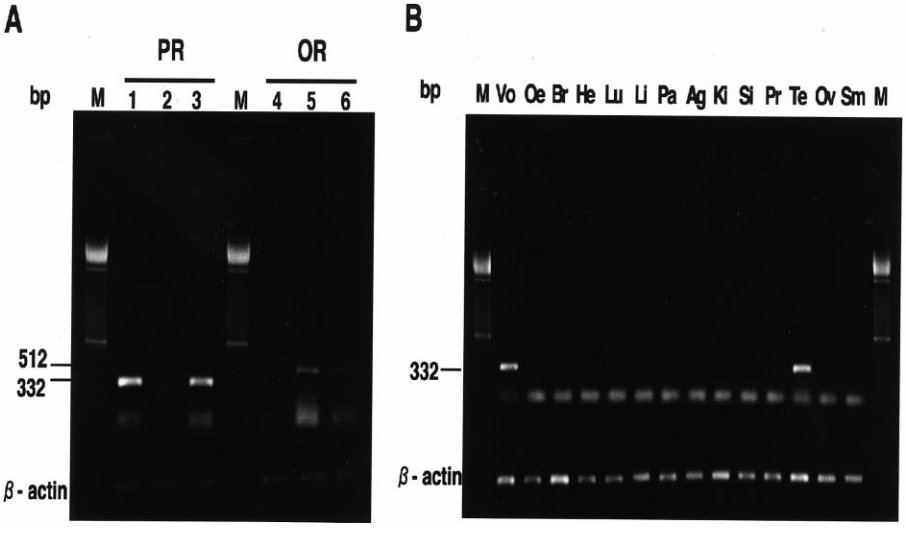

Fig. 1. RT-PCR analysis of V1R subset pheromone receptors and ORs. A: RT-PCR of V1R subset pheromone receptors (V1R) and ORs using total RNAs obtained from VNO, OE and testis (Te). Predicted sizes of pheromone receptor (322 bp) and OR (512 bp) were indicated by arrows. B: V1R pheromone receptors are expressed selectively in VNO and Te. RT-PCR of the pheromone receptors using cDNAs from various tissues. The results of β-actin RT-PCR using the same samples used in the upper panels were shown in the bottom. Br: brain, He: heart, Lu: lung, Li: liver, Pa: pancreas, Ag: adrenal gland, Ki: kidney, Si: small intestine, Pr: prostate, Ov: ovary, Sm: skeletal muscle, M: 100 bp ladder marker.

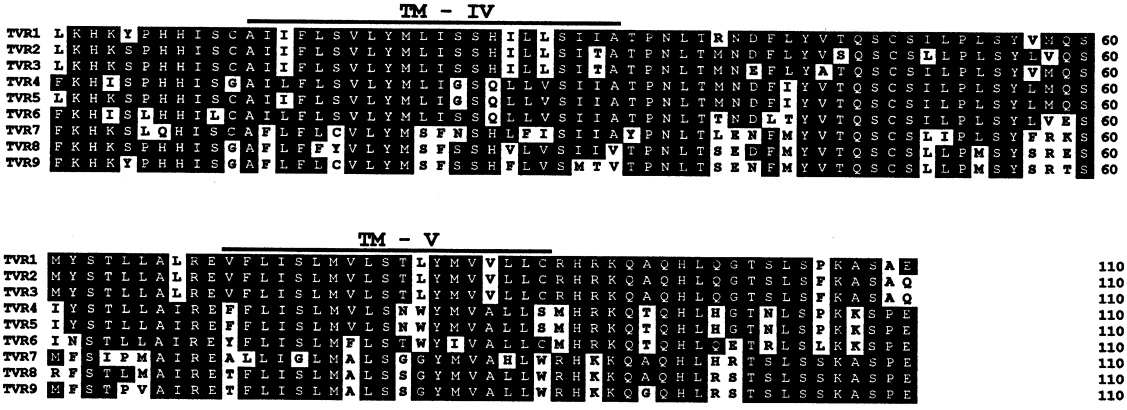

Fig. 2. Comparison of deduced amino acid sequences of PCR fragments of V1R subset pheromone receptor family expressed in the mouse testis. Amino acid residues conserved among more than four clones were indicated in white letters with black background. Nine kinds of clones were subdivided into two groups (group I: TVRs 1–6, group II: TVRs 7–9) based on their sequence similarity. Lines indicate the possible transmembrane domains, TM-IV and TM-V. Asterisks indicate clones used in the in situ hybridization analysis.

