title: „Czech-Japan Joint Symposium for Animal Reproduction“

subtitle: „From Gametes to Stem Cells“

date: **20th - 21st September, 2010**

venue: **Conference Centre AV CR, Chateau Liblice, Liblice 61, 277 32 Bysice, Czech Republic**

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Financial support:

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Dear Participants,

Welcome to the Czech-Japan Joint Symposium for Animal Reproduction under the title “From gametes to stem cells”.

Liblice castle, now the Conference Centre of the Czech Academy of Sciences, was traditional venue for animal reproduction meetings. I presented my first slides with maturing pig oocytes just here. So our symposium logically continues in this line. But why the castle silhouette on the poster is bellow “Fuji san”, asked me several friends? All basic knowledge in the field of physiology of reproduction flows into an essential concept of embryonic stem cells. Nearly two decades of concentrated effort focused to the mouse and human embryonic stem cells clarified several principles of mammalian development. However, only the pioneering studies coming from Kyoto University showed a new way to dedifferentiation of somatic cells. A unique property of mammalian egg, first demonstrated by Dolly, has been now proved under in vitro conditions. Professor Shiny Yamanaka’s results have brought our dreams about stem cell therapy much closer to the reality. The most beautiful lake bellow Mount Fuji is Yamanaka ko and I wanted to bring this allegory on the poster. However, a lake is not graphically optimal, so Fujisan represents this unique Japanese achievement. The last session of the conference will demonstrate that large animal models that represent an important step on the way to regenerative medicine. I hope that numerous discussions will bring a new insight in a position of reproductive biotechnology that is now at the interface between agricultural and biomedical research.

From my unique position as the conference organizer, I want to follow the Japanese tradition in respect to old people and to remind you one colleague building the solid background of animal reproduction before us. In this year, Luboš Holý, the emeritus professor of the University of Veterinary and Pharamaceutical Sciences in Brno will be octogenarian. Luboš Holý devoted his great effort in development of all new biotechnology methods in cattle reproduction and paved the way for practical application of cattle embryo transfer, embryo micromanipulation and cryopreservation. First of all he found the clever implementation of embryo transfer for improvement of herd production. I wish you, Luboš, good health and I wish us to listen to your unique voice either in wise advices or in Moravian songs.

We will also respect the tradition of opening the conference by the key note lecture. I am very pleased that Professor Takeo Kishimoto has taken this duty. The Czech Academy of Sciences recognized his concentrated scientific effort in the field of biology of reproduction and Professor Helena Illnerova will award him the Johan Gregor Mendel medal as a symbol of pioneering research in biology.

The whole Organizing Committee wishes you a productive and inspiring meeting. Our great hope is that you will leave this beautiful castle with fresh ideas, new collaborative connections and new friendships since a human factor plays very important role in our scientific effort.

Jan MOTLIK
Head of the Laboratory of Cell Regeneration and Plasticity
Institute of Animal Physiology and Genetics AS CR, v.v.i.
Libechov, Czech Republic

Programme:

Day 1st – **20th September, 2010**	
08:00 – 09:30	Registration
09:30 – 10:00	Opening the Syposium **Josef FULKA** *Institute of Animal Science, v.v.i., Prague-Uhrineves, Czech Republic*
10:00 – 10:30	Key note lecture **Takeo KISHIMOTO** *Tokyo Institute of Technology, Japan* „The cell cycle control: Lessons from starfish oocytes“
10:30 – 11:00	Johan Gregor Mendel medal for Takeo Kishimoto **Jan MOTLIK** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* **Helena ILLNEROVA** *Academy of Science of the Czech Republic, Prague, Czech Republic*
11:00 – 11:20	Coffe break
11:20 – 11:30	Opening topic 1: Oocytes Chair-men: **Takashi MIYANO**, **Radek PROCHAZKA**
11:30 – 11:55	**Jibak LEE**, Tatsuya HIRANO *Chromosome Dynamics Laboratory, RIKEN Advanced Science Institute, Wako, Japan* „Regulation of chromosome dynamics by cohesins in mammalian meiosis“
11:55 – 12:20	**Takashi MIYANO**, Mohammad MONIRUZZAMAN *Graduate School af Agricultural Science, Kobe University, Kobe, Japan* „Activation and inactivation of primordial oocytes“
12:20 – 12:45	**Koji SUGIURA**[1,2], You-Qiang SU[1], John J. EPPIG[1] [1]*The Jackson Laboratory, Bar Harbor, ME, USA.* [2]*Laboratory of Applied Genetics, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan* „Oocyte control of granulosa cell function and development“
12:45 – 13:10	**Yuji HIRAO**, Kosuke IGA, Kenji NARUSE, Naoki TAKENOUCHI *National Agricultural Research Center for Tohoku Region (NARCT), Morioka, Japan* „*In vitro* growth of bovine oocyte-granulosa cell complexes after vitrification“
13:10 – 14:10	Lunch break
14:10 – 14:35	**Radek PROCHAZKA** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „Regulation of pig oocyte maturation by EGF-like peptides“

14:35 – 15:00	**Sugako OGUSHI**[1], Yasuko KATO[1], Akira NAKAMURA[1], Mitinori SAITOU[2] *[1]Laboratory for Germline Development, RIKEN Center for Developmental Biology, Kobe, Japan.* *[2]Department of Anatomy and Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.* „Functional analysis of maternal nucleolus in mouse oocytes“
15:00 – 15:25	**Petr SOLC** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „Aurora-A regulates MTOCs (centrosome) biogenesis but it does not trigger G2/M transition in mouse oocytes matured in vivo“
15:25 – 15:50	**Martin ANGER** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „Age-related aneuploidy in mammalian oocytes“
15:50 – 16:10	Coffe break
16:10 – 18:30	Poster session Poster competition: **Jan MOTLIK, Kazuhiro KIKUCHI**
18:30 – 19:00	Free time
19:00 – 20:30	Galla dinner
20:30 – 21:00	„**Baldrián**“, the folklore group from Pardubice, Czech Republic
21:00	Drinks

Day 2nd – **21st September, 2010**

08:30 – 08:40	Opening topic 2: Fertilization Chair-men: **Jiri KANKA**, **Naomi KASHIWAZAKI**
08:40 – 09:05	**Junya ITO**[1,2], Rafael A. FISSORE[3], Naomi KASHIWAZAKI[1,2] *Laboratory of Animal Reproduction,* *[1]School of Veterinary Medicine,* *[2]Graduate School of Veterinary Science, Azabu University, Sagamihara, Japan.* *[3]Department of Veterinary & Animal Sciences, University of Massachusets, Amherst, USA* „Factors associated with calcium oscillations during fertilization in mammals“
09:05 – 09:30	**Konosuke OKADA** *Faculty of Applied Life Science, Nippon Veterinary and Life Science University, Masashio, Japan* „Vole sperm cryopreservation: assessmants of viability, sperm DNA integrity and fertilizing capacity“
09:30 – 09:55	**Tereza TORALOVA** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „The role of CENPF in bovine preimplantation development“
09:55 – 10:20	**Helena FULKA** *Institute of Animal Science, v.v.i., Prague-Uhrineves, Czech Republic* „From oocyte to embryo – remodeling the parental genomes“
10:20 – 10:40	Coffe break
10:40 – 10:50	Opening topic 3: Stem cells Chair-men: **Ales HAMPL, Noboru MANABE**
10:50 – 11:15	**Ales HAMPL** *Department of Biology, Medical Faculty of Masaryk University, Brno, Czech Republic* „Human embryonic stem cells“
11:15 – 11:40	**Zuzana KOLEDOVA** *Department of Biology, Medical Fac of Palacky University, Olomouc, Czech Republic* „Centrosomal decisions on self-renewal in mouse embryonic stem cells“
11:40 – 12:05	**Petr VODICKA** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „iPS cells in miniature pigs“
12:05 – 12:30	**Jana JUHASOVA-HLUCILOVA** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „Bone marrow-derived mesenchymal stem for the regeneration of cartilage and bone“
12:30 – 12:55	**Helena SKALNIKOVA** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „Proteomics of neural stem cells isolated from miniature pigs“
13:00 – 14:00	Lunch break

14:00 – 14:10	Opening topic 4: Genetics manipulation Chair-men: **Josef FULKA**, **Takashi NAGAI**
14:10 – 14:35	**Atsuo OGURA**, Kimiko INOUE *RIKEN BioResource Center, Tsukuba, Japan* „Improvement of somatic cell nuclear transfer in mice: Genetic and epigenetic approaches“
14:35 – 15:00	**Masahiro KANEDA**, Tamas SOMFAI, Seiki HARAGUCHI, Satoshi AKAGI, Shinya WATANABE, Takashi NAGAI *National Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization, Tsukuba, Japan* „Normal and abnormal epigenetic changes in the development of cloned cattle“
15:00 – 15:25	**Noboru MANABE**[1], Ichiro ONOYAMA[1], Junyou LI[1], Yutaka SENDAI[2], Yoshito AOYAGI[3] [1]*Animal Resource Science Center, The University of Tokyo, Kasama, Japan.* [2]*Central Research Institute for Feed and Livestock, Zen-Noh, Tsukuba, Japan.* [3]*Embryo Transfer Center, Zen-Noh, Kami-Shihoro, Japan.* „Characteristics of prion, bovine spongiform encephalopathy (BSE) pathogen, homo-knockout cow“
15:25 – 15:50	**Michiko NAKAI**[1], Kazuhiro KIKUCHI[1], Junya ITO[2,3], Naomi KASHIWAZAKI[2,3], Hiroyuki KANEKO[1] [1]*Division of Animal Sciences, National Institute of Agrobiological Sciences, Tsukuba, Japan.* *Laboratory of Animal Reproduction,* [2]*School of Veterinary Medicine,* [3]*Graduate School of Veterinary Medicine, Azabu University, Sagamihara, Japan.* „Xenotransplantation of gonadal tissue for utilization of genetic resources in pigs“
15:50 – 16:15	**Stefan JUHAS** *Institute of Animal Physiology and Genetics AS CR, Libechov, Czech Republic* „Miniature pig as a large animal model of Huntington disease“
16:15 – 16:30	Closing of the Symposium

Key-note lecture, invited by Czech Republic

Cell Cycle Control: Lessons from Starfish Oocytes

Takeo KISHIMOTO

Laboratory of Cell and Developmental Biology, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan

Cell cycle control is a molecular system that ensures faithful duplication of the cell, and hence underlies wide range of life science from basic biology to various diseases, particularly cancer and genetic disorders. Cell cycle studies at the end of the last century have established a concept that in eukaryotic cells, the cell cycle control system is consisted of two major elements, the cell cycle engine and the checkpoint. The cell cycle engine performs progression of the cell cycle through various cyclin-Cdk (cyclin-dependent kinase) complexes, while the checkpoint is negative feedbacks that ensure correct replication and segregation of chromosomes. Based on these accomplishments, the next subject would be to elucidate how the cell cycle control system is actually regulated in various living cells.

The pattern of the cell cycle in higher eukaryotes can be divided into two types, the somatic cell mitosis and the germ cell meiosis. The meiotic cell cycle, which is specialized to bring about haploidy through two consecutive M-phases, generally arrests twice in animal oocytes: the primary arrest at prophase of meiosis I, and the second arrest at a later particular stage of meiosis until fertilization. Although the primary arrest is released by various extracellular stimuli (typically by maturation-inducing hormone), the release from the primary arrest, or “meiotic reinitiation”, is consistently equivalent to G2/M-phase transition in somatic cells. In contrast, the stage of the second arrest varies with the species: typically, metaphase of meiosis I (meta-I) in insects and ascidians, metaphase of meiosis II (meta-II) in most vertebrates, or G1 phase (pronuclear stage) after completion of meiosis II in echinoderms. But the second arrest, or “post-meiotic reinitiation arrest”, always enables mature eggs to prevent parthenogenesis and to begin development as a zygote only after fertilization. It is an essential question how each of these different meiotic arrests is executed and released, and whether there is a common molecular principle. Model organisms to analyze these arrests are mouse, frog and echinoderm starfish. Here I would like to review signalling pathways that lead to the regulation of these primary and second arrests in starfish oocytes.

The release from the primary arrest in starfish oocytes is induced by 1-methyladenine (1-MeAde), which was identified first in the animal kingdom as a physiological maturation-inducing hormone by Kanatani and colleagues in 1969. Since then, the starfish oocyte has been for more than 40 years a model system to analyze the signalling pathway that leads to meiotic reinitiation. 1-MeAde acts on the oocyte surface and eventually causes the activation of cyclin B-Cdc2 kinase, the universal trigger of G2/M-phase transition, with no requirement of new protein synthesis. In this pathway, a putative surface receptor for 1-MeAde is coupled to a heterotrimeric Gi-protein, and the released $G_{\beta\gamma}$ is suggested to activate PI3 kinase to produce PIP3, thus providing an occasion in which PDK1, PDK2 and Akt/PKB meet each other. As a result, the phosphorylated and activated Akt/PKB directly phosphorylates and inhibits Myt1, and thus reverses the balance between opposing Cdc25 and Myt1 activities towards the activation of cyclin B-Cdc2. Downstream of cyclin B-Cdc2, mitotic choreographer kinases, such as Plk1 (polo-like kinase 1), Aurora, Gwl (Greatwall kinase) and MAPK, are activated to execute meta-I. I will update on our progress in the study of 1-MeAde signallings. Although this pathway might be unique to the starfish in some aspects, the starfish oocyte is the only system in which outline of the complete pathway from the maturation-inducing hormone to cyclin B-Cdc2 has been unravelled.

After meiotic reinitiation, starfish oocytes complete meiosis and then arrest at G1 phase unless fertilization occurs. The second arrest at G1 phase in mature starfish eggs relies on the Mos-MAPK pathway. While degradation of Mos upon fertilization is necessary and sufficient to release the

G1 arrest, downstream of MAPK two separate pathways are operating to maintain the G1 arrest. One is a Rsk-dependent pathway that leads to prevention of entry into S-phase, through suppression of loading onto chromatin of Cdc45 which is a major regulator of transition from pre-RC (pre-replicative complex) to pre-IC (pre-initiation complex). And the other is a Rsk-independent pathway that leads to prevention of entry into M-phase, through suppression of synthesis of mitotic cyclins A and B. Such a "dual-lock" downstream of MAPK is necessary for G1 arrest due to lack in functional checkpoint in eggs. So far, this is the first demonstration that the Mos-MAPK cascade separates into Rsk-dependent and Rsk-independent pathways, thereby arresting the cell cycle prior to fertilization. Originally, the Mos-MAPK pathway was first identified in unfertilized mature frog eggs as an essential component of CSF (cytostatic factor) to arrest the cell cycle at meta-II. However, the role of its core component, the Mos-MAPK pathway, has expanded for various second arrests other than meta-II arrest, particularly for G1 arrest in starfish and meta-I arrest in sawfly. Based on these, we now propose a conceptual extension in the definition of CSF. CSF can be considered as a common cytostatic arrest factor, irrespective of the particular stage of arrest, in metazoan eggs which are not fertilized after meiotic reinitiation. The core of CSF is universal Mos-MAPK, downstream of which there are variable elements. The rewiring downstream of Mos-MAPK produces cytostatic arrest at a particular stage, either meta-I, meta-II or G1.

