axons from the left eye always passes through the ventral side at the optic chiasm, while an axon bundle from the right eye runs on the dorsal side. The LR sidedness of midline crossing of RCG axons was randomized in medaka and zebrafish. Spontaneous mutants appeared in cultivated flounders, which show abnormality of metamorphic LR asymmetry such as left or right isomerism, showed wild-type crossing, suggesting that the LR sidedness of the optic nerve crossing is established independently of the metamorphic LR asymmetry. The molecular analyses using a left-specific gene *Pitx2* suggest that the LR sidedness of the crossing is established in parallel with other asymmetric morphogenesis mediated by *Pitx2*.

ROLE OF ANDROGEN RECEPTOR IN SEX DIFFERENTIATION IN JAPANESE FLOUNDER (*PARALICHTHYS OLIVACEUS*)

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Japanese flounder (*Paralichthys olivaceus*) is a teleost fish that has a XX(female)/XY(male) sex determination mechanism. The genetic females (XX) can be sex-reversed to phenotypic males by rearing the larvae at high water temperature or by treatment with androgens during the sex differentiation period. Therefore, we suggest that the flounder provides an excellent model to elucidate the mechanism of sex differentiation. In order to elucidate the role of androgen receptor (AR) in the sex differentiation in Japanese flounder, we isolated AR cDNA from the gonads, analysed the mRNA expression by RT-PCR, and examined the effect of flutamide (AR antagonist) on the gonadal sex differentiation. As a result, AR mRNA was expressed in the gonads before the onset of sex differentiation. Treatment of the genetic females with flutamide dose-dependently inhibited their masculinization, which is normally induced by rearing the larvae at high water temperature, suggesting the importance of AR in sex differentiation in the flounder.

SPERM TRANSPORTER IN AVIAN FERTILIZATION

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This study was done to elucidate the fertilization mechanism in quail *Coturnix japonica*. The infundibulum of the avian oviduct is cytologically divided into three segments, infundibulum 1 to 3, from anterior to posterior. We found a noble structure in the infundibulum 3 at the bottom of epithelial folds. It is a round structure of about 100 μm in diameter and its interior is arranged in concentric layers of composites. Since sperm are present on the structure, we tentatively labeled it a sperm transporter. There are 2 types of sperm transporters. They are the same but some transporters are surrounded by needles and look like a hedgehog. An X-ray analysis showed that the needles are composed of calcium carbonates. We noted a hole, the diameter of which is about 15 μm, in the vitelline membrane of a fertile egg in the uterus. At the hole, the sperm transports and sperm were always observed. We speculate from these observations that the sperm transporters adhere to the vitelline membrane when the egg is ovulated in infundibulum 3, release the sperm and help it penetrate the membrane, and finally close the hole the sperm made.

MODES OF SPERM PASSAGE THROUGH ZONA PELLUCIDA IN *SUNCUS*

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Suncus spermatozoa had a giant, flattened, shield-shaped head with a giant acrosome and a nucleus, and were characterized by the presence of a conspicuously long middle piece, its outer dense fibers arranged in a horsehoe fashion and well-developed satellite fibers found in association with the inner aspect of fiber Nos. 5 and 6. *Suncus* cumulus oophorus was ovulated as a compact, matrix free ball of cell linked by specialized junction. *Suncus* spermatozoa passed through the cumulus cell layer, reaching to the perizonal space. The acrosome-reacted spermatozoa bound by the tip of the apical body to the zona pellucida. The long middle piece had remarkably asymmetrical flagellar waves. On the other hand, the zona pellucida displayed a looser sherbet-like outer region and a denser inner region, and a bilayer organization of the zona was recognized by the binding pattern of WGA, which had an affinity for only the outer layer. The outline of the zona pellucida of the unfertilized egg had disappeared after exposure to trypsin. Such a morphological feature of the zona pellucida seems to provide a key with which to accelerate penetration of the motile spermatozoon adhering to the zona.

ATTEMPTS OF A MASS PRODUCTION OF THE RECOMBINANT VITELLINE-COAT LYSINS OF *TEGULA PFEIFFERI* USING *ESCHERICHIA COLI* AND *ASPERGILLUS ORYZAE*.

