### ANNEXIN IX PARTICIPATES IN THE DETERMINATION OF TIMING OF PCD

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Anterior silk gland (ASG) of the silkworm, *Bombyx mori*, undergoes programmed cell death (PCD) during pupal metamorphosis, which is triggered by 20-hydroxyecdysone (20E). To identify the 20E-inducible genes that could be involved in the PCD, we performed subtraction PCR using the ASGs incubated with or without 20E *in vitro* and obtained seven novel genes, one of which was annexin IX. Annexins are conserved throughout animals, but their precise functions remain to be seen. In the cultured ASGs, 20E up-regulated the annexin IX gene. By contrast, developmental profile of its expression in the fifth instar showed that annexin IX mRNA level was high when the ecdysteroid titer was low, and decreased with an increase in the titer occurring concomitantly with the time when the ASGs began the PCD sequence in response to ecdysteroid. The expression profiles of annexin IX were different between tissues, depending on whether they undergo PCD or not i.e., tissues destined to die initiated the PCD sequence after annexin IX was down-regulated. These results suggest that annexin IX participates in determining the timing of PCD.

### APOPTOSIS INHIBITING FACTOR PRODUCED BY *BOMBYX* ANTERIOR SILKGLANDS

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The anterior silk glands (ASG) of the silk worm, *Bombyx mori*, undergo programmed cell death (PCD) during pupation. The PCD is induced by 20-hydroxyecdysone (20E) in vitro. The glands first exhibit their responsiveness to 20E in late day 5 through early day 6 of the fifth instar. The glands before day 5 did not respond to 20E. Surprisingly, the conditioned medium after culturing day 5 ASGs inhibited the PCD of day 7 ASG that is competent to respond to 20E in vitro. The conditioned medium lost the inhibiting activity by the heating at 60 degrees for 5 min. Timed application of JH analogue (JHA) to the last instar larvae showed that the death commitment might occur between day 4 and 5. This timing is 1 day before the acquisition of 20E responsiveness. The inhibiting factor may account for the 1 day difference between the commitment and responsiveness. Silkworm hemolymph contains protein, called 30k protein, which possesses apoptosis inhibiting activity. We will discuss the connection of our newly found factor with 30k protein.

### AN APOPTOTIC PATHWAY INDUCED BY OVEREXPRESSION OF *DROSOPHILA KELCH2*

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*Drosophila kelch2* gene was identified as a gene encoding a novel Cortactin-binding protein, which contains BTB(broad complex, tramtrack, bric-a-brac)/POZ (poxvirus, zinc finger) domain-like structure in the predicted N terminus and "Kelch repeats" in the predicted C terminal domain. We reported, last year, the cytoplasmic Kelch2 protein was concentrated at cell-cell junction site in the imaginal disc epitherial cells, and the overexpression of *kelch2* induced apoptosis in the imaginal disc epitherium. Here, in order to elucidate whether *kelch2*-induced apoptosis is mediated by caspase or JNK pathway, we examined the rescuing activity of *baculovirus p35* gene, *Drosophila inhibitor of apoptosis protein 1* gene, known to inhibit caspase activation, or dominant negative form of *Drosophila JNK* gene. Using dpp-GAL4 driver, the coexpression of *kelch2* and each of these genes could prevent apoptosis, whereas the coexpression induced by GMR-GAL4 driver could not. Comparing these results, we will discuss the regulating pathway for kelch2-induced apoptosis.

### APOPTOSIS OF THE ORAL EPITHELIUM IN RELATION TO ADENOHYPOPHYSIAL DEVELOPMENT

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To investigate the possible involvement of apoptosis in the developing adenohypophysis, Rathke's pouch of 12.5-15.5 day old rat fetuses were examined either by the TUNEL method or immunohistochemistry with the use of an antibody to caspase 3. Apoptotic cells were few in number on day 12.5 when Rathke's pouch was still open to the oral cavity. On day 13.5, a high incidence of apoptosis was demonstrated in the oral ectoderm corresponding to the site of closed Rathke's pouch. Cell death was also observed along the median line of the oral epithelium beginning from the closed pouch to the rostral end of the maxillary process. The incidence of apoptotic cells was variable among different fetuses in the epithelial stalk whose degeneration is known to contribute to the complete separation of Rathke's pouch. After day 14.5, only a small number of apoptotic cells were observed. These results are discussed in relation to the hypothesis that cells undergoing apoptosis migrate from the anterior neural ridge but they are excluded without entering Rathke's pouch.