In conclusion, there should be meiosis-specific modulations in the signalling to cell cycle regulators. The starfish oocyte system provides insights into both common and unique modulations, thus contributing to the studies of cell cycle control in general.

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Lectures:

Topic 1: Oocytes

Regulation of chromosome dynamics by cohesins in mammalian meiosis

Jibak LEE and Tatsuya HIRANO

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Meiosis is a sexual reproduction process, in which haploid gametes (sperms and eggs) are produced from diploid germ cells (spermatocytes and oocytes). For the reduction of chromosome number, two successive meiotic divisions (meiosis I and II) follow a single round of DNA replication. Meiotic chromosomes display unique behaviors particularly in meiosis I: homologous chromosomes pair, synapse, and recombine with their partners during prophase I. As a result, homologous chromosomes are connected with their partners by inter-sister arm cohesion distal to chiasmata, thereby leading to the formation of bivalent chromosomes by metaphase I. At the onset of anaphase I, resolution of the inter-sister arm cohesion causes separation of homologous chromosomes while sister chromatids are kept attached at their centromeric regions. The sister chromatids eventually separate from each other when the centromeric cohesion is resolved in meiosis II.

Central to the regulation of the program of meiotic chromosome dynamics are cohesins, a class of multi-subunit protein complexes that play a main role in sister chromatid cohesion. In this presentation, we will introduce two topics about cohesins in mammalian meiosis. The first topic concerns recent advance in our understanding of the mechanism underlying the unique fashion of meiotic chromosome separation in mammals. This mechanism involves the meiosis-specific cohesin subunit REC8, a counterpart of RAD21, and its centromeric protector called shugoshin (SGO2). In mice, REC8 is localized along the inter-sister-chromatid axes at metaphase I. At anaphase I, REC8 is lost from chromosome arms, but remains at the centromeres (Lee et al., 2003). Disruption of separase, which cleaves RAD21 in mitosis, causes nondisjunction of homologous chromosomes at anaphase I, suggesting that cleavage of REC8 by separase is needed for homologous chromosome separation in meiosis (Kudo et al, 2006). On the other hand, REC8 at centromeric regions survives throughout meiosis I and remains until the onset of anaphase II. The protection of centromeric REC8 is achieved by SGO2, since depletion of SGO2 by siRNA in oocytes causes the loss of centromeric REC8 and precocious separation of sister chromatids at anaphase I (Lee et al., 2008). These studies, together with other reports in yeasts, reveal that the basic mechanism of sister chromatid cohesion, protection and separation in meiosis is essentially conserved from yeasts to mammals. The second topic is about an unpublished study concerning a new cohesin subunit that we have very recently identified as meiosis-specific one. Our results allow us to propose that multiple forms of cohesins coordinate proper progression of chromosomal events in mammalian meiosis.

Activation and inactivation of primordial oocytes

Takashi MIYANO and Mohammad MONIRUZZAMAN

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Ovaries of newborn animals contain a huge number of non-growing oocytes in primordial follicles (primordial oocytes). Some of them are selected to enter the growth phase (1st selection) and reach to their final size, while others become quiescent in the primordial follicles and are served as the future stockpile. After animals reach puberty, small number of oocytes in primordial follicles in the quiescent pool are activated to start to grow (2nd selection) and then some of the fully-grown oocytes resume meiosis by the stimulation of gonadotropins to mature to metaphase II. This recruitment of primordial oocytes continues throughout the female reproductive life. The mechanisms underlying the above two selections of primordial oocytes are not understood well. We have conducted a series of experiments of pig oocyte growth using *in vitro* or xeno-transplantation systems, and found that primordial oocytes in prepubertal pigs (6-month-old) took a longer time to initiate their growth comparing those in neonatal animals (10- to 20-day-old). We think that primordial oocytes in adults are different from those in neonatal animals.

A transcription factor FOXO3 has been implicated as one of the molecules responsible for the dormancy of primordial oocytes in mouse ovaries, since Foxo3$^{-/-}$ female mice exhibited a distinctive ovarian phenotype of global follicular activation leading to oocyte death [1]. In prepubertal pigs, FOXO3 was detected in almost all (94+/-2%) primordial oocyte nuclei, and in neonatal pigs, 42+/-7% primordial oocytes were FOXO3 positive. Further, FOXO3-knockdown primordial follicles from prepubertal pigs by the specific siRNAs developed to the antral stage accompanied by oocyte growth in the xenografts faster than those in the control [2]. The results suggest that ovaries of neonatal pigs contain a mixed population of quiescent and activated primordial oocytes, and almost all the primordial oocytes are quiescent in prepubertal/adult pigs, and that a transient knockdown of the FOXO3 activates the primordial oocytes to enter the growth phase.

Usually, all of the primordial oocytes do not start to grow at the same time in the mammalian ovary. Mammals have a long reproductive life span and the ovary is required to maintain a pool of non-growing oocytes throughout the female reproductive life. Most of the primordial oocytes become dormant by a FOXO3-related mechanism to establish a non-growing oocyte pool in the ovary for the future recruitment, and perhaps the oocytes that have escaped the mechanism enter the growth phase at the 1st selection. After puberty, the quiescent primordial oocytes are activated at the 2nd selection by certain stimulatory factors that inhibit or overcome the FOXO3-related inhibitory mechanism.

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Oocyte control of granulosa cell function and development

Koji SUGIURA[1,2], You-Qiang SU[1] and John J. EPPIG[1]

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Mouse oocytes play a critical role in regulating granulosa cell function and development by secreting several growth factors, including bone morphogenetic protein 15 (BMP15), growth differentiation factor 9 (GDF9), and fibroblast growth factors. In addition to the oocyte signals, estrogen signaling is required for normal development of granulosa cells; however, coordination of these signals during follicular development was ill-defined.

Expansion of cumulus cells, which requires expression of *Has2* mRNA, is a prerequisite process for normal ovulation. Participation of oocyte-derived factors and estrogen is required for acquisition and/or maintenance of the cumulus expansion competence. Therefore, to investigate coordination of oocyte and estrogen signaling during follicular development, effects of estradiol (E2) and oocytes on development and function of cumulus cells were assessed using cumulus expansion as a marker.

Exogenous E2 maintained competence to undergo expansion and to express full levels of *Has2* mRNA by cumulus cell-oocyte complexes cultured in vitro. In contrast, exogenous E2 could not maintain full expansion competence of isolated cumulus cells unless co-treated with denuded oocytes or recombinant GDF9 and BMP15. Furthermore, exogenous E2 promoted less-differentiated granulosa cells from preantral follicles to become competent to undergo expansion in vitro. Moreover, transcripts encoding NRIP1, a suppressor of estrogen receptor signals, were decreased in cumulus cells by oocytes or GDF9 in vitro.

Therefore, oocytes-derived GDF9 and BMP15 coordinate with the E2 to control granulosa cell function and development, and oocyte suppression of *Nrip1* expression in cumulus cells may be one of the mechanisms of oocyte and E2 signal crosstalk. Supported by grant HD23839 from the Eunice Kennedy Shriver NICHD.

In vitro growth of bovine oocyte-granulosa cell complexes after vitrification

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Introduction

The *in vitro* growth of oocytes and the cryopreservation of the growing oocytes are both important technologies to be fully developed in the near future. Then the combination of them will expand significantly the availability of mature oocytes. Studies of oocyte growth *in vitro*, and its application to the vitrificaiton of growing follicles, have been conducted largely with mice [1]; this tendency is probably due to the ease in handling and culturing mouse oocytes compared to the bovine oocytes. Cryopreservation of growing follicles in other species has been reported [2], but usually the oocytes were not followed up until they acquire the competence to mature. In the present study, we tested the possibility of preservation of growing bovine oocytes by using a step-wise vitrification, followed by a 14-day growth culture using a system developed for bovine oocytes [3].

Materials and Methods

Bovine oocyte-cumulus/granulosa cell complexes, hereafter referred to as simply complexes, were dissected out from early antral follicles (0.5-0.8 mm in diameter) in a Hepes-buffered modified Minimum Essential Medium, and subjected to a ten-step equilibration treatment as previously reported for fully grown immature mouse oocytes by Aono et al [4]. The eventual equilibration solution contained 3% (v/v) ethylene glycol and 3% (v/v) DMSO. The complexes were then transferred to a vitrification solution consisting of 15% ethylene glycol, 15% DMSO and 0.5 M sucrose, and were placed onto the membrane of Millicell plates with a minimum volume of the solution. After storage in liquid nitrogen, the complexes were thawed and washed twice in the medium described below.

In vitro culture of the complexes after thawing was performed as follows: the complexes were placed on Millicell culture inserts and cultured for 14 days in a modified TCM199 supplemented with 2 mM hypoxanthine, 5% fetal bovine serum, and 4% (w/v) polyvinylpyrrolidone (molecular weight**:** 360 000) [3]. The cultures were housed for 14 days in an atmosphere of 5% CO_2 at 38.5°C, but the O_2 tension was switched from 5% to 20% after the first 3 days of culture. The oocyte diameter was measured on Day 1 (the day of thawing designated Day 0); this measurement was to screen out the oocytes with any signs of degeneration and to culture only oocytes 92–106 μm in diameter. On Day 14, the oocytes showing no signs of degeneration were subjected to final size measurement and *in vitro* maturation for 23 h.

Results and Discussion

The overall efficiency of the oocyte (and complex) recovery after vitrification and thawing was 88% (72/82), as assessed by its morphology on Day 1. The viable complexes adhered to the substratum and grew outward. About a week later a characteristic dome-like structure of granulosa cells was formed, each containing a normal looking oocyte-cumulus cell complex.

The percentage of oocytes/complexes surviving through the 14-day culture period was 89% (64/72). The mean oocyte diameter on Day 1 was 100.0 μm, and the final size was 116.4 μm. This is a significant increase in size, but smaller than that attained by unvitrified control oocytes, whose mean diameter had increased from 101.3 μm on Day 1 to 119.4 μm on Day 14. The percentages of the viable oocytes progressing to metaphase II with a polar body emission after the subsequent 23-h culture were 52% (33/64) and 50% (19/38) for the vitrified group and unvitrified control group, respectively.

Several lines of evidence have indicated that the appropriate bidirectional interactions between oocytes and granulosa cells are essential for normal development of the oocytes. The results of the present study, exhibiting the oocyte viability, growth, maturation, and dome-like

structure formation, suggest that the functional associations between the oocytes and granulosa cells were successfully preserved, or recovered, after the vitrification and thawing.

Conclusion

The present study clearly indicates that the growing bovine oocytes, isolated from early antral follicles, can acquire the meiotic competence during the 14-day culture after the vitrification and thawing process

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Regulation of pig oocyte maturation by EGF-like peptides

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Recent data indicate that LH-induced events in preovulatory follicles are mediated indirectly: LH binds to its receptor on mural granulosa cells and induces expression of *Areg*, *Btc* and *Ereg*. By autocrine and paracrine mechanisms, these EGF-like peptides induce expression of prostaglandin-endoperoxidase synthase 2 (Ptgs2) in both mural granulosa and cumulus cells, leading to increased production of prostaglandin E2 (PGE2). PGE2 binds to its receptor on cumulus cells activating MAPK 14 and consequently stimulating production of AREG and EREG in cumulus cells. These peptides then bind on EGF receptor of cumulus cells and in the autocrine manner stimulate transcription of genes involved in regulation of meiotic resumption and cumulus expansion.

The aim of this work was to assess FSH-stimulated expression of EGF-like peptides in cultured cumulus-oocyte complexes (COCs) and to find out an effect of the peptides on activation of kinases in the COCs, cumulus expansion, oocyte maturation and acquisition of developmental competence in vitro. FSH significantly stimulated expression of *Areg* and *Ereg,* but not *Btc* in cultured COCs. Expression of *Areg* and *Ereg* reached maximum at 2 and 4 h after FSH addition, respectively, no significant increase in *Areg*, *Ereg* and *Btc* expression was found in COCs beyond 4 h of culture. FSH significantly stimulated in the COCs expression of expansion related genes (*Ptgs2*, *Tfnaip6* and *Has2*) at 4 and 8 h of culture, with a significant decrease at 20 h of culture. Both AREG and EREG also stimulated a prompt increase in expression of the genes, the dynamics of the expression was somewhat different for EREG-induced expression of *Tfnaip6* and *Has2,* that persisted high at 20 h of culture. In contrast to FSH, the AREG and EREG did not stimulate in the COCs expression of Cyp11A1 and an increase in progesterone concentration in culture medium. The EGF-like peptides stimulated a prompt activation of MAPk 3/1 and AKT kinase, but not activation of PKA. AREG and EREG stimulated maturation of the oocytes and expansion of the cumulus cells, although the percentage of oocytes that had reached metaphase II was significantly lower when compared to FSH-induced maturation. Parthenogenetic development to blastocyst stage of oocytes matured with AREG and EREG or with combination of FSH and the EGF-like factors was better then the development of oocytes matured with FSH only. This feature was well documented by comparison of the proportions of blastocysts among the cleaving activated oocytes.

We conclude that the EGF-like factors do not reproduce all events elicited in the cultured COCs by gonadotropins (FSH) – they do not stimulate activation of PKA, in vitro production of progesterone by cumulus cells and are not as efficient as the FSH in stimulating meiosis resumption and completion. However, the oocytes that complete maturation following the stimulation by AREG and EREG possess higher developmental potential than the oocytes stimulated by FSH only. Optimal conditions for maturation of pig COCs in vitro and subsequent parthenogenetic development were reached in medium supplemented by both FSH and the EGF-like factors.

Supported by GAČR No. 523/08/0111 and NAZV 101A166.