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The vitelline-coat(VC) lysins of marine Mollusk of the genus *Tegula* are released from acrosome of sperm in ferterization and can lyze the VCs of only the same species. The cDNA sequences of VC lysins from 12 species of the *Tegula* genus have already been determined by our group, and Hellberg and Vacquier.These sequences were very similar to each other. In Japanease teguline 4 species, the VC lysin cDNA sequences of *T. lischkei, T. carpenteri* A and B differ only 3 amino acid sites, 4 sites and 6 sites from the cDNA sequence of *T.pfeifferi*. Moreover, the X-ray structure of abalone (*Haliotis rufescens*) lysin has been revealed using natural product, but X-ray analysis of teguline lysin is very diffecult on use of natural protein. In order to enable the determination of the amino acid site(s) carried species-specificity and X-ray analysis, we tried a mass production of the recombinant VC lysins of *T.pfeifferi* using *Escherichia coli* and *Aspergillus oryzae*. These results will be reported on 2 expression systems.

THE EFFECT OF cGMP ON THE ACROSOME REACTION OF STARFISH SPERMATOZOA

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In the starfish, *Asterias amurensis*, three components of the jelly coat of eggs, i.e. ARIS, Co-ARIS and asterosap, act in concert on sperm to trigger the acrosome reaction. Experimentally, ARIS and asterosap are enough for the induction of acrosome reaction. However, when sperm are treated only with ARIS or only asterosap, sperm become irresponsive to the egg jelly. Asterosap causes a rapid and transient increase in the intracellular cGMP through the activation of the asterosap receptor, guanylate cyclase. When sperm are treated only with asterosap, the guanylate cyclase is irreversibly inactivated after a transient cGMP production. Recently, we have discovered that if sperm are treated only with ARIS, the intracellular cGMP elevation by following asterosap treatment is similarly suppressed. On the other hand, if sperm are pretreated with IBMX or zaprinast, inhibitors for phosphodiesterases, sperm undergo the acrosome reaction, even after asterosap treatment, in response to the egg jelly or ARIS alone. These results suggest that the preservation of the intracellular cGMP level is essential for the acrosome reaction induced by egg jelly components.

CHARACTERISTICS OF NON-MAMMALIAN SPERM FACTOR WHICH INDUCE EGG ACTIVATION

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During fertilization, calcium (Ca^{2+}) transient takes place prior to egg activation, which is a common phenomenon of mammalian and non-mammalian oocytes. Recently, mammalian sperm factor was characterized as new type of phospholipase C (PLC), PLC zeta (Saunders C.M. *et al*, 2002, Development 129). After active PLC was introduced by the fertilizing spermatozoon into the oocyte, it was involved in producing inositol 1,4,5-trisphosphate (IP_3) which released Ca^{2+} from Ca^{2+} pools in the oocyte through IP_3 receptors. However, the mechanism of non-mammalian oocyte activation by fertilizing spermatozoon is still unclear. The intracytoplasmic sperm injection (ICSI) is a usuful method for the characterization of sperm factor. It does not lose nor inactivate any factor(s) during preparation. I have suceeded in ICSI experiment of ascidian, *Ciona savignyi*. Injection of sperm from heterogonous ascidian induced similar calcium transient and oscillations as homologous one. Injection of sea urchin sperm into ascidian oocyte also induced calcium transient but was incomplete one. The result indicates that non-mammalian sperm have common factor(s) which induce Ca^{2+} transient in heterologous oocytes.