against sequences conserved among the known rat V1Rs [15]. As expected, amplified cDNA fragments of about 320 bp were obtained from the VNO, whereas no PCR product was obtained from OE (Fig. 1A, V1R). Using the same primer set, a clear band with corresponding size was detected from testis, suggesting that V1Rs are also expressed in testis. For comparison, we performed RT-PCR in the VNO, OE and testis using OR-specific primers. In agreement with previous reports [14], OR mRNA was expressed in the OE and testis, but not in the VNO (Fig. 1A, OR). RT-PCR examination using cDNAs from various tissues including the brain, heart, lung, and liver showed that the expression of V1R type pheromone receptor mRNAs was restricted to the VNO and testis (Fig. 1B).

3.2. Sequence analysis of testicular pheromone receptor gene fragment

We cloned the cDNA fragments obtained from the RT-PCR of the pheromone receptors in testis and analyzed their sequences. Among 33 clones analyzed, nine different cDNAs of V1R type pheromone receptors were distinguished. These fragments, possibly encoding 110 amino acids covering the region from transmembrane domain III to VI (Fig. 2), shared high amino acid identity (68–77%) with the corresponding portion of known rat V1Rs [15]. This indicates that the nine clones belong to the V1R subfamily of the pheromone receptors. We thus named them testicular vomeronasal receptors (TVRs). Amino acid identity within the nine clones was between 58% and 96%. As is the case for the rat V1Rs [15], the nine clones could be classified into two groups (TVRs 1–6 in group I and TVRs 7–9 in group II) according to their sequence similarity. For detailed analysis of pheromone receptor expression in testis, we selected two clones; TVR2 in group I and TVR7 in group II (shown by asterisks in Fig. 2). Pheromone receptors are expressed in developing germ cells.

The development of germ cells in testis proceeds from the immature spermatogonia distributed at the outer edge of the seminiferous tubules, through various stages of spermatocytes, to spermatids located in the inner portion facing to the lumen [25,26]. The spermatids undergo morphological differentiation into spermatozoa, which then migrate into the lumen of the seminiferous tubules. To examine whether germ cells in testis express pheromone receptors, we performed in situ hybridization analysis using the TVR probes on coronal sections of adult testicular seminiferous tubules. Strong hybridization signals of TVR2 (Fig. 3A,B) and TVR7 (Fig. 3D,E) were observed on cells distributed in the most interior portion of the tubules, suggesting that germ cells in a late developmental stage express these pheromone receptor mRNAs. The sense probes of TVR2 (Fig. 3C) and TVR7 (data not shown) labeled no cells in the testis.

In the testis, mRNA for protamine 2, a marker molecule for the germ cells, can be detected in developing germ cells from the middle stage of pachytene spermatocyte through round spermatids up to elongated spermatids [27]. Corresponding to the developmental stage-specific expression pattern, in situ hybridization analysis using protamine 2 cRNA probe showed hybridization signals distributed in the inner 2/3 of all the seminiferous tubule sections (Fig. 3F). Comparison of hybridization signals between protamine 2 and pheromone receptors suggests that mRNAs of TVR2 and TVR7 were selectively expressed by spermatids which appear in the late stage of the spermatogenesis. Morphological observation of TVR-positive cells with higher magnification confirmed that the mRNAs for the TVR2 and TVR7 were expressed selectively by round and elongated spermatids (data not shown). Other cell types including peritubular cells, Sertoli cells and Leydig cells did not express the pheromone receptor mRNAs.

We observed that mRNAs for TVR2 and TVR7 were present on a subset (about 1/3) of cross sections of the seminiferous tubules (Fig. 3A,D). Because all the cross sections of the seminiferous tubules contained round and elongated spermatids or elongated spermatids in the most inner portion, the lack of hybridization signals with TVR2 and TVR7 probes in a subset of the cross sections suggests that these pheromone receptors are not expressed by all spermatids. TVR2 and TVR7 may be expressed by selective subsets of spermatids.