Functional analysis of maternal nucleolus in mouse oocytes

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The nucleolus is the site of ribosomal RNA (rRNA) synthesis and processing and ribosomal subunit assembly. During growth in the ovary, the oocyte possesses a fibrillo-granular nucleolus with active transcription and ribosome biogenesis and stockpiles the proteins and RNAs for early embryonic development. When the oocyte reaches full size, indicating that it has acquired developmental competence, all its transcription ceases, including the transcription of rRNA. Apparently, its nucleolus no longer contains DNA at this point and shows a highly compacted, fibrillar morphology. As the nucleolus is eliminated during spermiogenesis, the spermatozoa do not have nucleoli. After fertilization, this maternally derived, transcriptionally inactive fibrillar nucleolus appears in both male and female pronuclei and in the embryonic nuclei of each interphase until the 4- or 8-cell stage; that is the time at which zygotic RNA polymerase I activity is fully established.

When we produced an embryo lacking this inactive nucleolus, the embryo failed to develop past the first few cleavages, indicating the requirement of the oocyte nucleolus for preimplantation development. However, the detail timing of this nucleolus requirement in early embryonic development and function of inactive nucleolus are not fully understood.

We reinjected the nucleolus into oocytes and zygotes without nucleoli at various time points to examine the timing of the nucleolus requirement during meiosis and early embryonic development. When we put the nucleolus back into oocytes lacking a nucleolus at the germinal vesicle (GV) stage and at second metaphase (MII), these oocytes were fertilized, formed pronuclei with nucleoli and developed to full term. When the nucleolus was reinjected at the pronucleus (PN) stage, most of the reconstructed zygotes cleaved and formed nuclei with nucleoli at the 2-cell stage, but the rate of blastocyst formation and the numbers of surviving pups were profoundly reduced. Moreover, the zygotes without nucleoli showed a disorder of higher chromatin organization not only in the female pronucleus but also, interestingly, in the male pronucleus. Thus, the critical time point when the nucleolus is required for progression of early embryonic development appears to be at the point of the early step of pronucleus organization.

To find oocyte nucleolus function, we focused on one protein, Nucleoplasmin2 (Npm2). Npm2 knockout mouse has defect in oocyte-nucleolus formation and in fertility. We found that the oocyte nucleolus contains Npm2 proteins, and the expression level of this protein is quite high in oocytes. To better understand oocyte nucleolus function, we sought to identify Npm2 associating proteins in nucleolus by yeast two hybrid screening, and found interacted proteins. Our progress in understanding the nucleolus function will be presented.

Aurora-A regulates MTOCs (centrosome) biogenesis but it does not trigger G2/M transition in mouse oocytes matured in vivo

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Aurora-A kinase (AURKA) is an important mitotic kinase involved in G2/M transition, centrosome separation and spindle formation in somatic cells. Almost completely all known information about a role of AURKA in cell cycle regulation is derived from in vitro cultured human cancer cells. Using transgenic models we provide insight on the physiological role of AURKA during meiotic maturation (meiotic cell cycle progression) in mice. By means of Cre-lox system we have generated mice with expression of both wild-type (wt) and kinase-dead (kd) aurka transgenes specifically only in ovarian oocytes.

Intrafollicularly, control oocytes are arrested at prophase I and they resume meiosis in vivo (G2/M transition) after LH/hCG stimuli. The first AURKA activation occurs shortly after LH/hCG peak. The initial activation of AURKA is CDK1 independent but full activation of AURKA requires CDC25B mediated CDK1 activation. The control oocytes contain 1-3 microtubule organizing centres (MTOCs; centrosome equivalent) at prophase I. At the time of nuclear envelope break down (NEBD), a first visible marker of meiosis resumption, MTOCs number increases up to several dozen (MTOCs multiplication). MTOCs multiplication requires full AURKA activation and in this way it is dependent on CDC25B.

Although expression of wt aurka transgene leads to premature activation of AURKA at prophase I oocytes they do not resume meiosis without LH/hCG stimuli. After wt aurka transgene expression in mouse oocytes, MTOCs multiplication occurs prematurely at prophase I without NEBD although both control and kd aurka mice exhibit usually 1-3 MTOCs per oocytes. At metaphase I stage both wt aurka and kd aurka oocytes have more gamma-tubulin. Both wt aurka and kd aurka transgenic mice have normal fertility during first 7 mouth of their life. It suggests that the AURKA induced premature MTOCs multiplication does not interfere with developmental competence of oocytes. In conclusion these data imply that AURKA activity is responsible for physiological MTOCs multiplication and that AURKA regulates independently of its kinase activity the amount of gamma tubulin. AURKA is not a trigger kinase for G2/M transition in mouse oocytes.

This study was supported by the Czech-U.S. cooperation grant ME08030.

Age-related aneuploidy in mammalian oocytes

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It has been shown that mammalian meiosis is associated with a high incidence of errors leading to chromosomal aberrations, which are more prevalent in female gametes and seem to increase with maternal age. Oocyte aneuploidy has severe consequences, including pregnancy loss or birth defects, and is probably originated in two steps during the oocyte development. According to this theory, first error, created early in meiosis, for example during recombination, might lead to aneuploidy because of a failure of checkpoint mechanisms controlling chromosome segregation after resumption of meiosis. Since our knowledge of chromosome segregation in meiosis is coming predominantly from studies on yeasts, any research aimed at the etiology of oocyte aneuploidy must be focused first on characterization of the basic molecular mechanisms controlling meiotic program in mammals.

In meiosis, the reduction of number of chromosomes is achieved by combining two nuclear divisions without DNA replication in between. Successful segregation of chromosomes during this process is crucially dependent on stepwise removal of cohesion between sister chromatids, mediated by cohesin complex. In order to ensure segregation of homologous chromosomes in meiosis I, and sister chromatids in meiosis II, cohesion between chromosome arms is removed during first meiosis, whereas centromeric cohesion is maintained until second meiosis. We were able to show that in mammalian meiosis, removal of cohesin complex during meiosis I, is dependent on proteolytic cleavage of its Rec8 subunit by Separase. Deleting Separase from the oocyte or replacing Rec8 with its uncleavable version would disrupt chromosome segregation. It was also hypothesized that the errors in chromosome segregation in female gametes might be caused by malfunction of Spindle assembly checkpoint (SAC), which in somatic cells is essential for monitoring of the correct attachment of the chromosomes to the spindle. We have shown that intact SAC is essential in mouse oocytes and its absence causes premature anaphase onset accompanied by massive chromosome segregation defects. We now focus on characterization and quantification of changes in oocyte meiosis specifically reflecting maternal aging. Using techniques, which can be applied on a single cell, such as live cell imaging or kinase assays, we are able to reveal subtle changes introduced by maternal aging into meiosis and to address which components of the meiotic cell cycle machinery are more susceptible to emphasize errors accumulated early during meiosis.

Lectures:

Topic 2: Fertilization

Factors associated with calcium oscillations during fertilization in mammals

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The activation of the egg is the first stage in the initiation of embryo development. It comprises a series of events that unfold soon after interaction of the gametes and that end with the completion of meiosis and progression onto the mitotic cell cycles. In all species studied to date, egg activation requires a fertilization-associated increase in the intracellular concentration of calcium ($[Ca^{2+}]i$). In mammals, the fertilizing $[Ca^{2+}]i$ signal consist of periodicals rises, which are also referred to as $[Ca^{2+}]i$ oscillations. It is well known that at least two molecules (oocytes: type 1 inositol 1,4,5-trisphosphate receptor [IP_3R1], sperm: phospholipase C zeta [PLC□]) are involved in the regulation of calcium oscillations.

In vertebrate oocytes, it has been reported that IP_3R1 is expressed at both mRNA and protein levels. IP_3R1 in mammals, or its homologue in other species, is responsible for the majority of Ca^{2+} release during fertilization. Production of inositol 1,4,5-trisphosphate (IP_3), the IP_3R1 ligand, is detected during fertilization, and entails hydrolysis of phosphatidylinostitol (4,5)-bisphosphate by the action of a phospholipase C (PLC). We previously reported that during maturation in mouse oocytes the IP_3R1 becomes phosphorylated at an MPM-2 epitope, and that this phosphorylation persists until the fertilization-associated $[Ca^{2+}]i$ responses cease. We also found a role for the ERK pathway, as oocytes matured in the absence of ERK activity showed decreased IP_3R1 MPM-2 reactivity and oscillatory ability. From these results, it is suggested an interdependence between the M-phase kinases active during maturation, IP_3R1 MPM-2 phosphorylation and $[Ca^{2+}]i$ release. We recently examined which kinase is responsible for IP_3R1 MPM-2 phosphorylation and how the ERK pathway contributes to this phosphorylation. We found that IP_3R1 is a novel target for Polo-like kinase 1 (Plk1), an M-phase kinase with broad functions during meiosis/mitosis and cytokinesis. Plk1 phosphorylates IP_3R1 at an MPM-2 epitope, and these proteins interact in an M-phase preferential manner. IP_3R1 and Plk1 co-localize on the meiotic spindle poles, and on other sites in the ooplasm, and this overlap, as well as IP_3R1 MPM-2 phosphorylation is reduced in oocytes matured without ERK activity; these data demonstrate a novel regulatory role of the ERK pathway on IP_3R1 reorganization. We therefore propose that IP_3R1 phosphorylation by Plk1, and possibly by other M-phase kinases, makes possible the delivery of spatially and temporally regulated $[Ca^{2+}]i$ signals to ensure faithful progression/completion of meiosis/mitosis and cytokinesis.

In addition, we focused on molecular mechanism of another factor which was derived from sperm, is a sperm-specific PLC isoform, PLCζ. During mammalian fertilization, PLCζ, is reportedly responsible for IP_3 production. After the discovery in the mouse, PLCζ has been identified in not only mammalian species (rats, pigs, cows, monkeys, humans) but also non-mammalian species (medaka, chickens, quails). Injection of PLCζ mRNA and protein induced Ca^{2+} oscillations in mouse oocytes. On the other hand, it has been demonstrated that PLC□-deficient sperm in humans could not induce Ca^{2+} oscillations. These results lead us to the possibility that PLC□ is a sole factor which is required for Ca^{2+} oscillation. In domestic animals, injection of PLC□ into oocytes clearly showed induction of Ca^{2+} oscillations in the pig and the cattle. During fertilization, releasing of PLCζ from sperm was rapidly (probably within a first hour) occurred after sperm-oocyte fusion. By using fluorescence-tagged PLCζ, it has been showed that PLCζ is concentrated to the pronucleus (PN) formed and Ca^{2+} oscillations were terminated in most of the mouse eggs before initiation of the accumulation. However, contrary to mice, PLCζ in other species is never concentrated to PN. This result strongly suggests that species-specific difference is existed and molecular mechanism of PLC□ in other species is required to be clarified. We recently succeeded in molecular cloning of PLCζ in the horse. Equine PLCζ showed much higher activity than those in other species have been reported up to date.

In this symposium, we will show recent results about IP_3R1 and PLC□ and discuss how Ca^{2+} oscillation is regulated by these molecules.

Vole sperm cryopreservation: assessments of viability, sperm DNA integrity and fertilizing capacity

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Recently, a number of animals/plants have been rapidly threatened and the number of threatened animals is more increasing. With regard to mammalian species described previously (total 5490 species), 20.8% (1142 species) of them are threatened and 1.4% (78 species) had already become extinct according to Red List (the International Union for Conservation of Nature; IUCN, 2009). In addition, half of mammals listed are small species, including rodents. Except for experimental animals, however, small species have attracted less notice than larger species for a study of conservation-technology, although smaller animals are thought to be more vulnerable to environmental changes. We hence think that even small animals the research and maintenance of them might indirectly contribute to conservation of biological diversity, since the loss of biological diversity would lead serious disadvantage for human life in future.

Vole (*Animal*; *Chordata*; *Vertebrate*; *Mammallia*; *Rodentia*; *Muridae*; *Arvicolinae*; *Microtus*), small animal, widely inhabit from the Frigid Zone to the Temperate Zone and total 64 strains have been discovered so far. Ten of sixty-four strains have been classified into the endangered category in Red List (IUCN, 2009); *M. bavaricus*、*M. oaxacensis*、*M. umbrosus*、*M. breweri, M. schelkovnikovi, M. quasiater, M. cabricus, M. sachalinensis, M. guatemalensis,* and *M. kikuchi*. In our laboratory, three strains have been maintained; *M. montebelli* (Japanese grass vole), *M. arvalis* (common vole), and *M. rossiaemeridionalis* (southern vole).

Microtus has been expected as a model for metabolic disease in herbivorous animal, since this species is also herbivorous and possesses a complex stomach that is similar to ruminant animals, although field vole dose not ruminate. Moreover, the chromosome number differs with strains; for instance, Japanese grass vole, common vole, and southern vole that are maintained in our laboratory possess 30, 46, and 54 chromosomes, respectively, and the analysis of speciation has been also performed utilizing this variation of chromosome number. On the other hand, there are only a few reports involved with its reproduction, characteristic of gametes, and related reproductive technologies.

Our final goal is to establish the methods of preservation and regeneration in this species as an animal genetic resource. To attain this objective, we firstly attempted to cryopreserve spermatozoa isolated from Japanese grass vole and common vole using a conventional protocol for mouse sperm cryopreservation. After frozen-thawed, the rate of vole sperm viability assessed by nigrosin-eosin staining was similar to control mouse, although the viability of frozen-thawed spermatozoa was significantly lower than that of fresh and such decline was equally to control. Secondly, an alkaline comet assay was used to evaluate whether vole sperm DNA integrity was protected after frozen-thawed. In both *Microtus* species, the rate of sperm DNA with a comet tail (this means damaged DNA) significantly increased after cryopreservation. Moreover, the rate of damaged sperm DNA from Japanese grass vole was higher than a result from common vole. Finally, we used the interspecies ICSI procedure with mouse oocytes to test the fertilizing capacity of cryopreserved vole spermatozoa, because of unstable collection of vole oocytes matured *in vivo*. Most of mouse oocytes successfully injected with a single vole spermatozoon resumed meiosis, extruded the second polar body, and formed female and male pronuclei. Taken together, vole spermatozoa can be successfully cryopreserved using a conventional protocol in mouse, although the effect of freezing-thawing on vole sperm DNA may differ with strains. In addition to above results, characteristic of female gametes in *Microtus* will be illustrated in this symposium.