3D-STRUCTURE OF THE SPERM-ACTIVATING AND -ATTRACTING FACTOR (SAAF) FROM THE ASCIDIAN, *CIONA INTESTINALIS*

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Formerly, we purified the sperm-activating and -attracting factor (SAAF) from eggs of the ascidian, Ciona intestinalis, and identified it as a novel sulfated steroid, 3,4,7,26-tetrahydroxycholestane-3,26-disulfate (Yoshida et al. PNAS 99, 14831). In this study, in order to confirm the sulfated steroid really has the SAAF activities and identify 3D-structure of SAAF, we synthesized SAAF. Synthesis of SAAF and its C-25 epimer (25S- and 25R-SAAF) was performed from chenodeoxycholic acid. The ^1H NMR spectra of the synthetic samples were compared with that of the natural SAAF. Whereas the chemical shifts of 27-methyl group and H-26a in 25R-SAAF are unmatched with those of the natural SAAF, those of 25S-SAAF are identical with SAAF including other portion of the steroid framework. Therefore, structure of SAAF is determined as (3R,4R,7S,25S)-2,4,7,26-tetrahydroxycholestane-3,26-disulfate. 25S-SAAF (native SAAF) could activate and attract the ascidian sperm at 3.7 - 10 nM.

It is noteworthy that 25R-SAAF (SAAF epimer) possess the same two activities as 25S-SAAF. Thus, epimerism at C-25 is no effect on the SAAF activities.

THE ACROSOME REACTION-INDUCING SUBSTANCE ON THE VITELLINE ENVELOPE IN *XENOPUS LAEVIS*

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Although the acrosome reaction (AR) in sperm is indispensable for fertilization of many animals, its molecular mechanism remains to be investigated in amphibians. To identify an acrosome reaction-inducing substance in *Xenopus* (ARISX), a monoclonal antibody against ARISX was raised by immunization with a pars recta extract (PRE) containing ARISX. The anti-ARISX antibody which recognizes the carbohydrate moiety inhibited the AR induced by PRE or on the VE in a dose-dependent manner. The ARISX in PRE was lost the AR-inducing activity by periodate oxidation. The treatment of PRE with several lectins precipitated ARISX but SBA and DBA inhibited the activity of ARISX. The activity of ARISX was not inhibited by the treatment with several proteases. These results indicate that terminal GalNAc residues in ARISX are impotant for the acrosome reaction-inducing activity.

SEA URCHIN SPERM FLAGELLA CONTAINS GLYCOPROTEIN WITH α2→9-LINKED POLYSIALIC ACID CHAINS

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Previously, we showed enrichment of a sulfated ganglioside HSO_3→8Neu5Acα2→8Neu5Acα2→6GlcCer in membrane microdomains of sea urchin sperm. We also found that the HSO_3→8Neu5Ac structure was expressed on certain glycoproteins of sea urchin sperm. To elucidate the structure of HSO_3→8Neu5Ac-containing glycans, the major sialic acid-containing glycopeptide fraction (designated SGP) was prepared from sea urchin sperm by exhaustive Actinase E digestion. The structure of glycan chains of SGP was determined by various chemical analyses and established as follows: HSO_3→8Neu5Acα2→9(Neu5Acα2→9)$_{n-2}$Neu5Acα2→6GalNAc1→Ser/Thr (n = about 15). This novel polysialic acid chain was shown to be localized on sea urchin sperm flagella. This suggests the involvement in sperm activation or sperm motility at early stages of fertilization rather than in acrosome reaction or sperm-egg fusion. This is the first evidence for the occurrence of polysialic acids in animal sperm.

THE APOPTOTIC SIGNAL FROM SEA URCHIN SPERM DURING FERTILIZATION

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We studied the interaction between egg and sperm of sea urchin, *Anthocidaris crassispina*. After treatment on ice more than three hours, the unfertilized eggs lost the capacity of fertilization, but were able to bind the sperm. This new egg model, "cooled egg", is available to observe adhesion of sperm to egg surface using fluorescent dyes. We used as a fluorescent probe annexin V-FITC which reacted with phosphatidyl serine translocated to the cell surface in the early stages of apoptosis. Some of the sperm adhered to the surface of the cooled egg showed the fluorescence of annexin V-FITC. The cooled egg or sperm alone did not show any signal of the fluorescence. Presence of jelly around cooled egg did not affect the fluorescence of the sperm. We discuss the meaning of the apoptotic signals of the sperm during fertilization.

