Abstracts of the presentations selected for 2007
JSAR Outstanding Presentation Award

Metastin may Induce Apoptosis of Luteal Cells *via* Gonadotropin Releasing Hormone in the Corpus Luteum of Rats

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ABSTRACT. Corpora lutea formed during the estrous cycle regress without significant production of progesterone in rats. Once uterine cervical stimulation is given on proestrus (P), twice daily prolactin (PRL) surges are induced and the corpus luteum becomes functional. We have already reported that gonadotropin releasing hormone (GnRH) and annexin 5 (AX5, a biomarker of GnRH signal) expressed in corpus luteum and local GnRH is involved in the luteal regression. Recently, it has been reported that metastin, the ligand of GPR54, induces GnRH release in the hypothalamus. We investigated, in this study, a putative functional relationship among luteal metastin, GnRH, AX5 and luteolysis. Cycling Wistar-Imamichi rats were used. Corpora lutea were harvested at 1000 h and 1700 h of each day of estrous cycle. Expression levels of mRNA were measured by real-time PCR respectively. All mRNA levels of metastin, GnRH and AX5 were significantly high from the afternoon of D2 to the afternoon of P. When PRL (10 IU/head) was administrated twice daily from estrus, the expression of metastin, GnRH and AX5 mRNA were inhibited at 1700 h of D2. When metastin was administrated into the ovarian bursa using an osmotic mini pump (10^{-6} M/μl/h) from five days after uterine cervical stimulation, the mRNA levels of metastin, GnRH, AX5, FasL and Fas were significantly augmented after two days. TUNEL (TdT-dUTP nick end labeling) analysis showed apoptotic cells in the corpora lutea. Corpora lutea harvested at 1000 h of D2 were cultured in the medium containing metastin with/without Cetrorelix (GnRH antagonist) under the condition of 40% O_2 and 5% CO_2. Metastin (10^{-7} M) stimulated the expression of luteal metastin, GnRH, AX5, FasL and Fas mRNA levels during 6 h incubation, while simultaneous treatment of Cetrorelix (10^{-7} M) with metastin blocked the augmentation of metastin, GnRH, AX5, FasL and Fas mRNA expression. In addition, estrogen (10^{-10} to 10^{-8} M) given for 6 h increased metastin and GnRH mRNA levels in a dose-dependent manner. These results suggest that luteal metastin is synthesized from the afternoon of D2 to P and it stimulates GnRH expression. These change may result in the apoptosis of luteal cells. PRL is suggested to make corpus luteum functional, at least partly, by inhibiting the expression of metastin, GnRH and AX5. It could be possible to postulate that estrogen produced by a crop of new follicles could trigger a sequence of events from metastin to luteolysis.

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JSAR Outstanding Presentation Award

A Unique Role of Maternal Nucleolus in Early Mammalian Development

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ABSTRACT. Fertilization is a dynamic process of the transition from two highly specialized cell—the oocyte and spermatozoon—into the totipotent zygote. Paternal and maternal contributions to the zygote are not only introducing a half set of the nuclear DNA for the next generation. They introduced a complementary arsenal of structures and molecules necessary for fertilization, and make up for each other after fusion to be functional zygotes. In mammals, mitochondria originate exclusively from the oocyte, whereas the centriole in mammals, except rodents, is of paternal origin. Another organelle that can be found only in the oocyte is the nucleolus. The nucleoli in fully grown oocytes are compact and transcriptionally inactive, and chromatin, ribosomal RNAs and proteins are undetectable level inside of the nucleoli. It is not known how or to what extent this nucleolar material contributes to the formation of nucleoli in newly formed zygotes and early developing embryos. Considering that the oocytes have the ability to mature, to be fertilized and then to develop as embryos, we may suppose that their nucleoli (or nucleolar material) play certain roles in maturation, fertilization, and development. Thus, we remove the nucleoli from the pig and mouse GV-oocytes microsurgicaly (enucleolation), and cultured in vitro. The oocytes without nucleoli (enucleolated oocytes, ENL) progressed maturation as similar time course as control oocytes and form the pronuclei (PNs) after activation. There was, however, no nucleoli inside of both male and female PNs. Re-injection of the oocyte nucleolus into ENL oocytes restored the formation of nucleoli in both pronuclei. In contrast, injection of the nuclei of somatic cells containing nucleoli into ENL oocytes was unable to rescue the nucleolus reformation in pronuclei of zygotes derived from ENL oocytes. ENL oocytes after maturation and activation were cultured up to the blastocysts, and they hardly progressed to the blastocyst stage in both pig and mouse. Our results suggest that the oocyte nucleolus provides the molecules to pronuclei in zygotes and even to pseudo-pronuclei formed after somatic cell nuclear transfer to assemble their nucleoli. Moreover, the oocyte original nucleolar material is absolutely essential for successful early embryonic development.