### ON THE CHORION PROTEASE PRODUCED DURING THE HATCHING PROCESS OF QUAIL

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It is known that in quail *Coturnix japonica* the limiting membrane of the shell membrane decreases in width from 74 nm on Day 3 of egg incubation to 35 nm on Day 10 during the hatching process. This study was done to find agents that affect the limiting membrane. Zymography tests on the extracts from extraembryonic tissues, yolk sacs and/or chorioallantoic membranes showed proteolytic activities during Days 4 to 10. Localization studies of these activities, performed on Day 5 eggs, indicated that they were located in an avascular chorion. Electron microscopic studies showed there were secretory cells specifically located in the avascular chorion. After partial purification of extracts of the Day 5 chorion through QA52, Sephadex G200 and arg-Sepharose column chromatographies, a single proteolytic activity of 20 kD was isolated, which we named the chorion protease. The chorion protease had an optimum pH of 8.5 and was inhibited by bestatin. The enzyme digested the limiting membrane. It was concluded that it is the chorion protease secreted from the avascular chorion that affects the limiting membrane.

### VIDEO MICROSCOPY ON THE FUNCTIONAL DEVELOPMENT OF CARDIA BIFIDA OCCURRED IN CAUTERIZED EMBRYOS OF A TELEOST, *ORYZIAS LATIPES*

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Functional development of embryonic heart of *Oryzias* in association with its three-dimensional anatomy is not yet closely analyzed, and contribution of functional development of the embryonic heart to the establishment of embryonic circulation needs further study.

When the anterior end of the embryos at the embryonic bud stage was cauterized, cardia bifida occurred as V-shaped structure at the ventral side of the embryo, each arm of which is blind tube, which subsequently changes into balloon-shaped. Conformational change in the contractile cardia bifida at an interval of 1/90 second was analyzed from still images of the heart in each frame of video records. This revealed peristalsis in these straight tubes; their rhythm was not synchronous. Peristalsis was also observed in balloon-shaped cardia bifida on several occasions; their synchronicity was not confirmed. Development of perfect three-dimensional anatomy in embryonic heart seems not necessary to induce peristalsis in *Oryzias*.

Embryonic blood circulation was not observed when cardia bifida occurred; this must be as due to lack of communication between cardia bifida and vitelline vein.

### THE ROLE OF ACTIN IN THE ANCHORING OF SPINDLE POLE IN GRASSHOPPER NEUROBLASTS

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The grasshopper neuroblast (NB) repeats asymmetric division to produce small ganglion mother cells (GMC). To perform the asymmetric division, it is necessary that the spindle pole anchors to the GMC-side cortex at late anaphase. After the anchoring of the spindle pole, the contraction of the GMC-side cortex distributes different amount of cytoplasm to the two daughter cells. The electron microscopic observation disclosed that, a small contraction other than a contractile ring appeared at the GMC-side cortex. This seemed to be a structure to grip the GMC-side spindle pole at the beginning of late anaphase. By using a fluorescence dye (rhodamine conjugated phalloidine) a contractile ring was clearly stained in the cleavage furrow area, and at the same time, fibrous actin was detected on the GMC-side cortex apart from the furrow region. The structure of a grip, the small contraction, in GMC-side cortex was located in the actin-detected area. These results suggest that the presence of the actin networks in GMC-side cell cortex may be closely related to the anchoring of the spindle body in unequal NB cytokinesis.