The role of CENPF in bovine preimplantation development

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CENPF (centromeric protein F; mitosin) is a large multifunctional protein that plays a key role during cell division. It is expressed and localized in cell-cycle-dependent manner with peak at the G2/M phase boundary and is degraded at the end of mitosis. CENPF is one of the earliest proteins associated with kinetochores and helps to form the correct kinetochore–microtubule interactions. The embryonic CENPF mRNA expression is started during the major genome activation at late 8-cell stage. At the beginning of mammalian embryonic development all mRNAs are of maternal origin, the embryonic genome is activated during an event called embryonic genome activation (EGA), which takes place at late 8-cell stage in cattle. The genes that are activated at this stage are supposed to be important for the preimplantation development.

Identification of these genes is essential for understanding early mammalian embryogenesis. Up to now, several genes activated during EGA were identified, but only a small portion of them has been studied more thoroughly to uncover their exact role during preimplantation development. In order to investigate the importance of CENPF, we have silenced the CENPF mRNA in bovine zygotes by CENPF dsRNA injection and monitored the developmental competence in the embryos.

We showed that the embryos are able to develop without any developmental impairment until the 8-cell stage (EGA). However, the developmental competence rapidly decreased then, and only 28.1% of 8-cell embryos with silenced CENPF mRNA were able to develop to 16-cell stage or beyond (72% in control groups).

In order to understand what enables the development until the 8-cell stage we used a translational inhibitor cycloheximide (CHX). The CHX treatment of embryos before EGA does not changed the intensity of CENPF immunofluorescence staining. However, in CHX treated post-EGA embryos, the intensity of staining was markedly decreased. This suggests that in pre-EGA embryos, CENPF is not degraded at the end of cell cycle, while in post-EGA embryos, the protein behaves in the same manner as in somatic cells, i.e. is degraded at the end of cell cycle. The latter statement was further confirmed by immunofluorescent analysis, which showed the somatic-cell-like localization and expression of CENPF in post-EGA embryos.

These results show that microinjection of CENPF dsRNA leads to mRNA and protein silencing in preimplantation embryos. The inhibition of CENPF mRNA does not influence the development of embryos until 8-cell stage, however the development thereafter is considerably deteriorated and the majority of embryos arrest before reaching the 16-cell stage. Hence, it shows that the expression of CENPF mRNA is necessary for proper preimplantation development.

Supported by GACR 523/09/1035 and GACR 204/09/H084

From oocyte to embryo - remodeling the parental genomes.

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Gametogenesis and early embryonic development in mammals are characterized by extensive epigenetic remodeling. Although many issues concerning such processes remain unresolved, it is clear that a functional link exists between this remodeling, gamete quality and/or developmental potential of embryos. Thus, major epigenetic remodeling events take place during four main phases – gametogenesis, fertilization, early embryonic development up to the blastocyst stage and during the first differentiation at the blastocyst stage. During male gametogenesis, most histones are replaced by protamines and the sperm DNA is packaged to almost crystalline state. On the other hand, the oocyte genome is organized by histones throughout the whole phase of gametogenesis – the only clear difference when compared to somatic cells is the absence of somatic type linker histones. The above described changes go hand in hand with transcriptional silencing in both male and female gametes. After fertilization both parental genomes decondense and the sperm protamines are replaced by histones stored in the oocyte cytoplasm. At this phase, the parental genomes are epigenetically unequal: the maternal genome retains high levels of DNA methylation and shows the presence of repressive histone modifications whereas the paternal genome undergoes active DNA demethylation and exhibits the lack of repressive histone modifications and the presence of the histone variant H3.3. Thus, although both parental genomes share a common environment (zygotic cytoplasm) clear epigenetic asymmetry between parental genomes can be detected. During subsequent divisions the parental genomes are combined and become intermingled - the epigenetic asymmetry is probably largely lost and equalized. This period is characterized by passive DNA demethylation (replication dependent DNA demethylation) and global loss of certain repressive histone modifications. On the other hand, some genes (imprinted genes) or specialized structures (X chromosome) escape this phase of epigenetic equalization. Finally, a third phase of epigenetic remodeling during early embryogenesis takes place during the blastocyst formation when the differentiation of the trophectoderm (TE) and of the inner cell mass (ICM) occurs. These two lineages differ in the mode of X chromosome inactivation (random in ICM vs. imprinted in TE), in the level of DNA methylation and in the presence of certain histone modifications. Although we can currently only speculate about the impact of individual remodeling steps, it is possible that these event influence processes such as embryonic genome activation and thus successful embryonic development. Moreover, it is clear that the epigenetic remodeling and erasure of epigenetic marks during early embryogenesis is necessary for epigenetic resetting leading to totipotency of the early embryo as both gametes are highly specialized and terminally differentiated cells.

Lectures:

Topic 3: Stem cells

Human embryonic stem cells suffer from abnormal metabolism of centrosomes

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When propagated in culture human embryonic stem cells (hESCs) tend to harbor alterations to their karyotype, which may limit their prospective use in patients. Chromosomal instability of some malignancies is considered to be driven, at least in part, by centrosomal overamplifications perturbing balanced chromosome segregation. Here we for the first time report very high frequency of mitotic cells with supernumerary centrosomes in cultures of hESCs. This inadequate situation features strictly undifferentiated state of hESCs and becomes progressively suppressed upon prolonged propagation in culture. We demonstrate that improved attachment to culture substratum as well as inhibition of key molecular regulators of centrosomal metabolism, CDK2 and Aurora A, diminish the frequency of multicentrosomal mitoses. In other words, attenuated cell attachment and deregulation of machinery controlling centrosome numbers both contribute to centrosomal overamplification in hESCs. Still, linking the number of centrosomes to the ploidy indicated that not only overduplication within a single cell cycle but also mitotic failure were involved in generation of numerical abnormalities of centrosomes in hESCs. Collectively, our data point to the supernumerary centrosomes as to be a significant risk factor for cultured hESCs in terms of maintenance of integrity of their genome, and as such should be evaluated when new culture conditions are being implemented.

Supported by: MSM0021622430, AV0Z50390512, AV0Z50390703, 1M0538

Centrosomal decisions on self-renewal in mouse embryonic stem cells

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Mouse embryonic stem cells (mESCs) have a unique cell cycle structure with a peculiarly short G1 phase that lacks functional G1 checkpoint; *i.e.* mESCs do not stop in G1 phase after DNA damage. In our study, we attempted to investigate causes of G1 checkpoint non-functionality in mESCs and the relevance of rapid G1 phase progression for pluripotency of mESCs.

In somatic cells, in response to DNA damage in G1 phase, G1 checkpoint pathways (the Chk1/Chk2–CDC25A and p53–p21 pathways) are activated to stop cell cycle progression by downregulating CDK2 activity. However, as our studies revealed, in mESCs CDK2 activity is refractory to DNA-damage. We found out that G1 checkpoint pathways are not functional in mESCs due to centrosomal sequestration of Chk1/Chk2 proteins and, importantly, localization of a large proportion of CDK2 molecules to centrosomes. Although alternative G1 checkpoint pathways, including p53–p21 and GSK-3β–CDC25A pathways, were activated in mESCs after DNA damage, they did not impinge on CDK2 activity, suggesting that centrosomal localization shelters CDK2 from checkpoint mechanisms. Furthermore, our studies furnished evidence on CDK2 activity in underpinning rapid G1 phase progression during unperturbed mESC cycles, driving escape from G1 phase in the conditions of DNA damage, as well as maintenance of self-renewal: Downregulation of CDK2 activity slowed down G1 phase progression in mESCs and, importantly, induced somatic cell-like cell cycle, differentiation-associated changes in mESC morphology and expression of differentiation markers.

From our observations, we conclude that centrosomal CDK2 might have a crucial function in maintenance of self-renewal in mESC in the conditions of DNA damage and that centrosomes might play an important, mediatory role in the cell fate game in mESCs, providing an interface for crucial cell cycle and cell fate decisions to take place.

This work was supported by grants NR/9508 (Ministry of Health), MSM 6198959205 and 2B06077 (Ministry of Education, Youth and Sport), Palacky University grant LF 2010 013, and by funds of the Deutsche Krebshilfe (108560) and Deutsche Jose Carreras Leukämie Stiftung (DJCLS R 06/04).

iPS cells in miniature pigs

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Pigs and miniature pigs are steadily gaining importance as large animal models in the field of regenerative medicine and in stem cell research. Several somatic stem cell populations, including mesenchymal stem cells or neural stem cells were successfully isolated in pigs, but establishment of proper embryonic stem (ES) cell line, fulfilling all classical ES cells landmarks, including germ-line transmission remains so far elusive. Porcine ES cells would be useful both for production of gene-modified animals and for testing potential stem cell therapies. Discovery of induced pluripotency makes preparation of pig induced pluripotent stem (iPS) cells an attractive alternative to ES cells. We have taken advantage of our experience with the isolation and culture of fetal porcine neural progenitors (NPC) and used them as a starting population for preparation of minipig iPS cells. NPC express Sox2, one of the factors required for reprogramming into pluripotent state. We have transduced several porcine NPC lines with either the combination of all four "Yamanaca factors" (Oct4, Sox2, Klf4 and c-Myc), 3 factor combination without Sox2 or with Oct4 only. Using all these factor combinations, we were able to derive cell clones displaying morphology similar to human ES cells, but proliferating on feeders in LIF containing medium, similar to mouse ES cells. We have chosen one of the clones transduced with 3 factor combination (B8) for further characterization and the cells displayed alkaline phosphatase positivity, expression of Nanog, endogenous Sox2 and Oct4. B8 minipig iPS cells formed embryoid bodies when placed in a suspension culture, and further differentiated when placed onto gelatin coated dishes. Detailed characterization of more cell lines is currently under way and we hope to use minipig iPS cells as model system for stem cell therapies.

Acknowledgements
This study was supported by the AS CR project M200450971, Centre for Cell Therapy and Tissue Repair (1M0538), Grant Agency of the Academy of Sciences of the Czech Republic (grant No. KJB400500801) and Institutional Research Concept IAPG No. AV0Z50450515.

Bone marrow-derived mesenchymal stem for the regeneration of cartilage and bone

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Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into a variety of cell types (osteoblasts, chondrocytes, tenocytes and adipocytes). They are also the primary cell source for cartilage and bone formation and so may be optimal for cell-seeded scaffolds and can be used for bone tissue repair (Sumanasinghe et al., 2006). Several studies reported that porcine or rabbit MSCs are a valuable model system for mesenchymal basic research and tissue engineering (Ringe et al., 2002; Zhou et al., 2010).

The aim of our research was to obtain essential information on biological properties of mesenchymal stem cells combined with newly synthesized polymeric biomaterials and nanofibres in vivo as well as in vitro. We were focused mainly on development of effective methods of controlled differentiation of MSCs into chondrocytes, osteocytes and fibrocytes and on testing compatibility between MSCs and above-mentioned materials. Composites of biomaterials and MSCs-derived cells were implanted into appropriate sites of musculoskeletal system of optimal animal models (miniature pigs and rabbits) to test their effectiveness in the treatment of serious diseases of joints and bones. Osteogenic and chondrogenic ability of porcine and rabbit MSCs was tested by various evaluation methods in different cultivation conditions and systems.

We performed an experimental study on New Zealand's white rabbits and miniature pigs to find differences in the results of treating the distal physeal femoral defect by the transplantation of autologous or allogeneic mesenchymal stem cells (MSCs) combined with scaffolds (collagen I - chitosan). The results showed that the transplantation of both autogenous and allogeneic MSCs into a defect of the growth plate appears as an effective method of surgical treatment of physeal cartilage injury. The next study evaluated macroscopically, histologically and immunohistochemically the quality of newly formed tissue in iatrogenic defects of articular cartilage of the femur condyle in miniature pigs treated with the clinically used method of microfractures in comparison with the transplantation of a combination of a composite scaffold with allogeneic mesenchymal stem cells (MSCs) or the composite scaffold alone. The newly formed cartilaginous tissue filling the defects of articular cartilage after transplantation of the scaffold with MSCs had in 60% of cases a macroscopically smooth surface. In all lesions after the transplantation of the scaffold alone or after the method of microfractures, erosions/fissures or osteophytes were found on the surface.
Our experiments are essential for development of safe and effective technology prior to initiation of clinical studies or prior to potential clinical use of these biomaterials as a substitutes for irreversibly damaged cells and tissues in people.

This work was supported by AV0Z50450515, 1M0538, and 2B06130

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Proteomics of neural stem cells isolated from miniature pigs

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The mechanisms that regulate the stem cell self-renewal and differentiation are complex and remain mostly unknown. Understanding neurogenesis and neural cell differentiation presents a unique challenge for the treatment of nervous system disorders.

To characterise molecular mechanisms governing neural stem cell differentiation we combined the advantage of porcine fetal neural stem cells *in vitro* differentiation model and complementary proteomic analyses. To monitor changes in cellular proteins, we applied 2-dimensional gel electrophoresis for protein separation followed by mass spectrometry for protein identification. We demonstrated that differentiation of neural cells was accompanied by changes in the expression of proteins involved in DNA and RNA binding, mRNA processing and transport, stress responses, iron storage and redox regulation. To access signalling proteins that are present in cells in very low copies, we utilised the Kinex^{TM} Antibody Microarrays. The neural stem cells exhibited high levels of G-protein coupled receptor kinase 2 (GRK2), several members of apoptotic FAS pathway and phosphorylations of αB-crystallin at Serin 45. For the differentiated neural cells were typical enhanced integrin, platellet-derived growth factor and MAP kinase signalling and increased levels of heme-oxygenase 2. In-depth studies using immunoblot and immunocytochemistry verified quantitative changes and localisation of selected proteins in neural cells. In addition, expression of potential biomarkers identified in porcine model was studied in human postmitotic neurons (hNT cells) and during differentiation of human spinal cord stem cells (566RSC cells).

We anticipate that characterisation of protein changes related to stem cell differentiation will help define suitable biomarkers that might be necessary for partially differentiated cells to optimally progress to a fully differentiated stage of neurons and glial cells. These biomarkers might be then applied in selection of cells optimal for transplantation treatment of neurological disorders.

This study was supported by the Ministry of Education, Youth and Sports (projects 1M0538 and ME10044) and Institutional research concept IAPG No. AVOZ50450515.