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The Role of Arcuate Metastin Neurons in Regulation of Pulsatile GnRH/LH Secretion in the Female Rat

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ABSTRACT. Pulsatile secretion of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) has a critical role in stimulating follicular development and steroidogenesis to maintain normal reproductive functions in mammals. Metastin, a peptide coded by KiSS-1 gene, was found as a natural ligand for GPR54, a G protein-coupled receptor. We have been focusing on the metastin-GPR54 signaling in the brain, because accumulating evidence suggests that the signaling in the brain plays a critical role in the regulation of reproduction in several mammalian species including human, monkey and rodents. In the present study, we report that prolonged neonatal estrogen treatment abolishes pulsatile LH secretion and metastin neurons in the hypothalamus of female rats. Wistar-Imamichi strain female rats were treated with daily injections of estradiol 3-benzoate (EB) or peanut oil for 10 days after birth. The vaginal smears in the animals were daily examined after vaginal opening. The ovaries were removed and weighed when the rats reached 11–13 weeks of age. The animals were bled for 3 h at 6-min intervals to determine the pulsatile LH release. The number of the metastin-immunoreactive cells was determined in the hypothalamic arcuate nucleus (ARC) in the neonatally EB- or vehicle-treated ovariectomized (OVX) rats. Neonatally EB-treated female rats showed persistent vaginal diestrus, while the vehicle-treated controls showed regular 4-day estrous cycles after the puberty. Ovary weight in EB-treated rats was significantly lower than vehicle-treated controls. Pulsatile LH secretion was profoundly inhibited in OVX rats with neonatal EB treatment, resulting in significantly lower mean LH levels and frequency and amplitude of LH pulses compared with those in neonatally vehicle-treated controls. Few metastin-immunoreactive cells were found in the ARC in the neonatally EB-treated OVX rats, whereas many metastin- immunoreactive cells were present in the ARC of the vehicle-treated controls, and the number of the cells in the EB-treated animals was significantly lower than those in controls. The present results indicate that prolonged neonatal EB treatment reduces the number of ARC metastin neurons with inhibited GnRH/LH pulses and ovarian follicular development. Considering the stimulatory effect of metastin on GnRH/LH release, the ARC metastin neurons may play a role in generating pulsatile GnRH/LH release to maintain the ovarian function in female rats.

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JSAR Outstanding Presentation Award

Analysis of the Determinants of Meiotic and Developmental Competence in Mouse Oocytes

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ABSTRACT. Mammals have two types of full-grown oocytes: those with germinal vesicles (GVs) in which the chromatin is condensed and surrounds the nucleolus (SN type), and those in which the chromatin is less condensed and does not surround the nucleolus (NSN type). Although SN oocytes possess higher meiotic and developmental competence than NSN oocytes, the factors underlying this difference are unknown. To determine whether cytoplasmic or nuclear factors are involved, the GVs of murine SN and NSN oocytes were exchanged by nuclear transfer and the nucleus/cytoplasm of each reconstructed oocyte was classified as follows: SN/SN, NSN/SN, SN/NSN, or NSN/NSN. After reconstruction, the meiotic maturation and preimplantation development of the oocytes were analyzed. Few mature SN/NSN and NSN/NSN oocytes were observed (20–26%). In contrast, 88% of the NSN/SN oocytes matured; however, they rarely developed to the blastocyst stage after fertilization (4%), whereas most of the SN/SN oocytes matured (84%) and reached the blastocyst stage (83%). These results suggest that the differences in meiotic and developmental competence between SN and NSN oocytes are determined by factors in the cytoplasm and nucleus, respectively.