### THE CHANGE OF THE DIVIDING AXIS IN GRASSHOPPER GANGLION MOTHER CELLS

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Grasshopper neuroblasts (NBs) divide along the dorso-ventral axis, producing ganglion mother cells (GMC) through asymmetric cell divisions. By using indirect

immunofluorescence staining, α-tubulin in a spindle body, and γ-tubulin in centrosomes were detected in NBs and GMCs. In NBs, centrosomes were located in the ventral side of nucleus until early prophase. One of the two centrosomes migrated to the dorsal side of the nucleus. At prometaphase, a spindle body was formed along the dorso-ventral axis. On the other hand, GMC centrosome was located at the dorsal side of the nucleus until early prophase, and was divided in two. One of the centrosomes remained in the dorsal side and the other one migrated to the ventral side of the nucleus. At prometaphase, a spindle body was formed along the dorso-ventral axis, as observed in NB. At metaphase, however, the spindle axis turned for 90° to the perpendicular position against the NB dividing axis. These results suggest that the spindle axis at the early stage of spindle formation in GMCs is determined by the position of NB centrosomes, while the final dividing axis is regulated by some other factors.

**INCREASE OF TYROSINE HYDROXYLASE-POSITIVE CELLS AND NT-4 PROTEIN IN THE FOREBRAIN OF RAT EMBRYOS FROM PROPYLTHIOURACIL-TREATED DAMS**

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Thyroid hormone is essential for embryonic brain development. We administrated propylthiouracil (PTU), a thyroid hormone synthesis inhibitor, to pregnant rats on 13.5 days gestation. Embryos were removed after 24 hours and the brains were examined immunocytochemically. The tyrosine hydroxylase-positive cells were fewer in the diencephalon of control embryos than embryos from PTU-treated dams. The brains were subdivided into the forebrain, midbrain, hindbrain and region containing dorsal root ganglion (DRG) and concentrations of neurotrophins in each dissected tissue were measured using two-site enzyme immunoassay system. NT-4 protein was lower in the forebrain and hindbrain of control embryos than embryos from PTU-treated dams, while it was not different in the midbrain and region containng DRG. As to concentrations of NGF, BDNF, and NT-3, there was no difference in all dissected regions between control and the experiment.

**CHROMOSOMAL STATUS OF EMBRYOS GENERATED BY TRANSPLANTATION OF CULTURED CELL NUCLEI FROM THE CAUDAL FIN TO NON-ENUCLEATED OOCYTES OF MEDAKA**

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The potential of nuclei from actively proliferated cells in primary cultures for reprogramming and development have been investigated. Cells from the caudal fin of adult fish carried the green fluorescent protein (GFP) gene were cultured for 3-4 days and transplanted into non-enucleated oocytes. The orange-red variety of the medaka was used as donor and recipient fish. Out of 620 operated eggs 190 (31%) developed to blastula stage and two of them hatched out. All nuclear transplant embryos and fry were characterized by a reduced level of GFP expression compared with that of control embryos. Twenty GFP positive nuclear transplant embryos with minor abnormalities in the body formation were used for chromosome examination. They consisted of mixed cell populations of different ploidy. Three groups of nuclear transplant embryos were obtained: haploid-diploid, haploid-triploid and haploid-diploid-triploid. The part of triploid cells did not exceed 53%. These data suggest that donor and recipient nuclei fused at least after the first division of cleavage. These embryos were characterized as chimeras of cells with the different ploidy.

**FERTILE AND DIPLOID MEDAKA GENERATED FROM BLASTULA AND 4-SOMITE-STAGE EMBRYONIC CELLS BY NUCLEAR TRANSPLANTATION TO NON-ENUCLEATED OOCYTES**

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Fertile and diploid nuclear transplants were generated by using nuclei from blastula and 4-somite-stage embryonic cells as donors for transplantation in medaka (*Oryzias latipes*). Two series of experiments were made. In the first experiment, nuclei from blastula cells were transplanted into non-enucleated eggs. Out of 1722 transplanted eggs 26 fish survived to the adult stage. Three of them were diploid and fertile. In the second experiment, dissociated cells from 4-somite-stage embryos were transplanted into non-enucleated oocytes. Three out of 1688 operated eggs were reached maturity. One of them was fertile and diploid. The donor nuclear markers of melanophores in the first experiment and GFP fluorescence in the second experiment were transmitted to F1 and F2 offspring by Mendelian fashion.The mechanisms leading to generation of the diploid nuclear transplants are under discussion. This observation that nuclear transplant fish could be obtained without enucleating the recipient eggs, may have an important implications for nuclear transplantation in medaka.