For comparison, we made in situ hybridization analysis using a testicular OR probe (TOR9). As shown in Fig. 3G,H, TOR9 mRNA was found in cells between late stage of pachytene spermatocytes and elongated spermatids. The comparison between TVRs and TOR9 of the distribution of hybridization signals in the cross sections suggests that the OR mRNA begins to be expressed before the start of the pheromone receptor mRNA expression. TOR9 hybridization signals were detected in about 90% of sections of the semi-

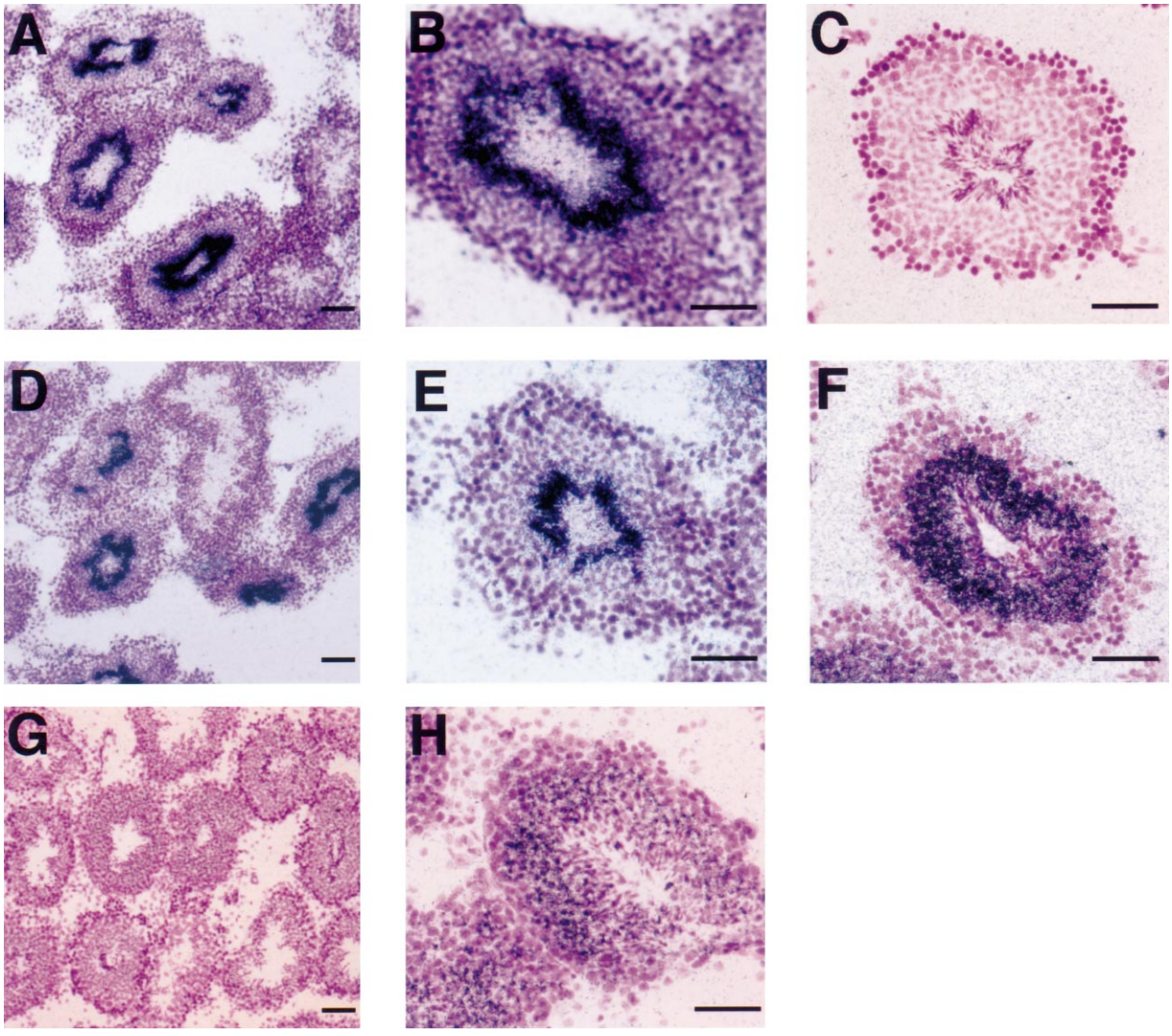

Fig. 3. In situ hybridization analysis revealed that TVR2 and TVR7 mRNAs were expressed by subsets of spermatid in the testis. Panels show the sections of the testis hybridized with TVR2 antisense probe (A, B), TVR2 sense probe (C), TVR7 antisense probe (D, E), protamine 2 antisense probe (F), and testicular OR (TOR9) antisense probe (G, H). A, D, G: low magnification view. B, C, E, F, H: high magnification view. Notably, hybridization signals of TVR2 and TVR7 were observed in the subset of seminiferous tubules (A, D) whereas TOR9 mRNA was expressed by the spermatids in almost all seminiferous tubules (G). Scale bar: 100 μm.

niferous tubules (Fig. 3G), suggesting that most of the spermatids express the OR. This is in contrast to the expression pattern of pheromone receptors; TVR2 and TVR7 are expressed by smaller subsets of spermatids.