Lectures:

Topic 4: Genetics manipulation

Improvement of somatic cell nuclear transfer in mice: Genetic and epigenetic approaches

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Cloning mammals by somatic cell nuclear transfer (SCNT) has been successfully applied to more than 16 animal species. However, it is highly inefficient because of erroneous reprogramming of the donor genome, the nature of which has not been fully understood yet. Recently, with growing evidence for epigenetic alterations in SCNT embryos, agents used in modifying chromatin structure or DNA methylation status in donor cells or reconstructed embryos have attracted attention as possible treatments to overcome their developmental deficiency. One of the most successful measures is the treatment of the recipient mouse oocytes with histone deacetylase (HDAC) inhibitors, such as trichostatin A (TSA) and scriptaid, which led to significant increase in the cloning efficiency for this species. As histone acetylation is a central factor regulating chromatin accessibility, HDAC inhibitors are thought to facilitate the accessibility of putative ooplasmic factors to the transferred nuclear genome, thus enabling potent genomic reprogramming. Therefore, it is probable that they exert their effects on correcting random reprogramming errors in a global rather than a specific manner. It is known that there are many HDAC inhibitors and each has its specific inhibitory effect on several classes of HDACs. Although TSA and scriptaid work for cloned mouse embryos very well, we may find other HDAC inhibitors that can be universally used for improvement of mammalian SCNT.

Besides the non-specific reprogramming errors described above, the presence of many SCNT-specific phenotypes (e.g., placental abnormalities, obesity, immunodeficiency, and perinatal death) suggests that SCNT inevitably induces specific epigenetic errors in the donor genome. These might be non-random and definable characters, perhaps caused by fundamental epigenetic differences between the somatic and germ cell genomes. However, no evidence for this assumption has been proposed. To define the epigenetic characters specific for SCNT, we have recently analyzed global gene expression patterns in mouse embryos cloned from different donor cells under strictly standardized conditions in our laboratory. When the relative expression levels of filtered genes in cloned embryos taken from a 44k oligo DNA microarray were plotted on the 20 chromosomes (except for Y), genes on the X chromosome were specifically downregulated. This phenomenon was sex- and genotype-independent because the average X:autosome (X:A) expression ratio in the four types of cloned embryos (two males and two females) was consistently lower than in the corresponding control embryos. Interestingly, treatment of reconstructed oocytes with TSA could not ameliorate the X-linked downregulation of cloned embryos. The chromosome-wide gene downregulation on the X chromosome in cloned embryos was reminiscent of X chromosome inactivation (XCI). As XCI is established by *Xist* RNA coating *in cis*, we next examined whether *Xist* was expressed excessively in cloned embryos. As expected, the *Xist* expression level was significantly higher in male and female cloned embryos than in corresponding IVF embryos. RNA fluorescent *in situ* hybridization (RNA FISH) revealed ectopic expression of *Xist* from the active X chromosome (Xa) in cloned embryos of the both sexes. Interestingly, deletion of *Xist* on Xa resulted in normalization of the expression levels of the majority of downregulated genes, not just on this chromosome but also on autosomes. Embryo transfer experiments are now under way.

Reprogramming mechanisms exist in the ooplasm primarily to modify the epigenetic status of gametes into that of zygotes. The SCNT technique somehow utilizes this mechanism to reprogram the donor somatic cell genomes, which are epigenetically different from those of gametes. Probably, they might reflect fundamental differences in the nature of germ cells and somatic cells. Thus, we can classify the epigenetic errors in the cloned embryos broadly into two categories: one is random and can be overcome to some extent by enhancing the so-called genomic reprogramming (e.g., by HDAC inhibitor treatment), while the other is more specific and probably beyond the ability of the putative reprogramming factors. We demonstrated that the latter can be overcome at least to some extent by correcting the aberrated gene expression pattern by a genetic modification. The ultimate

goal of cloning researchers is to increase the efficiency of mammalian SCNT to a practical level (e.g., > 20% per embryos transferred), because it is undoubtedly a promising technique with many potential applications in biological drug manufacture, regenerative medicine and agriculture. To this end, we need to know the fundamental differences between somatic and germ cell genomes. We expect that SCNT will become more practical in future, by specifically targeting nonrandom epigenetic errors associated with SCNT.

Normal and abnormal epigenetic changes in the development of cloned cattle

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Since the first cloned sheep Dolly was born in 1996, lots of cloned animals in many species have been produced by somatic cell nuclear transfer (SCNT). However, the success rate of SCNT is still very low, less than 5-10 % in all species. The reason is not yet revealed, but it is suggested that the incomplete reprogramming during SCNT causes epigenetic errors and abnormal gene expression. Actually, there are lots of reports showing abnormal DNA methylation, histone modifications and gene expression patterns in cloned animals that die before or after birth. However, once the cloned animals were born and healthily grew up to adulthood, their fertility is normal and their offspring do not show any defects. This can be explained by the presumption that the first reprogramming step (SCNT) is insufficient and abnormal epigenetic changes cause abnormal gene expression, whereas the second reprogramming step (only in germ cells) is sufficient and epigenetic errors should be erased during germ cell development. Consequently, spermatozoa or oocytes from cloned animals are epigenetically normal and therefore, offspring from cloned animals should be healthy. To test this hypothesis in cloned cattle, we compared DNA methylation levels of unique genes and repetitive elements in cloned embryos, cloned bull somatic cells and spermatozoa. We observed aberrant DNA methylation patterns in cloned embryos, however, there was no difference of DNA methylation in somatic cells and spermatozoa between cloned and non-cloned bulls. These results suggest the occurrence of proper reprogramming during cloned bull spermatogenesis leading to the normality of cloned cattle offspring.

Characteristics of prion, bovine spongiform encephalo- pathy (BSE) pathogen, homo-knockout cow

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Abstract

Prion (Pr) protein plays a pivotal role in the infection of bovine spongiform encephalopathy (BSE). The aim of our research was to make Pr gene homo knockout (KO) Japanese black cows and to reveal their characteristics. Firstly, hetero Pr gene was knockouted in embryonic fibroblastic cells. Somatic cell nucleus with hetero Pr gene KO was transferred into oocyte. After activation and *in vitro* culture, somatic cell nucleus of blastocyst cell was transferred into oocyte to make homo Pr gene KO embryo. The homo Pr gene KO embryos were transferred in to the uterus. Detailed data of Pr distribution in organs of wild-type and somatic cell nuclear cloning Japanese black cows were demonstrated as follows: Pr protein were shown in peripheral organs: 7 skeletal muscles, 5 areas of intestinal tracts and 5 areas of tongue (5- to 31- fold less Pr protein than the brain where high levels of that, 9.0-11.0 μg/g, were noted). Histochemistry showed that Pr protein was expressed in nerve cells not only in brain but also in the tongue, skeletal muscles and intestines, indicating that these organs may serve as potential sources of BSE infection. No positive reaction for Pr mRNA or for Pr protein was noted in Pr gene homo KO cows, indicating that the KO cows have no possibility of BSE infection. In conclusion, to produce the KO cattle is the only way to make safe bovine derived foods, drugs and medical materials.

Keywords: *Bovine spongiform encephalopathy (BSE), Japanese black cow, Prion (Pr) protein, Pr gene homo knockout (KO)*

Introduction

Misfolding of the Pr protein is involved in the pathogenesis of BSE [1, 2], but the mechanisms of prion transmission remains unresolved. One emerging hypothesis advocates that the abovementioned conversion phenomenon occurs at the site where the infectious agent (abnormal Pr protein) binds with cellular Pr protein. Abnormal Pr protein has the potential to accumulate in peripheral tissues as well as in specified-risk materials (SRM). In Japan, SRM were immediately removed at slaughterhouse. Abnormal Pr protein was recently detected in the muscles of sheep [3-5] and humans infected with Creutzfeldt-Jakob disease [6, 7]. To assess the risk of abnormal Pr protein accumulation in peripheral tissues, it is important to understand the distribution and expression levels of cellular Pr protein in these tissues. To date, although studies have been performed in sheep [8, 9] and humans [10], Pr protein levels in the peripheral tissues of cattle remain to be determined. To perfectly take away the risk and hazard factor for BSE infection, moreover, we produced Pr gene homo knockout (KO) Japanese black cows.

Materials and Methods

Production of Pr KO cows: The cows lacking prion protein were produced using modified methods reported previously [11]. Briefly, $PRNP^{-/-}$ fetal fibroblastic cell lines were made. Then, we used somatic cell nuclear transfer (SCNT) technique to produce the cloned fetuses and calves. Eight wild-type and seven $PRNP^{-/-}$ KO cows were produced.

Biochemical and histochemical analyses: RT-PCR and *in situ* hybridization for Pr mRNA and Western blotting, ELISA, protein misfolding cyclic amplification (PMCA) procedure and immunohistochemistry for Pr protein in $PRNP^{+/+}$ and $PRNP^{-/-}$ cows were performed as previously described [12].

Results and Discussion

Distribution of Pr mRNA and protein in organs: In situ hbridization and immune-histochemistry showed that Pr mRNA and protein was expressed in nerve cells not only in brain but also peripheral tissues (tongue, skeletal muscles and intestines). Such data raise the possibility of Pr accumulation in these tissues, indicating that these organs may serve as potential sources of BSE infection.

Pr mRNA and protein levels in organs: Western blotting, ELISA and PMCA analyses revealed that high levels of Pr (9.0-11.0 μg/g wet tissue) was demonstrated in brain of normal and SCNT cows but not in Pr KO cows. Lower levels of Pr were shown in other organs [in decreasing order as follows: skeletal muscles (longissimus, iliocostalis thoracis, splenius, femoris biceps, triceps brachii, longissimus thoracis and omotransversarius muscles), intestinal tracts (ileum, jejunum, duodenum, colon and cecum) and tongue (apex, posterior, anterior and radix areas) (5.0 - to 31.0-fold less Pr than the brain).

Conclusion: We success to produce Pr gene homo KO Japanese black cows, which lack Pr mRNA or protein. The Pr KO cows have no possibility of abnormal Pr protein infection. Thus, to produce the KO cattle is the only way to make safe bovine- derived foods (beef and milk), drugs and medical materials and equipments.

Acknowledgements. This study was supported in part by Grant-in-Aid for Food Safety Assessment from the Food Safety Commission of Japan, by Grant-in-Aid for Creative Scientific Research 13GS0008 from the Ministry of Education, Culture, Sports, Science and Technology, and by Grants-in-Aid for Challenging Exploratory Research 18658105 and 21658092 and Scientific Research B18380164, B22380148 and S16108003 from the Japan Society for the Promotion of Science.

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Xenotransplantation of gonadal tissue for utilization of genetic resources in pigs

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Abstract

In vitro fertilization of *in vitro* matured oocytes in pigs has become the most popular method of studying gametogenesis and embryogenesis in this species. In addition, because of recent advances in *in vitro* culture of those embryos, *in vitro* embryo production now enables us to generate viable embryos as successfully as for *in vivo*-derived embryos and with less cost and in less time. In addition to those *in vitro* production technologies, more advanced technologies such as xenografting of gonadal (testicular and ovarian) tissue into immunodeficient experimental animals have been developed in recent years, to help conservation and utilization of gamete resources. We have already shown fertilizing ability and developmental ability of porcine embryos from gametes grown in mice. Here, we discuss the possibility of conservation and utilization of pig resources by xenografting to mice.

Testicular tissues xenografring

The possibility of spermatogenesis by the transplantation of germ cells into mouse testis has been suggested in mice [1, 2] and pigs [5]. In these cases, germ cells, including spermatogonial stem cells, were injected directly into the seminiferous tubules. Spermatozoa from the introduced cells would be expected to be produced in the ejaculate. However, this technique requires special skills for the injection and also accurate separation procedures to select the spermatozoa derived from the introduced cells. Although germ cell transplantation into mice has promoted complete donor-derived spermatogenesis in rodents, it has not yet been applied in large domesticated animals such as cattle and pigs, and also wild animals. Xenografting of testicular tissues can be performed into immunodeficient mice ectopicaly, such as under the skin of the back [6]. They succeeded in the production of mammalian sperm grown in nude mice. Several years later, successful embryo production by using these sperm cells from xenografted testicular tissues has been reported but limited to rhesus monkeys [7] and pigs [8, 13]. In our previous study [13], blastocysts could be produced after intra-cytoplasmic sperm injection (ICSI). Furthermore, we have also reported the successful live piglet production when the embryos just after the ICSI using xenogeneic sperm were transferred to the recipients [14]. Although the efficacy of piglet production remains low, the results suggests clearly that oocytes injected with a sperm differentiated from the gonocytes within the xenografts have the ability to develop to viable offspring in a large mammal. In combination with cryopreservation of testis tissue, this procedure will be one of useful methods for conservation of male genetic recourses.

Ovarian tissue xenografting

Primordial follicles act as stores of ovarian follicles and are potential resources of oocytes for medical, agricultural and zoological purposes. It was reported that full-term culture of primordial follicles to the mature stage and birth of viable offspring after *in vitro* fertilization and embryo transfer have been achieved only in mice [3]. However, ovarian xenografting is a potential method of maturing the oocytes in the primordial follicles (primordial oocytes) of large mammals. To date, ovarian tissues could be prepared from some species phylogenetically distant from mice (including pigs [9, 10]), and then been xenografted into immunodeficient mice. To our knowledge, only our previous studies, in which neonatal pig ovarian tissues were xenografted, had proven that primordial oocytes can develop in the host mice and acquire *in vitro*-fertilizing ability [10, 12] and also that viable embryos can be generated after *in vitro* fertilization [11]. We further reported that the acceleration of follicular growth from the primordial stage and also the developmental ability of

oocytes can be improved by treating with gonadotrophins (eCG or FSH) to the host mice [11]. However, it is also suggested that those oocytes derived from primordial follicles, even after *in vivo* growth and maturation, seem to have difficulty achieving cytoplasmic maturation. Transfer of oocyte chromosomes to an enucleated cytoplast with full developmental ability seems to be one possible method for improving the ability. Fusion of an ooplasmic fragment(s) prepared by 'Centri-Fusion' method [4] to the oocytes obtained from xenografts may be the other possibility. In combination with cryopreservation of ovarian tissue, this procedure will be one of useful methods for conservation of female genetic recourses.

Acknowledgements

This study was supported in part by a Grants-in-Aid for Scientific Research (22380153 to K. K. and 21380715 to H.K.) from the Japanese Society for Promotion of Science.