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JSAR Outstanding Presentation Award

A Novel Gonad-Specific Gene *DD2-2* Involved in Early Zygotic Gene Activation (ZGA) in Mouse Pre-Implantation Embryos

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ABSTRACT. ZGA take place at the G2 phase of the 1-cell stage in the mouse. However, its molecular mechanism has not been fully elucidated yet. Thus, we have investigated molecular functions of many gene clusters, i.e., DD clones obtained by Differential-Display analysis between the ovulated eggs at the MII stage and the 1-cell stage embryos at the G2 phase. This way, we have identified a novel gene (*DD2-2* gene), whose function was unknown and further whose DNA sequence did not match any known transcripts in the Gene Bank. Here, we report the expression profile and knockdown analyses of the *DD2-2* gene in the mouse pre-implantation embryos. We have detected specific expression of *DD2-2* gene in mouse gonads (testis and ovary) carrying out RT-PCR and Western blot analyses of mouse main tissues (the brain, lung, liver, heart, spleen, kidney, testis, ovary, and skeletal muscle). In pre-implantation embryos, *DD2-2* gene showed the unique expression profile: maternal *DD2-2* transcripts were detected in oocytes at GV and MII stages, whereas embryonic *DD2-2* transcripts in early 2-cell embryos were expressed approximately 3-fold more than that of oocytes at GV and MII stages; however, the expression dramatically decreased at the later stages and could not be detected in the stages of morulae and blastocysts. These results indicated that DD2-2 gene was specifically expressed during ZGA. Western blot analyses with polyclonal antibody against specific peptides of DD2-2 amino acids showed that the expression profile of DD2-2 protein was in parallel to that of *DD2-2* transcript, implying that the expression of *DD2-2* gene was not regulated under translational control. Furthermore, we have performed immunofluorescence cytochemical staining of DD2-2 in pre-implantation embryos using the polyclonal antibody. The results obtained showed that DD2-2 localized in both the nuclei and cytoplasms of oocytes or of embryos from GV stage to 2-cell stage at 36 hpi (hours post insemination). Further, to investigate *DD2-2* involvement in the development of pre-implantation embryos, we have examined knockdown effects of *DD2-2* gene expression using antisense technology (*pβ-act/antisense DD2-2/IRES/EGFP*) on the development of mouse pre-implantation embryo. The injected antisense expression vector in EGFP-positive 1-cell embryos significantly inhibited their development to the 2-cell and 4-cell embryos as compared with EGFP-positive embryos injected with a negative control expression vector (*pβ-act/luc+/IRES/EGFP*)(54% (71/131) vs 72% (94/130), $p<0.05$). Further, in parallel to these phenomena, the amount of DD2-2 protein synthesized in EGFP-positive 1-cell embryos or 2-cell embryos which had been injected with antisense expression vector significantly decreased at 24 hpi by 52% when compared with that of EGFP-positive 2-cell embryos injected with the negative control expression vector. Thus, these results suggest that *DD2-2* could be specifically involved in the transition from maternal gene activation to embryonic gene activation in mouse early pre-implantation embryos.

Abstracts of the presentations selected for 2007
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Purification of Fish Spermatogonia Using Flow Cytometer