3.3. *Pheromone receptors present in testis are expressed also by sensory neurons in the vomeronasal epithelium*

Previous study in dog showed that a few members of the OR gene family are preferentially expressed in testis, with little or no expression in the olfactory sensory epithelium [13]. To examine whether the pheromone receptors found in testis are expressed in sensory neurons of the vomeronasal epithelium, in situ hybridization was performed on coronal sections of the VNO. Fig. 4 shows that each probe of TVR2 and TVR7 hybridized to a specific small subset of sensory neurons (about 1% of the cells in the sensory epithelium) that are distributed in the apical zone of the epithelium. Within the apical zone, TVR expressing sensory neurons were scattered widely along lateromedial and rostrocaudal axes. This pattern of TVR expression is consistent with that of V1Rs [15], suggesting that these TVRs are expressed as pheromone receptors by specific subsets of vomeronasal sensory neurons in the accessory olfactory system. The vomeronasal sensory epithelia of both male and female mice expressed the TVR2 and TVR7, and the overall expression pattern of the TVRs in the vomeronasal sensory epithelium did not differ between male and female mice (Fig. 4). These results suggest that TVR2 and TVR7 are expressed in both the vomeronasal sensory epithelium and testis.

4. Discussion

The following results of the present study strongly suggest that TVR cDNAs cloned from testis encode the V1R subset of pheromone receptors. Firstly, TVR mRNAs are detected also in the VNO in which known pheromone receptors are expressed [15]. TVRs were not detected in the OE nor in other tissues examined (Fig. 1). As is the case with known V1R

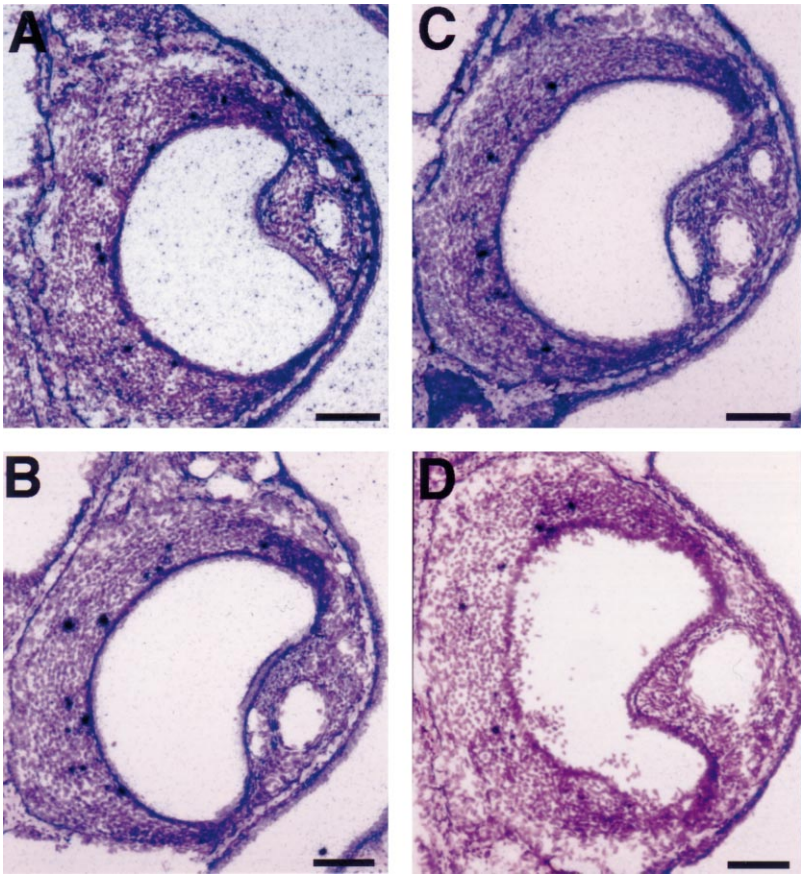

Fig. 4. In situ hybridization analysis using TVR2 and TVR7 probes in the VNO. TVR2 (A, B) and TVR7 (C, D) expressing cells were located in the apical zone of the vomeronasal sensory epithelium of 2 week old mice. Hybridization signals of TVR2 and TVR7 were observed in both male (A, C) and female (B, D) mice. Scale bar: 100 μm.

subset pheromone receptors, TVRs were expressed by a highly restricted subset of sensory neurons in the apical zone of the vomeronasal sensory epithelium. Secondly, TVRs show high amino acid identity with known V1R subset pheromone receptors.