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Miniature pig as a large animal model of Huntington disease

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Huntington Disease (HD) is the most common and well-studied polyglutamine neurodegenerative disorder and is caused by an expansion of cytosine-adenine-guanine (CAG) repeats in the huntintin gene, which leads to neuronal loss in the striatum and cortex and to the appearance of neuronal intranuclear inclusions of mutant huntingtin. Huntingtin plays a role in protein trafficking, vesicle transport, postsynaptic signaling, transcriptional regulation, and apoptosis. Thus, a loss of function of normal protein and a toxic gain of function of the mutant huntingtin contribute to the disruption of multiple intracellular pathways. Excitotoxicity, dopamine toxicity, metabolic impairment, mitochondrial dysfunction, oxidative stress, apoptosis, and autophagy have been implicted in the progressive degeneration observed in HD. Nevertheless, despite the efforts of multidisciplinary scientific community, there is no cure for this devastating neurodegenerative disorder (Gil and Rego, 2008).

Research in this field is primarily based on rodent models which are, however, insufficient in conversion of the basic research to the human clinic comparing with the increasingly restricted research on primates. Looking for a suitable and publicly acceptable large animal model for study of neurodegenerative diseases, we have chosen the pig (miniature pig of our own breed) which has several advantages over the rodents and can serve as a primate substitute (the pig is genetically well characterized and has a large gyrencephalic brain with a primate-like neuroarchitecture that allows conventional brain imaging). In addition to general advantages of miniature pigs, our own breed (cross of Minnesota, Göttingen, Cornwall and Large White breed) is fully sexually matured as early as 4 months after birth and it has very small frontal sinus comparing to Göttingen miniature pig which allow us to perform more precise neurosurgical operations (Hruška, 2009).

Our effort was targeted on the HD transgenic minipig generation and on the HD acute minipig generation. HD transgenic minipig will be invaluable in the long-term follow-up examinations which could lead to better understanding of the neurodegeneration mechanisms in the HD brain. In addition to the transgenic model which can vary in the extent of gene expression, brain regions involved, neuropathology, and behaviors, we decided to develop a rapid-onset porcine model of HD, in which timed neuropathology and motor impairments would be predictable, robust, and experimentally testable.

This work was supported by CHDI, 1M0538, AV0Z50450515 and EMBO short term fellowship ASTF 308.00-2009

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Poster session

Expression and function of nucleophosmin/B23 during the preimplantation bovine development

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Identification of genes that are differentially expressed during bovine preimplantation development is crucial for proper understanding of early mammalian embryogenesis. Genes that are differently expressed during preimplantation development are supposed to be important for normal preimplantation development. Nucleophosmin, the significant nucleolar protein, functions as a transporting protein between cytoplasm and nucleoli. It is concerned in duplication of centrosomes, formation of ribosomes and functions as a chaperoning protein. Localization of this protein is cell cycle dependent. It is not known how important is embryonic expression of nucleophosmin for subsequent development.

At the beginning of preimplantation development embryo lacks functionally active nucleoli. Ribosomes from maternal oocyte are used for protein synthesis. Nucleoli become structurally recognizable towards the end of the third and fourth post-fertilization cell cycle - at the time when the embryonic genome activation begins. The localization of nucleolar proteins reflects in the development of the nucleoli. Nucleophosmin, one of these nucleolar proteins, may be involved in shuttling other proteins and in the assembly of pre-ribosomal particles.

Bovine zygotes were microinjected with nucleophosmin dsRNA (800ng/ul) 20h post fertilization. Two control groups were established – the uninjected group and a group injected with GFP dsRNA. After micronjection, embryos were cultivated in vitro and collected at specific developmental stages (late 8-cell stage, 16-cell stage, morula, blastocyst). The number of embryos that reached each developmental stage was counted and the quality of each embryo was determined. The level of nucleophosmin mRNA was measured by a real-time RT-PCR; the expression of protein was characterized using immunofluorescence.

We did not observe significant differences in development of control and experimental groups. The embryos with silenced nucleophosmin mRNA were able to develop until the blastocyst stage. The localization of protein in injected and uninjected embryos did not seem to be different. Our study showed that the maternal nucleophosmin protein is probably stored even after the activation of the embryonal genome and it is sufficient for embryonal development at least until blastocyst stage.

In conclusion, maternal nucleophosmin protein seems to substitute embryonal protein in the preimplantation development with no crucial defects on the embryo.

This study was supported by by a grant GAUK 43-251133 and GA CR 523/09/1035.

Polo-like kinase 1 and Aurora A cooperate in spindle assembly during meiotic maturation of mouse oocytes

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Polo-like kinase 1 (Plk1) and Aurora A (Aurka) are Ser/Thr kinases specifically activated at G2/M transition and involved in centrosome maturation and spindle assembly. Aurora A is known to be the primary activator of Plk1 early in mitosis: in association with Bora cofactor it directly phosphorylates Plk1 promoting mitotic entry. In later stages activated Plk1 mediates Bora degradation and phosphorylates Tpx2. This enables the switch to Tpx2 –Aurka association which triggers conformational changes of Aurka, stimulating its autophosphorylation and association with microtubules.

Loss of function of either Aurka or Plk1 during mitosis results in spindle abnormalities and misaligned metaphases. However, the difference is that Aurora A disruption does not lead to SAC activation and some cells enter mitosis with defects, whereas Plk1 disruption induces metaphase arrest and cell apoptosis.

In our studies we used BI2536 Plk1 inhibitor and MLN8054 Aurka inhibitor to elucidate the roles and interplay of these two kinases during meiotic maturation of mouse oocytes.

BI2536 treatment induced GVBD delay and consequently MI arrest as referred before on somatic cells. Plk1 inhibition led to premature MTOC amplification in GV and their breakdown after GVBD. In MI, MTOCs disappeared completely and gamma tubulin was not detected. Although in principle bipolar spindle formed, we observed some spindle defects and abnormal chromosome congression.

Aurora A inhibition did not reduce the resumption of meiosis as markedly as Plk1 inhibition. Neither the combination of two inhibitors increased the GVBD delay induced by Plk1 inhibition only. However, the inhibition of Aurka in MI induced mislocalization of gamma tubulin and chromosomes. Furthermore, when both kinases were inhibited, no spindle was polymerized.

We also revealed that Plk1 inhibition blocked Aurka activation in maturing oocytes. Moreover, it prevented phosphorylation of Aurka substrate, Tacc3 protein which is involved in meiotic MTOCs regulation. Our results suggest similar roles of Plk1 in mitosis and meiosis. Plk1 probably participates in resumption of meiosis, process analogous to recovery from G2/M checkpoint in mitotic cells, where Plk1 plays an important role. It also regulates spindle assembly and inhibition of its function leads to MI arrest of oocytes. According to our data Plk1 and Aurka cooperate in meiotic spindle assembly, however, their functions seem to be different. In contrast to mitosis, Plk1 mediated AURKA activation may occur from the beginning of meiotic maturation.

Paper referred:
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Development of twin blastocysts derived from single blastomeres of the 2-cell stage embryos in pigs

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In the present study, the developmental competence of intact and zona-free porcine 2-cell embryos and single blastomeres obtained from 2-cell embryos (IN, ZF and SB groups, respectively), and the quality of twin blastocysts derived from the sister blastomeres of 2-cell embryos was compared. Expression of transcripts related to developmental competence in blastocysts derived from IN, ZF and SB groups was also assessed. There was no difference in blastocyst formation rates (of the total embryos cultured) among three groups. The blastocyst yield of SB group reached 90.2% of the original number of 2-cell embryos from which the blastomeres had been separated, and significantly higher than those of IN and ZF groups. However, the total cell number in SB blastocysts (24.7 ± 1.7) was significantly lower than those in IN and ZF blastocysts (36.2 ± 2.5 and 35.0 ± 2.7, respectively). The percentages of the sister blastomere pairs both developed to blastocysts (36.6 ± 5.3%) or both degenerated (46.3 ± 10.3%) were significantly higher than that of the pairs in which one developed to blastocyst while the other degenerated (17.1 ± 7.8%). In the pairs both developed to blastocysts one blastocyst had a significantly higher cell number (31.6 ± 2.9) than that of the other one (19.1 ± 1.9). The expression of transcripts associated with cellular organization, TUBA1 and TUBB, was significantly reduced in SB blastocysts compared with IN and ZF blastocysts. However, the expression of transcripts associated with response to stresses (HSPE1, HSPD1, and HSPCA) and catabolism of glucose (ENO1, COX6C, COX7B, NDUFA4, NDUFA13, UCRC, and UQCRFS1) in blastocysts was not different among 3 groups. In conclusion, blastomere separation at 2-cell stage remarkably increased the blastocyst yield obtained from IVP embryos and might be an approach for conservation of rare pig breeds, in which the issue of lacking embryos for transfer is necessary to overcome.

Calcium-free medium including ethylene glycol for oocyte vitrification improves survival and embryonic development of vitrified rat oocytes

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Successful cryopreservation of unfertilized oocytes is one of the most valuable technologies for efficient production of animals. Even though the laboratory rats are important experimental animals and have been used for various research fields, information about cryopreservation of the oocytes is very limited. Since we recently reported successful vitrification of rat embryos at the pronuclear stage by minimum volume cooling procedure (Cryotop), the objective of the present study was to improve the vitrification protocol for unfertilized rat oocytes. Cumulus oocyte complexes were collected from the oviducts and cumulus cells were removed. To determine the optimal equilibration time, the oocytes were equilibrated in 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) + 20% fetal calf serum (FCS) for 1, 4, 7 or 10 min and then 15% EG + 15% DMSO + 0.5 M sucrose + 20% FCS for 1 min before being plunged into liquid nitrogen on Cryotops when room temperature around at 24 C. Oocytes exposed to equilibration medium for 4 min showed higher survival and cleavage rates after artificial activation. The survival and cleavage rates of vitrified oocytes after activation were 98.3 and 78.4%, respectively. Oocytes vitrified in calcium-free medium including EG showed high survival rates after warming (79.4 ± 4.3%). After artificial activation, the cleavage and blastocyst formation rates of the oocytes were also high (72.8 ± 4.0 and 23.1 ± 4.2%, respectively). The inhibitory effect of calcium and cryoprotectants (EG vs DMSO) in vitrification medium on cortical granule exocytosis (CGE) was examined. In most of the oocytes vitrified in calcium-free medium, CGE was strongly suppressed independent of cryoprotectants. The zona penetration rate of vitrified/warmed oocytes was improved by using calcium-free medium including EG after in vitro fertilization. Thus, our data suggest that calcium-free medium including EG improve survival, embryonic development and zona penetration of vitrified rat oocytes by spermatozoa.

Single blastomeres within mouse 4-cell embryo expresses different amounts of mRNA

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The aim of this study was to detect the expression patterns and levels of mRNAs in single blastomeres from the same embryo. In mammalian preimplantation embryos, the study of quantitative gene expression has been difficult because of the limited amount of starting material. Although semi-quantitative measurements of mRNA levels can be performed using several ways, these are not applicable when working with single blastomere. Here, we show the comparative quantitative analysis of mRNA from single blastomeres of mouse 4-cell embryos. Four-cell embryos were mechanically separated into single blastomeres and subjected to RT-PCR with a *Gfp* cRNA as an external control. After optimal number of cycles, in which only the *Gfp* signal could be detected, PCR products were applied for Southern blotting analysis using a specific internal probe. The large variation from cell to cell in the expression of mRNA levels was observed for all genes of interest; *ß-actin, Gapdh, CyclinB, Cateninß, Psen1, Oct4,* and *Nanog*. These results clearly show that different amount of mRNAs are expressed in single blastomere within a mouse 4-cell embryo, implying that housekeeping genes such as *ß-actin* and *Gapdh* are not suitable for endogenous control. Moreover, in order to know whether there is a correlation between the expression pattern and the location of blastomeres, each blastomere was marked with a dye and examined. However, the signal of mRNAs was randomly detected, showing no correlation with the cell allocation. The biological meaning of the phenomenon that blastomeres express high/low amounts of mRNA will be discussed.

Analysis of chromosome segregation errors in porcine oocytes using comparative genomic hybridization

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Although numerical chromosome errors are known to be prevalent and are likely to be a considerable factor influencing the mortality of early embryos and implantation failure, in farm animals, data about the frequency and nature of errors is rather limited.

Studies concerning chromosome errors in gametes and embryos of animals utilized so far FISH technique or karyotyping of chromosome spreads. However, internal drawbacks of the above mentioned methods are significant - FISH is capable of analysis of only a few chromosomes and karyotyping provides reliable data only on ploidy errors due to poor chromosome quality.

Aim of our work was to implement reliable technique in order to investigate the whole chromosome set of single cells, therefore suitable for aneuploidy study of gametes or blastomeres. We accomplished this task using methods of Whole Genome Amplification (WGA) and Comparative Genomic Hybridization (CGH). WGA-CGH is capable of detecting not only abnormalities of all chromosomes in the entire chromosome set but also determines chromosome breakages. On the other hand, WGA-CGH is not able to detect ploidy errors and balanced structural abnormalities.

In our subsequent study, we focused on porcine oocyte aneuploidy analysis. Using WGA-CGH approach we would like to provide new insight into oocyte aneuploidy and establish the frequency of chromosome errors in the examined group of oocytes.

This study was supported by Grants GACR 523/09/0743 and GAAV IAA501620801.

Inhibition of Polo kinase 1 (Plk1) affects resumption of meiosis in porcine oocytes.

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The aim of the study was to investigate the implication of Plk1 in meiosis resumption during *in vitro* maturation of porcine oocytes. When oocytes were treated with 1.5 μM of Plk1 inhibitor BI2536, phosphorylation of Plk1 at its activation site Thr210 decreased and MPF activity was inhibited as well. Moreover, resumption of meiosis was substantially suppressed as only 11% of oocytes underwent GVBD during 24 h of treatment. However, this inhibition was fully reversible as 92% of oocytes passed GVBD and majority of them reached metaphase II -stage when BI2536-treatment was followed by culture in control conditions. In the case when oocytes were subjected to BI2536 only after 8h pre-culture in control conditions the incidence of GVBD increased up to 62%. These results indicate that Plk1 plays an important role in meiosis resumption, nevertheless, the importance of Plk1 is decreasing when chromatin condensation and other events leading to GVBD are already initiated.

Role of selected kinases in CPEB-dependent cytoplasmic polyadenylation

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Cytoplasmic polyadenylation of specific mRNAs is a mechanism of translational regulation important for oocyte maturation and further development. This mechanism is mainly controlled by phosphorylation of cytoplasmic polyadenylation element binding protein (CPEB).