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ABSTRACT. The sorted population of spermatogonia is a powerful tool to study molecular events regarding gametogenesis and surrogate broodstock technology by germ cell transplantation. We previously succeeded to produce trout offspring from triploid salmon parents by germ cell transplantation. In this experiment, testicular cell suspension containing spermatogonia, together with large quantity of gonadal somatic cells and meiotic germ cell, was transplanted. Therefore, in order to improve transplantation efficiency, we purified spermatogonia showing the green fluorescence from pvasa-Gfp transgenic rainbow trout using fluorescence-dependent flow cytometer. However, the resulting offspring, derived from transgenic spermatogonia became also transgenic individuals. This fact is not favorable for aquacultural applications nor conservation usages. In this study, we purified fish spermatogonia by a flow cytometer using forward and side light-scatter from non-transgenic individuals. First, to characterize size and granularity of A-type spermatogonia we analyzed their forward light-scatter (FS) and side light-scatter (SS) using immature p*vasa-Gfp* transgenic rainbow trout possessing testes that contain only A-typed spermatogonia. FS property is an indicator of relative cell size and SS property is determined by cell shape and granularity. As a result, we found GFP-positive cell population (A-type spermatogonia) showed high FS and low SS values. Second, cell fraction showing high FS and low SS values was sorted from testicular cell suspension prepared from non-transgenic trout having only A-type spermatogonia. The resulting cell population was applied to RT-PCR analyses with primer sets for germ cell marker (*vasa*) and somatic cell marker (*Gsdf*). The population only expressed vasa but not *Gsdf*. Further, in order to check the purified cells have an ability to migrate and to be incorporated into the recipient's genital ridges, the sorted cells labeled with PKH26 were transplanted into the peritoneal cavity of rainbow trout embryos. The transplantation rate and the number of donor cells which were incorporated into the recipient's genital ridges were 82 ± 7.0% and 8.4 ± 1.5 cells/fish, respectively, in case the sorted cells were used. On the other hand, they were 29 ± 4.0% and 3.6 ± 0.6 cells/fish in case unsorted testicular cell suspension was transplanted. Further, the same sorting condition could also be applied to fishes other than rainbow trout, such as masu salmon, Japanese char and nibe croaker. Thus, we succeeded to purify A-type spermatogonia, which can be transplanted into the recipient embryos, from non-transgenic fishes.

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ISG-15 Gene Expression Patterns Derived from Peripheral Blood Leukocytes during the Pre- and Post-breeding Period in Dairy Cattle

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ABSTRACT. Early non-pregnancy diagnosis is essential to improve reproductive performance in dairy cattle. Although mobile equipment of ultrasonography has ability to diagnose pregnant cows from 25 days of pregnancy, it is difficult to diagnose them less than that stage. In recent years, however, various interferon-stimulated genes (ISGs) have been shown to express in the endometrium in response to the secretion of interferon at the time of pregnancy recognition in the ruminant. Increase in the expression of ISG-15, one of the ISGs, in peripheral blood leukocytes (PBL) of pregnant cows has also been reported. To clarify whether monitoring survivability of the embryos aged at less than 23 days is possible or not, we compared mRNA expression patterns of ISG-15 in PBL before and after breeding between pregnant and non-pregnant animals. A total of 13 animals, comprising of 12 Holstein and Jersey cows and one Holstein heifer, were used. All the animals were either inseminated on day 0 (day of estrus) or day 1, or served as a recipient for non-surgical embryo transfer on day 7 or 8. Blood was taken on day 0 and every other day from days 12 to 22. Additional blood samples were collected from two of the pregnant animals every other day from days 22 to 30 and day 50. All the samples were collected from the coccygeal vein into vacuum EDTA-2K tubes. PBL was separated from 1 ml of whole blood within six hours after the collection, and was stored at –80 C until total RNA extraction. cDNA was synthesized based on the extracted total RNA, and the mRNA expression of ISG-15 in PBL was examined by RT-PCR. Pregnancy was confirmed by ultrasonography either day 24, 25 or 26. An ISG-15 specific PCR product was observed as a clear 87-bp band in the samples collected from days 18 to 22 or from days 20 to 22 in seven out of the 13 animals. All of these seven were confirmed to be pregnant and the other six animals were confirmed to be non-pregnant by ultrasonography. The clear band was also shown in the samples collected from days 24 to 30 in the two pregnant animals. In contrast, the clear band was not detected consistently in the samples collected from days 0 to 16 in all the 13 animals, and no band was observed on day 50 in the two pregnant animals. In conclusion, the results of our study suggest that it is possible to monitor the survivability of bovine embryo by examining the expression of ISG-15 mRNA in PBL taken serially from days 18 to 22, and that a clear 87-bp band is not detected before day 15 and after day 50.