In situ hybridization study demonstrated that the TVR mRNAs were expressed selectively by developing germ cells during the periods of round and elongated spermatids, but not by germ cells at earlier stages nor by other types of cells in the testis. The present study also demonstrated that TOR9 mRNAs are expressed selectively by developing germ cells during the period spanning from pachytene spermatocytes to elongated spermatids. Using an antiserum against an OR, Vanderhaeghen et al. demonstrated that OR protein was expressed by round and elongated spermatids and on the tail midpiece of mature spermatozoa [13]. These observations suggest that OR mRNAs may be translated into receptor proteins during the period of spermatids and remain to be expressed in the mature spermatozoa even after all mRNAs in the spermatozoa had been destroyed. Similarly, it can be assumed that pheromone receptor proteins may be produced during late period of spermatids and may remain to be expressed by mature spermatozoa. However, because of the lack of specific antibodies against the TVRs, it is not possible at present to determine whether spermatids or mature spermatozoa express the pheromone receptor protein on their surface membrane.

Present results showed that TOR9 mRNA is expressed by most spermatids. This suggests that individual spermatozoa that express TVR may co-express TOR9 OR. In addition, the existence of at least nine different TVR clones in testis suggests that a large number of different pheromone receptors are expressed by populations of developing germ cells. Based on the observation that each of the TVR2 and TVR7 mRNAs was expressed by about 1/3 of spermatids, it can be speculated that individual spermatozoa may express multiple pheromone receptors. These observations are in contrast to the expression pattern of ORs or pheromone receptors in sensory neurons of the olfactory or vomeronasal sensory epithelia. ORs are selectively expressed by olfactory sensory neurons but not by vomeronasal sensory neurons [11]. Individual olfactory sensory neurons express just one or at most a few type(s) of ORs among a large repertoire of the receptors [28–33]. In the vomeronasal sensory epithelium, sensory neurons express pheromone receptors but not ORs. Individual vomeronasal sensory neurons express one or only a few types of pheromone receptors [15–18]. The difference in the receptor expression pattern between sensory epithelia and the testis may be due to the difference in the transcription of the receptor genes. For example, testicular OR genes in rat undergo 5′ splicing [14,34]. In mouse, transcription initiation site of an OR in testis is different from that in olfactory sensory neurons [34].

The expression of pheromone receptor mRNAs in the developing spermatids suggests that the receptor proteins might function as sensors of pheromone molecules involved in the sperm maturation, sperm migration, or fertilization. The co-expression with ORs and the possible expression of multiple

pheromone receptors by individual sperm suggest that a number of different molecules function as chemical cues for migrating sperm at different portions of the testis and females genital tract. For example, human ovulation ordinarily occurs in one side of ovary. Thus sperm at the uterus may be guided by specific molecules to ensure swimming to the fallopian duct during ovulation. In fact, fallopian fluid has been shown to contain chemokinetic and chemoattractive substances [4]. Some of the pheromone receptors might function as receptors for these substances during the guided swimming of sperms. Just prior to fertilization, sperms change their movement in order to break the jelly layer of eggs effectively through chemokinesis [4], implicating possible function of pheromone receptors at this stage also.

Present results showed that TVR2 and TVR7 are expressed by specific subsets of spermatids. This suggests that spermatids can be classified into several subsets by the expression pattern of TVRs. It has been suggested that mature sperm may be classified into at least three functional subsets; 'egg getter' sperm, 'killer' sperm and 'blocker' sperm. The egg getter sperms are able to approach and interact with the egg [35]. The killer sperm protects other sperm from obstructions such as leukocytes or bacteria. The blocker sperms block other person's sperm which invade the cervix of uterus. This raises an interesting possibility that subsets of sperms defined by expression of specific combinations of pheromone and ORs might correspond to the above mentioned functional subsets. Further work with specific antibodies against TVRs is necessary to examine this possibility.

The disturbance of sperm chemotaxis may be one of the causes of infertility. If TVRs and TORs are involved in the control of sperm chemotaxis to the egg, further analysis of functional roles of these receptors might provide a clue to understanding yet unknown reasons for male infertility.

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