We focused on determination of kinases involved in CPEB phosphorylation and consequently the CPEB-dependent polyadenylation during mammalian oocyte cell cycle. Therefore we cultivated porcine oocytes in the presence of specific kinase inhibitors (roscovitine, KN-93, ZM447439, PD98059 and MLN8054). We employed poly(A)-test PCR to monitor the effect of inhibitors on poly(A)-tail extension of cyclin B1 and mos mRNAs as the markers of CPEB-dependent cytoplasmic polyadenylation. In addition, the effect of inhibitors on CPEB phosphorylation was observed by 2D–PAGE western blot.

Our results show that CPEB is modified already in the GV-stage porcine oocytes with intense phosphorylation at the time of GVBD. This late phosphorylation, assumed responsible for CPEB degradation in metaphase I, is sensitive to roscovitine implying it is mediated by CDK1 in porcine oocytes. Furthermore, the inhibition of CDK and MAPK pathways from the beginning of maturation affected the cyclin B1 mRNA polyadenylation. Thus these pathways may play a role upstream the regulatory cascade leading to the CPEB-mediated polyadenylation. The CaMKII and Aurora kinase inhibitors (KN-93, ZM447439 and MLN8054) affected later process of maturation indicating different role of these kinases in meiotic maturation of porcine oocytes.

Financial support from specific university research (MSMT no. 21/2010), Institutional Research Programme (IAPG No. AV OZ 50450515) and Czech Science Foundation GACR 204/09/H084 and P502/10/0944.

Enucleolation of growing and full-grown pig oocytes

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Recent research has shown that the maternal nucleolus is essential for embryonic development in mammals. The morphology of the nucleolus in growing oocytes differs from that in full-grown oocytes. We examined the ability of nucleoli from growing oocytes to substitute for nucleoli of full-grown oocytes in terms of supporting embryonic development in this study (Experiment 1). We also examined the effect of growing oocyte nucleoli on the maturation of full-grown oocytes (Experiment 2). Pig growing oocytes (around 100 μm in diameter) and full-grown oocytes (120 μm) were collected from small (0.6–1.0 mm) and large antral follicles (4–5 mm), respectively. Nucleoli were aspirated from full-grown and growing oocytes by micromanipulation. Since the chromatins of growing oocytes were aspirated with the nucleolus during the enucleolation process, growing oocytes were treated with actinomycin D before enucleolation. The nucleoli collected from growing oocytes were transferred to the enucleolated full-grown GV oocytes (Experiment 2) or enucleolated and matured full-grown oocytes (Experiment 1). In the Experiment 1, enucleolated full-grown oocytes matured to metaphase II (MII). After being activated by electro-stimulation, nucleoli were formed in pronuclei of sham-operated oocytes, and in the enucleolated oocytes that had been injected with nucleoli from either full-grown or growing oocytes. However, enucleolated oocytes did not form any nucleoli in the pronuclei. No enucleolated oocytes developed to blastocysts, whereas enucleolated oocytes injected with nucleoli from full-grown oocytes (15%) or growing oocytes (18%) developed to blastocysts. In the Experiment 2, enucleolated growing oocytes underwent germinal vehicle breakdown (45% vs. control 12%). On the other hand, enucleolated full-grown oocytes which had been injected with growing oocyte nucleoli stopped maturation (MII: 21% vs. control MII: 91%). These results suggest that the growing oocyte nucleolus inhibits oocyte maturation, although it can substitute for the full-grown oocyte nucleolus for early embryonic development.

Characteristics of mitochondria in bovine oocytes related to their meiotic competence

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It is generally accepted that meiotic competence of oocytes increases with increasing follicle size but it can be impaired by physiologic atresia at any time of follicular development. The present study was designed to characterize mitochondria in oocytes that were recovered from different sized follicles and showed different signs of atresia. Cyclic cows, examined for ovarian status, were used as oocyte donors. Oocytes recovered separately from medium (MF) or small follicles (SF) were categorized on the basis of morphology of both cumulus and ooplasm as healthy, light-atretic and mid-atretic oocytes. Heavily atretic oocytes were excluded from the study. A half of the oocytes of each category was matured for 24 hours according to a standard protocol. The immature (GV) or mature (MII) oocytes were denuded from cumulus cells, stained with MitoTracker Orange and fixed. The morphology of active mitochondria (diffused, granulated or clustered), their distribution (peripheral or central) were evaluated by confocal microscopy. Expression of the mtDNA replication related gene, mitochondrial transcription factor A (TFAM), was detected by real-time RT-PCR. The results were analyzed by ANOVA.

Regardless of oocyte atresia level, a significantly higher ($P < 0.05$) percentage of GV oocytes with granulated mitochondria or MII oocytes with clustered mitochondria was found for MF than for SF oocytes (55.3 or 39.4% vs. 34.1 or 19.6%). Similarly, a significantly higher percentage of oocytes with central distribution of mitochondria was determined for MF than for SF-GV oocytes. The difference in percentage of oocytes with centrally distributed mitochondria was not found between MF and SF-MII oocytes. Contrary to mitochondrial morphology and distribution, significantly higher ($P < 0.05$) level of TFAM transcript was detected for SF compared with MF-GV oocytes. This difference remained valid only for mid-atretic SF-MII oocytes.

In MF GV and MII oocytes, a significantly higher ($P < 0.05$) percentage of those with granulated mitochondria and those with clustered mitochondria respectively, was detected in healthy than in light-atretic and mid-atretic oocytes (73.3 vs 44.4 and 38.7%, and 60.5 vs 35.5 and 8.0%). In contrast to MF oocytes, a significantly higher ($P < 0.05$) percentage of SF oocytes with granulated and clustered mitochondria respectively, was found for light-atretic than for healthy and mid-atretic oocytes at GV (53.8 vs. 32.9 and 20.4%) and MII stage (36.6 vs. 17.2 and 2.9%).

At GV stage, a significantly higher ($P < 0.05$) percentage of oocytes with peripherally distributed mitochondria was observed in healthy and light-atretic, as compared with mid-atretic MF oocytes (36.6 and 20.0 vs. 4.5%). But on the other hand, a significantly higher ($P < 0.05$) percentage of oocytes with peripherally distributed mitochondria was determined in light-atretic and mid-atretic in comparison with healthy SF oocytes (62.5 and 63.2 vs. 37.8%). Very high percentages of oocytes with centrally distributed mitochondria, without differences among healthy, light-atretic and mid-atretic oocytes, were detected in both MF and SF oocytes at MII stage.

It can be concluded that bovine oocytes with different meiotic competence differ in proportions of those with diffused, granulated or clustered mitochondria. Healthy oocytes from the medium follicles and light-atretic oocytes from the small follicles seem to be more competent in terms of mitochondrial granula and cluster formation before and after maturation, respectively. The morphology of mitochondria can characterize the cytoplasmic maturity of oocytes better than mitochondrial distribution or quantification of TFAM.

This study was supported by Grants QI 91A018 and 0002716202 of the Ministry of Agriculture of the Czech Republic.

Generation of Functional Mouse Oocytes and Spermatids from Isolated Primordial Germ Cells Following Ectopic Transplantation in Adult Animals

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Primordial germ cells (PGCs) are undifferentiated germ cells in developing fetuses. As they give rise to definitive oocytes and spermatozoa that contribute to new life in the next generation, their development must be under strict control regarding genetic and epigenetic aspects. However, we do not know to what extent their development depends on the specific milieu. In this study, we transplanted mouse PGCs isolated from male and female gonads at 12.5 days post coitum under the kidney capsule of adult mice, together with gonadal somatic cells. The transplanted cells constructed testis-like and ovary-like tissues, respectively, under the kidney capsule within 4 weeks. Normal-looking round spermatids and fully grown germinal vesicle (GV) oocytes developed within these tissues. Ectopic spermatogenesis continued thereafter, while oogenesis consisted of only a single wave. The injection of these round spermatids directly into mature in vivo-derived oocytes led to the birth at term of normal pups. PGC-derived GV oocytes were isolated, induced to mature in vitro, and injected with normal spermatozoa. The injected oocytes were successfully fertilized and developed into normal pups. Our findings demonstrate the remarkable flexibility of PGC development, which can proceed up to functional gametes under a spatially and temporally non-innate condition. This transplantation system may provide a unique technical basis for the induction of the development of early germ cells of exogenous origins, such as those from embryonic stem cells.

Nuclear and nucleolar remodelling during the first cell cycle in bovine somatic cell nuclear transfer and tetraploid embryos

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Keywords: SCNT, tetraploid embryos, bovine embryo, reprogramming, UBF, fibrillarin

Cloning by the transfer of somatic nuclei into unfertilized eggs requires a dramatic remodeling of chromosomal architecture. These radical changes are unambiguously related with wide range reprogramming of somatic cell genome. Even highly differentiated somatic nuclei can be dedifferentiated in egg cytoplasm to acquire the totipotency essential for supporting normal development to reproductive adulthood. In spite of this reprogramming process, somatic cell nuclear transfer does not always result in ontogeny in mammals, and development is often associated with various abnormalities and embryo loss with a high frequency.

The study was aimed on evaluating influence of ooplasm on introduced somatic genome in tetraploid (TP) embryos compared with somatic cell nuclear transfer (SCNT) embryos during the first cell cycle. Following 20-22 h of IVM, couplets of MII cytoplasts (SCNT), MII oocytes (TP) and starved bovine fibroblasts were, after 2 h co-culture, electrically fused, chemically activated by 5 µM ionomycin for 5 min, followed by 3-4 h incubation in 2 mM 6-DMAP, and fixed at 4 and 12 hours post activation (hpa). The SCNT and TP embryos were processed for immunofluorescence localization of the nucleolar proteins: upstream binding factor (UBF) and fibrillarin at 4 and 12 hpa.. Embryos were examined on confocal laser scanning microscope (Zeiss).

At 4 and 12 hpa, UBF was formed in discrete clusters surrounded by shell-like structure labeled by fibrillarin in SCNT embryos. This kind of localization of nucleolar proteins is distinguished by nucleolus with large fibrillar centers (FC) segregated from the dense fibrillar component (DFC). At 4 and 12 hpa, all of the TP embryos displayed localization of fibrillarin to large spherical bodies. During the first 4 hpa only 29.4 % of TP embryos was labelled for UBF. At 12 hpa, UBF displayed the increased pattern of labelling in TP embryos (more than 65%).

Discrete localization of UBF in analysed SCNT and TP embryos can be explained by residual association of this protein with rDNA characteristic for somatic cells during mitosis. This hypothesis is enhanced by partial recovery of nucleolus in SCNT embryos.

Acknowledgments
The work was supported by Marie Curie Intra-European Fellowships (contract MRTN-CT-2006-035468, Clonet), VEGA 1/4323/07, VEGA 1/0057/08, CGA VI/7/2008, DFG and Alexander von Humboldt fellowship.

Involvement of EGFR and Smad2/3 activation in FSH-induced porcine oocytes-cumulus cell complex maturation

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It has been shown that the activation of both EGF receptor and SMAD2 and SMAD3 in mouse cumulus cells is essential for their preovulatory maturation. In the present study we investigated whether these changes are also required for the final differentiation of pig cumulus cells. Porcine oocyte-cumulus complexes (OCCs) were stimulated in vitro with FSH in the absence or in the presence of the following inhibitors: AG1478 (an EGFR tyrosine kinase inhibitor), SB431542 (a specific inhibitor of SMAD2 and SMAD3 activation) and SIS3 (a specific inhibitor of SMAD3 activation). We found that EGFR inhibitor AG1478 completely blocked FSH-induced cumulus expansion by preventing hyaluronic acid production and its incorporation within the extracellular matrix. In addition, in the presence of AG1478, most of FSH-stimulated OCCs were in germinal vesicle stage (GV) and did not mature. Conversely, AG1478 did not affect the stimulation of progesterone synthesis by FSH. In agreement, EGF was unable to stimulate progesterone synthesis by OCCs cultured in vitro.
The SB431542, that inhibits the activation of SMAD2 and SMAD3, only partially inhibited cumulus expansion by slightly decreasing both total production and matrix accumulation of hyaluronic acid. Surprisingly, SIS, that inhibits only SMAD3, produced an inhibitory effect higher than SB431542. A divergent effect of the two inhibitors was observed on steroid synthesis: FSH-induced progesterone synthesis increased in the presence of SB431542 and decreased in the presence of SIS. Both inhibitors did not affect the induction of oocyte meiotic resumption promoted by FSH. These results suggest that in pig, like in mice, FSH stimulates cumulus cells to produce EGF that acting in an autocrine way induces the formation of the expanded matrix and the signaling for oocyte meiotic resumption. The results also suggest that SMAD2 and SMAD3 activation is required for optimal stimulation of hyaluronic acid and proteins involved in the organization of this polymer in the pig OCC matrix. Finally, FSH stimulation of progesterone synthesis seems to be independent from EGF, while it appears to be counteracted by SMAD2, but not SMAD3 activation, thereby suggesting that specific SMAD2 signaling pathway is probably indispensable to prevent luteinization of cumulus cells.
Supported by grant GACR 523/08/0111 and VEGA 2/0153/08.

Gametegenesis following transplantation of primordial germ cells in domestic fowls

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In avian species, unlike in mammal, techniques for cryopreservation of ova or nuclear transfer are not available because of the large size of yolk structures. Therefore, primordial germ cells (PGCs), the precursors of gametes, have been attracted attention as one of the powerful materials for reproductive biology in chicken.

The first priority is clarification of the timing and migration pathway of chicken PGCs. PGCs were scattered in the central zone of the area pellucida of blastoderm just after the eggs were laid. Following the formation of primitive streak, PGCs moved anteriorly to the edge of the extra-embryonic region, so called that germinal crescent region. After enter of the developing vascular system, PGCs circulate temporarily in bloodstream and then migrate to the future gonadal region.

The unique mode of chicken PGCs migration during early development enables to combine the cryogenic preservation of PGCs from early chicken embryos with the conservation of live animals. PGCs were collected from the blood of early embryos, and cryopreserved in liquid nitrogen for over 6 months until transfer. Manipulated embryos were cultured until hatching; fertility tests indicated that they had normal reproductive abilities. Frozen-thawed PGCs were transferred into the bloodstream of recipient embryos. Viable offspring derived-from the frozen-thawed PGCs were obtained by crossing germline chimeras with manipulated chicken (efficiency was 12.6 %).