ANALYSIS OF TCBP-25 (*TETRAHYMENA* CA^{2+}-BINDING PROTEIN OF 25 kDa) DURING SEXUAL REPRODUCTION IN *TETRAHYMENA THERMOPHILA*

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TCBP-25 is localized around both the migratory and stationary gametic pronuclei at the pronuclear exchange stage during conjugation. The function of TCBP-25 during sexual reproduction was not elucidated. To understand the function of TCBP-25, we analyzed the localization of TCBP-25 in conjugation mutants and executed the reduction in gene expression of TCBP-25, and then analyzed the phenotypic effects. According to the localization of this protein and its timing, three possible roles of TCBP-25 are proposed. TCBP-25 plays roles in 1) the distinction between the two functional gametic pronuclei and the other three degenerative nuclei, 2) the migratory pronuclear exchange and 3) the pronuclear fusion. To verify these hypotheses, the localization of TCBP-25 in conjugation mutants (*cnj10, cnj7* and *bcd2*) was examined. The results suggest that TCBP-25 should play a role in the migratory pronuclear exchange. We succeeded in the reduction in gene expression of TCBP-25 using the antisense ribosome system, and analyzed the phenotype of the transformants. The knock down of TCBP-25 function equally suggests that TCBP-25 takes part in the migratory pronuclear exchange.

MULTIPLE FORMS OF CATHEPSIN L IN *HEMICENTROTUS PULCHERRIMUS*

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Previously we purified cathepsin L-like proteinase in the egg of sea urchin, *Hemicentrotus pulcherrimus*, as a MYP-degrading enzyme. As the participation of other proteinases was suggested, developmental changes of proteinase activity and zymographic pattern were examined. Cathepsin L-like activity was decreased after fertilization and increased again at and after MBT. Zymography showed that 32kDa activity was dominantly contained in the unfertilized eggs. Interestingly 40-42kDa activity was transiently increased at blastula stage and ovary extract dominantly expressed about 50-52kDa activity. These results indicated the presence of multiple forms of cysteine proteinases and they were differently used depending on tissues and developmental stages. Therefore, we investigated the mRNAs encoding cathepsin L-like proteinases in the total RNA of ovary of H. pulcherrimus, by RT-PCR with degenerate primer sets. Partial DNA sequence analyses revealed the presence of five different kinds of cathepsin L cDNA fragments. His residue in the active site and all 4 Cys residues in the fragments were conserved. These cDNAs are homologous to that of human pro-cathepsin L.

AN INHIBITOR FOR H^+/K^+-ATPase DISRUPTED THE LEFT-RIGHT ASYMMETRY IN THE ECHINOID EMBRYO

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The molecular basis in establishment of left-right asymmetries has been studied mainly invertebrate development. In chick and frog, it was reported that specification of left-right asymmetry is initiated by a differential ion flux created by H^+/K^+-ATPase activity in very early developmental stages. We treated embryos of the sea urchin *Hemicentrotus pulcherrimus* with an inhibitor of H^+/K^+-ATPase, and found that the sided pattern of an asymmetrically expressed gene was disrupted. Our results suggest that H^+/K^+-ATPase has a role in establishment of LR patterning in echinoid embryos.

A SEA URCHIN *HEMICENTROTUS PULCHERRIMUS* HOMOLOGUE *HpSu(H)* IS INVOLVED IN THE DIFFERENTIATION OF SECONDARY MESENCHYME CELLS

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Suppressor of hairless (Su(H)) is a key component of the Notch signalling. It is a transcription factor that acts as a repressor in the absence of Notch signal. If Notch signal is activated, it binds the released intracellular domain of Notch and acts as an activator of transcription. Notch signal mediates communication between adjacent cells and is employed for binary fate decisions. In order to examine the role of Notch signalling in sea urchin development, first we identified the sea urchin *Hemicentrotus pulcherrimus* homologue of Su(H). *HpSu(H)* mRNA exists from unfertilized egg to hatched blastula stage, then increases significantly after mesenchyme blastula stage. To understand the function of HpSu(H) we designed the experiment to perturb the embryo by inducing ectopic overexpression of dominant negative form Su(H), which does not bind DNA but interacts with Notch, by injecting mRNA to fertilized eggs. The overexpression of dominant negative Su(H) specifically repressed the differentiation of secondary mesenchyme cells (SMCs) such as pigment cells and blastocoelar cells. This result suggests that Su(H) is involved in the differentiation of SMCs in sea urchin development.