**PLOIDY ESTIMATION OF THE PLANARIAN BY DNA MICRODENSITOMETRY**

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An asexual clonal population (OH strain) of the planarian, *Dugesia ryukyuensis* is known to be triploid. The worms lack sexual organs and undergo fission. However, if they are fed with sexually mature worms of the planarian *Bdellocephala brunnea*, they are sexualized and produce fertile sperm and eggs. Although mitotic cells so far examined were all triploid even after sexualization, typical bivalent meiotic images were observed in gonads of sexualized worms. This fact suggests that both eggs and sperm are haploid and derived from diploid primordial germ cells. In planarians, only totipotent stem cells named neoblasts are believed to be mitotic. However the mitotic index of the worms were only few percent even during regeneration. Therefore, we hypothesized that besides triploid cells, diploid interphase cells might exist in the asexual triploid OH worms. In order to estimate the ploidy of non-mitotic and non-meiotic cells, we measured DNA contents in the cells by microdensitometry.

**STANDARDIZED *SILURANA TROICALIS* MAINTENANCE———FOOD**

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Use of *S. tropicalis* has only just recently begun. When using amphibian tadpoles to screen for endocrine disruptors, because most endocrine disruptors contain estrogen activity, care must be taken to eliminate estrogen activity containing substances, not only from water and tray, but also from the diet itself. The present experiment investigates the effects of low estrogen or estrogen-free diets on *S. tropicalis* tadpole development and growth. NF stage 45 tadpoles were fed Sera micron (group 1), boiled spinach (group 2), casein-based formula "No. 5" containing no soybean or fish meal (group 3), and No. 5 plus an additional amount of poly unsatsurated fatty acid (PUFA). Food levels were adjusted to allow normal tadpole development and maximum growth. After one week, group-1 tadpoles showed the greatest total length, group-2 tadpoles the least, with group-3 and -4 tadpoles intermediate. It was concluded that a diet of Sera micron enhances the development and growth of *S. tropicalis* tadpoles, and should be considered an important food for standardized *S. tropicalis* tadpole maintenance in endocrine disruptor research.

**THE EFFECT OF HIGH GRAVITY ON AMPHIBIAN DEVELOPMENT**

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The present study was conducted to help clarify the effects of high gravity environments on amphibian development. Uncleaved Xenopus laevis eggs at 10 and 20 minutes after insemination, and embryos at cell stage 2 and at the gastrula stage were raised in 2G and 5G for 4 days. Controls were raised in normal gravity. Programmed cell death (PCD) was analyzed for body surface and inner structures of developing embryos using a more sensitive in situ endolabelling technique that identifies fragmented nuclear DNA in dying cells. PCD increased in embryos treated to high gravity. PCD abnormalities were not seen in the gastrula stage, but were first observed at the neurula stage. Under 5G conditions, serious abnormalities were observed in all embryos starting treatment before cell stage 3. Abnormal PCD was observed in various parts of the body that included microcephally, eye deformities, contortion of the notochord and incomplete gastrulation. Embryos treated at 2G appeared more normal than those undergoing 5G, although hatched tadpoles showed a higher number of dying cells in the surface compared to controls.

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**AN ANATOMICAL AND PHYSIOLOGICAL STUDY OF THE HEART DURING LARVAL DEVELOPMENT OF THE SHRIMP *METAPENAEUS ENSIS***

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We studied anatomical and physiological changes in the heart during development of a shrimp, *Metapenaeus ensis*. The heart is first seen as a globular structure located in the cephalothorax when it begins to beat at the end of the naupliar stage 6. In the mysid stage 1, a swelling appears adjacent to the posterior end of this original heart. This develops into a second, more muscular pumping structure (equivalent to the adult heart) while the original heart is reduced gradually during mysid stages and disappears completely in the following postlarval stages. We recorded intracellularly from cardiac muscle of both mysids and postlarvae. In mysids each cardiac muscle potential included a slow depolarization phase. The application of saline containing TTX did not stop either the cardiac muscle potentials or heart beats. In postlarvae,