To obtain only donor-derived offspring from germline chimeras, a novel technique for almost complete replacement of the recipient germline with donor germ cells has been developed. Following application of busulfan solublized in a sustained-release emulsion (100 μg/50 μl) into the yolk of fertile eggs before incubation, nearly total depletion of the endogenous PGCs was achieved (0.6 % of control value). This was applied for preparing partially sterilized embryos to serve as recipients for the transfer of donor PGCs. Immunohistochemical analysis showed that the proportion of donor PGCs in busulfan-treated embryos was significantly higher than in controls (98.6% vs. 6.4%). Genetic cross-test analysis revealed that the germline transmission rate in busulfan-treated chickens was significantly higher than in controls (99.5% vs. 6.0%). Of 11 chimeras, 7 produced only donor-derived progenies, suggesting that these produced only donor-derived gametes in the recipient's gonads.

These studies provide powerful tools for studying germline differentiation, for generating transgenic individuals, and for conserving genetic resources in avian species.

Sperm preincubation for intracytoplasmic sperm injection (ICSI) improves developmental ability of ICSI oocytes to the blastocyst stage in rats

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Although *in vitro* fertilization (IVF) enables lots of oocytes to be fertilized at the same time, IVF protocol using cryopreserved sperm has been very recently developed in rats. Intracytoplasmic sperm injection (ICSI) is an alternative way to produce fertilized oocytes derived from cryopreserved sperm. However, contrary to mice, it is known that rat ICSI-oocytes show low developmental ability. We previously reported that removal of acrosomal membrane from sperm head improves development of ICSI-oocytes in rats. Therefore, we next examined whether injection of capacitation-induced sperm was effective in improving developmental ability of rat ICSI-oocytes.

The cumulus-oocyte complexes (COCs) were collected from the oviducts and used for IVF. After insemination for IVF, the rest of sperm was collected and cryopreserved into liquid nitrogen using a cryotube. The thawed sperm was separated into heads and tails by sonication and then injected into the oocytes (IVF-ICSI group). On the other hand, a part of sperm was incubated without COCs. After collection and freezing-thawing, the sperm was used for ICSI (Pre-ICSI group). As control group, fresh sperm was immediately used for ICSI. In control group, the proportion of 2 pronuclei formation, the 2 cell stage and the blastocyst stage was 82.6%, 48.8% and 20.2%, respectively. In IVF-ICSI group, these proportions were 82.1%, 66.0% and 34.7%, respectively. In Pre-ICSI group, these proportions were 91.0%, 65.8% and 42.4% , respectively. The rate of the blastocyst stage in Pre-ICSI group was tended to be higher than that in control group ($p<0.1$).

In conclusion, we demonstrated that the pre-incubated sperm was effective in improving developmental ability of rat ICSI-oocytes, which suggests that induction of capacitation may be also important for ICSI. Theses information will be useful for efficient production of rats via ICSI using cryopreserved spermatozoa.

Fertile bull sperm aneuploidy and chromatin integrity in relationship to fertility

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This work was supported by the Grant Agency of the Czech Republic (523/07/P077) and by the Grant Agency of the Ministry of Agriculture (1B44018).

Aneuploidy is associated with spontaneous abortions, perinatal mortality, mental retardation and with embryonic and foetal mortality. Most of these abnormalities originate as a result of meiosis errors during gametogenesis. The main purpose of the study was to analyse frequency of aneuploidies of sex chromosomes and chromosome 6 by three-colour FISH in 47 young bulls, candidates for artificial insemination program with cryopreserved semen and to investigate the influence of aneuploidies and disturbed sperm chromatin integrity on non-return rates, the frequencies of abortions, perinatal mortality and stillbirths.

The average frequencies of sperm with disomy for chromosomes X, Y, XY and 6 were 0.032%, 0.005%, 0.003% and 0.039%, respectively. The incidence of XX66, YY66 and XY66 diploidy was 0.017%, 0.006% and 0.015%, respectively. Frequencies of meiotic II errors were significantly higher than meiotic I errors ($P<0.01$). More X bearing sperm than Y bearing sperm were detected (5151 vs. 5022; $P<0.01$). Sperm chromatin damage expressed by DNA fragmentation index (DFI) was 5.3±3.7 and percentage of cells with defective chromatin condensation (HDS) was 1.4±0.8. No correlation was found between sperm aneuploidy and basic sperm analysis. The relationship was found between non-return rate and total aneuploidy ($r=-0.310$; $P=0.036$). Significant correlation was found between sex disomy, total aneuploidy (disomy of chromosomes 6, X, Y and XY sperm and diploidy) and stillbirths ($r=0.390$; $P=0.013$; and $r=0.331$; $P=0.037$). Chromosome 6 disomy correlated with perinatal mortality ($r=0.317$; $P=0.047$). HDS correlated significantly with total aneuploidy ($r=0.449$; $P=0.002$). Our study indicated that aneuploidy frequencies in young fertile bull sperm are relatively low. Nevertheless, there exists a variability in aneuploidy frequencies amongst bulls, which appears to be able to have an influence on the fertility of these animals.

Isobutyl-methylxanthine facilitates capacitation-associated protein tyrosine phosphorylation of frozen-thawed C57BL/6J mouse sperm

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In vitro fertilization (IVF) using cryopreservation of mouse sperm is routinely applied for efficient production of animals. Since lots of transgenic and gene-targeting mice have been generated, IVF using cryopreserved sperm will become more important. However, it is well known that cryopreservation of mouse sperm in C57BL/6J strain which is commonly used for production of transgenic and gene-targeting mice, decreases the fertility markedly. We recently reported that treatment of isobutyl-methylxanthine (IBMX) dramatically improved capacitation-associated protein tyrosine phosphorylation of frozen/thawed rat sperm, succeeded in IVF for the first time using cryopreserved sperm in rats. Therefore, in this study, it was examined whether IBMX treatment also improved capacitation-associated protein tyrosine phosphorylation of frozen/thawed mouse sperm in C57BL/6J strain. Cauda epididymal sperm was collected from ICR and C57BL/6J strains and cultured in TYH medium up to 3 h. Frozen C57BL/6J sperm was thawed in TYH medium supplemented with or without IBMX (0, 100, 200, 400, 800 □M) up to 3 h. At every 1 h, sperm samples were collected and used for detection of capacitation-associated protein tyrosine phosphorylation by western blotting. In both ICR and C57BL/6J fresh sperm, capacitation-associated protein tyrosine phosphorylation was induced in a time-dependent manner. On the other hand, capacitation-associated protein tyrosine phosphorylation in frozen/thawed C57BL/6J sperm without IBMX treatment was completely inhibited even at 3 h. However, IBMX treatment dramatically improved capacitation-associated protein tyrosine phosphorylation of frozen-thawed C57BL/6J sperm in a dose-dependent manner. Taken together, our results suggest that IBMX treatment is effective for improvement of capacitation-associated protein tyrosine phosphorylation in frozen-thawed C57BL/6J mouse sperm. Now we are trying to clarify whether this IBMX treatment can also improve fertility of oocytes fertilized in vitro using cryopreserved sperm in C57BL/6J strain.

Telomerase (pTERT) mRNA expression in the pig granulosa cells in vitro

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Cell proliferation in the ovary is critical to the ovarian functions of producing and releasing germ cells (oocytes) and hormones for reproduction. During the development of oocytes, folliculogenesis is accompanied by significant proliferation of granulosa cells, which is tightly controlled in a temporal and spatial manner.

Telomerase plays an essential role in cell viability and has been defined as a key factor for the regulation of cell proliferation and senescence. Under various physiological and pathological conditions the dynamics of telomerase expression and activity in proliferating, regenerating or differentiating tissue is regulated by cellular microenvironment and growth factors (Bayne and Liu, 2005). We described telomerase enzymatic activity in pig granulosa cells in vitro and we suggested that its activity and function might be differentially regulated in small and large developing ovarian follicles (Tomanek et al., 2008). The aim of presented work was to develop a new RT-PCR method of pig telomerase (pTERT) mRNA quantification and its use for the study of pTERT mRNA expression in the pig follicular granulosa cells in vitro.

Granulosa cells (GC) were isolated from small (SF-GC) and large (LF-GC) follicles and cultured for 96 h in vitro in DMEM/F12 medium supplemented with 2% FBS, ITS, gentamycin, testosterone (10^{-8}M), EGF (10 ng/ml) and pFSH (50 ng/ml). Telomerase activity was assayed with the modified protocol based on the TRAPEZE® Telomerase Detection Kit, (Chemicon). For RT-PCR, the total RNA was isolated from granulosa cells with the use of Total RNA Chemistry method and 6100 Nucleic Acid PrepStation (Applied Biosystems). Primers and TagMan MGB probes for pTERT were designed with the Primer Expres ver. 3 Program. The mRNA was reverse transcribed into cDNA with the High Capacity cDNA Archive Kit and RT-PCR was performed on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems).

Freshly isolated SF-GC expressed higher levels of pTERT mRNA than LF-GC (3.363 vs. 1.834 RU) as well as higher levels of telomerase activity (112.11 vs. 71,20 TPG/prot.). During in vitro culture, pTERT mRNA levels in SF-GC increased in control and FSH treated cells (5.815 and 6.405 RU) and slightly decreased in EGF treated cells (2.723 RU). On the other hand, LF-GC expressed significantly lower pTERT mRNA levels (1.112, 0.78 and 0.95 for control, EGF and FSH) as well as the levels of telomerase activity (8.32, 9.66 and 11.12 TPG/mg prot. for control, EGF and FSH, respectively).

In conclusion, we suggest that telomerase expression and activity in pig follicular granulosa cells is related to their proliferative potential and function in developing ovarian follicles and pTERT mRNA expression and telomerase activity decline in highly differentiated, luteinized granulosa cells.

Supported by MZE CR project 0002701402

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Isolation, cultivation and characterization of porcine cell lines derived from blastocysts produced *in vitro* and *in vivo*

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The pig represents an interesting animal model for several branches of human medicine including stem cell therapies, however, the full-functional methodics for derivation of embryonic stem cells from porcine blastocysts has not been exactly established yet. The present study was designed to compare the potential to generate porcine embryonic stem (ES) cell lines from blastocysts produced under *in vivo* and *in vitro* conditions.

We found that significant proportion of inner cell masses (ICMs) from *in vivo* obtained blastocysts attached to the feeder layer and started to grow in a monolayer. In particular, proliferating cells formed three types of colonies – i/ tightly packed colonies of small cells resembling human ES cells – ES-like, ii/ flattened colonies of polyhedral shaped, weakly packed cells – epithelial-like, and iii/ big polyhedral shaped cells revealing large intracellular area with abundant lipid droplets in the cytoplasm – trophectodermal cells. Immediately after attaching, ES-like cells revealed markers of pluripotency. However, during cultivation cells derived from *in vivo*-produced blastocysts (age 8-11 days) spontaneously differentiated into neural-like or beating cells. In contrast to *in vivo*-produced blastocysts, the proportion of attached *in vitro* obtained blastocysts was low, moreover, proliferating cells revealed mainly trophectodermal or epithelial-like phenotype.

In summary, our results suggest that *in vivo* produced blastocysts represent more suitable material for derivation of porcine cell lines with various phenotype including ES-like cells.

Comparative transcriptomic analysis of in vivo and in vitro produced bovine embryos using a custom bovine embryo-specific microarray

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Pre-implantation embryos derived by in vitro fertilization differ in their developmental potential from embryos obtained in vivo. In order to characterize changes in gene expression profiles caused by in vitro culture environment, we employed microarray constructed from bovine embryo-specific cDNAs (BlueChip, Université Laval, Québec). The analysis revealed changes in level of 133 transcripts between in vitro derived and in vivo derived 4-cell stage embryos and 96 transcripts were differentially expressed between 8-cell stage in vitro and in vivo embryos. The expression profiles of 10 transcripts (BUB3, Fibrillarin, Nopp140, PCAF, Sf3a, GPBP, GABPα, TAF7, Cul1 and CNOT4) were studied in detail by quantitative RT-PCR during whole in vitro culture period (oocyte - hatched blastocyst). Protein level and localization of Cul1 and Fibrillarin in GV and MII oocytes and 2-cell – morula stage embryos was also studied by immunofluorescence. Several identified genes can be potentially useful as markers of embryo quality in future attempts to optimize in vitro culture conditions.

Acknowledgements
This study was supported by the GA CR project No. 523/06/1226, GACR project No.204/09/H084, MSM grant 6215712403, CLONET project MRTN-CT-2006-035468, DFG and VEGA 1/0012/10 and Institutional Research Concept IAPG No. AV0Z50450515.

Comparison of media for culture of matured and fertilized rat oocytes

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Recently, embryonic stem cells have been successfully established in rats. Therefore, it seems that *in vitro* manipulation and culture of rat oocytes/embryos become more important. Although rat oocytes/embryos can be cultured *in vitro*, developmental ability of the oocytes/embryos is generally low compared to mice regardless *in vitro* manipulation. This result suggests that development of more optimal medium is required for *in vitro* manipulation and culture of rat oocytes/embryos. The objective of the present study was to clarify the factors involved in developmental ability of rat oocytes/embryos. Since it is known that rat oocytes are spontaneously activated *in vitro* and this spontaneous activation is involved in the decrease of developmental ability of the oocytes, we compared the development of oocytes cultured in different media which are usually used for *in vitro* culture of mouse and rat oocytes/embryos. Cumulus-oocyte complexes were collected from the oviducts in superovulated female rats and then cumulus cells were removed. The oocytes were cultured in different media (R1ECM, mR1ECM, KSOMaa, TYH or CZB). Extrusion of second polar body was investigated as indicator of spontaneous activation. At the result, the proportion of the extrusion in oocytes cultured in KSOMaa and TYH was lower than those in other media. When calcium was removed from KSOMaa, the extrusion was further suppressed. To clarify the *in vitro* developmental ability of fertilized oocytes cultured in different media, *in vivo* fertilized oocytes were collected from the oviducts from mated rats and then cultured in different media (R1ECM, mR1ECM, KSOMaa, TYH). As for *in vitro* development of fertilized oocytes to the 2 cell stage, the proportion of the oocytes cultured in KSOMaa was higher than those in other media. However, only fertilized oocytes cultured in R1ECM could be developed to blastocysts. In conclusion, our result suggests that calcium-free KSOMaa seems to be more optimal media for *in vitro* manipulation of rat oocytes. Although further improvements will be required, KSOMaa may be also effective for *in vitro* culture of fertilized oocytes in rats.

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Content

Published by the Institute of Animal Physiology and Genetics AS CR, v.v.i., Libechov, Czech Republic 2010

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