

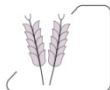


University of Thessaly



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# Maternal Interaction With Gametes and Embryos GEMINI

9-11 October 2008

Volos, Greece

Abstract Book

1<sup>st</sup> General Conference



Action FA0702

**Chair of the Action:**

*Dr Alireza Fazeli, UK*

**Vice Chair of the action:**

*Prof Fulvio Gandolfi, Italy*

**COST Science Officer:**

*Dr Ioanna Stavridou*

**Organising Committee**

Chair: Dr Georgios S. Amiridis, University of Thessaly  
Vice Chair: Dr Theodora Tsiligianni, NAGREF  
Member: Dr Alireza Fazeli, University of Sheffield  
Member: Prof Ann Van Soom, University of Ghent  
Member: Dr Jean-Pierre Ozil, INRA UMR  
Member: Prof Tom Fleming, University of Southampton



# GEMINI

This Action is aimed to promote further understanding of mechanisms of interactions of gametes and embryos with their maternal environment by joining laboratories from different countries with excellent expertise in the fields of reproductive biology. A full description of the action's activity can be found in the memorandum of understanding.

The acronym of GEMINI (Gametes and Embryos Maternal Interactions Network International) has been selected for this action.

Working groups are at the heart of our COST Action program. Through different working groups we hope to achieve the aims and objectives of our action.

There are three main working groups. These are:

- **Working Group I:** Experimental models; In vivo and In vitro
- **Working Group II:** Tools, Technologies and Systems Biology
- **Working Group III:** Maternal Nutrition

# 1st GEMINI MEETING PROGRAM

Volos, Greece, 9<sup>th</sup>-12<sup>th</sup> October 2008

## Thursday 9<sup>th</sup> October 2008:

18.00: Registration open and Reception

## Friday 10<sup>th</sup> October 2008:

8.30: Registration open

8.45-9.00: Chairman opening, **Dr. Alireza Fazeli**, *The University of Sheffield, UK*

9.00-9.50: Plenary lecture, **Dr. Thomas E. Spencer**, *Texas A&M University, USA*

## 9.50-12.50: Morning session WG 1 - Experimental models

### On the standardization of in vitro models

9.50-9.55: Introduction

Which are the functions of the oviduct that we should mimick in vitro?

**Dr. Ann Van Soom**, *The University of Gent, Belgium*

9.55-10.55: Short communications

9.55- 10.10 An *in vitro* model for transcriptome and proteome studies of oviduct-embryo interactions. **Wolf Eckhard, Regine Rottmayer, Karina Zitta, Fred Sinowatz, Marc Boelhave, Stefan Hiendleder.** *Gene Center, Munich, 25, Germany*

10.10-10.25 **Fazeli A** *University of Sheffield UK*

10.25-10.40 Sperm adhesion and release from oviduct in vitro. **Gualtieri R. and Talevi R.** *University of Naples, Italy*

10.40-10.55 Hormonal and seminal plasma influences on the pig oviduct *in vitro* and *in vivo*. **Adam J. Ziecik and Monika M. Kaczmarek.** *Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland*

10.55-11.25 Coffee break

11.25-12.00: Discussion workshop: What should a good experimental model look like?

Moderators: **Dr. A. Van Soom & Prof. A. Dinnyes**

12.00-12.50: Progress in tissue engineering, **Prof. Colin E. Bishop**, *The Wake Forest Institute for Regenerative Medicine, USA*

12.50-14.30: Buffet Lunch & Poster Session 1

14.30: **Afternoon session WG 2. Tools, Technologies and Systems**

**Biology: From tissues to models**

14.30-14.35: Introduction

Conceptional and Technical Challenges for Modelling Complex Interactions in the Reproductive Tract

**Prof. Eckhard Wolf**, *The University of Munich, Germany* &  
**Dr. Jean-Pierre Ozil**, *INRA, France*

14.35-15.15: Principles of systematic sampling and stereological investigation of complex tissues

**Dr. R. Wanke**, *Institute of Veterinary Pathology, LMU Munich, Germany*

15.15-15.30: Tea & Coffee Break

15.30-16.15: Holistic proteome studies of minimal samples: immature vs. in vitro matured oocytes. **Dr. G. J. Arnold**, *Gene Center, LMU Munich, Germany*

16.15-17.00: Mathematical modelling and control of physiological systems: a Systems Physiology approach of the gonadotrope axis

**Frédérique Clément**, *INRIA Paris- France*

17.00-17.45: Discussion workshop: What are the next steps to facilitate modelling of maternal interactions with gametes and embryos?

Moderators: **Dr. Jean-Pierre Ozil**, *INRA, France* & **Prof. Eckhard Wolf**, *The University of Munich, Germany*.

17.15-17.30: Procedural Reconstruction of a Three Dimensional Graphical Model of an Oviduct. **Mark Burkitt, Daniela M. Romano, Dawn Walker and A. Fazeli**, *University of Sheffield, UK*.

17.30-17.45: Embryo-Maternal communication in bovine oviduct: A transcriptomic Approach. **Barbara Schmaltz, P. Debey, Y. Locatelli, S. Uzbekova, C. Perreau and P. Mermillod**

17.45-18.00: Break

18.00-18.30: Discussion workshop: How we can make the best out of the COST action. Moderator: **Dr. Alireza Fazeli, GEMINI Chairman**

19.00: Dinner

**Saturday 11<sup>th</sup> October 2008:**

8.30: Registration open

8.45-9.35: Plenary lecture, "How Europe Research is organized?"  
**Dr. John Williams, INRA, France**

9.35-12.35: **Morning session WG 3. 'Maternal Nutrition Effects on Embryo and Long-Term Development'**

9.35-9.40: Introduction  
**Prof. Tom Fleming, University of Southampton, UK & Dr. Pascale Chavatte, INRA, France**

9.40-10.25: Preimplantation environment and postnatal cardiovascular and behavioural consequences. **Adam J. Watkins, Christopher Torrens, Jane K. Cleal, Clive Osmond, Judith J. Eckert, William P. Gray, Mark A. Hanson and Tom P. Fleming, Southampton UK**

Periconception maternal low protein diet: embryo responses and long-term consequences **J.J. Eckert, S. Brooks, E. Burt, R. Sihota, R. Porter, H.J. Leese, T.P. Fleming, Southampton UK**

10:25-11.00 Tea & coffee break (plus posters)

11.00-11.50 Dietary and environmental factors: effects on embryos and long-term development' **Dr. Karen Kind, University of Adelaide, Australia**

11.50-12.35 Short communications

High non-esterified fatty acid concentrations during murine follicle culture are detrimental for the oocyte's developmental competence **JLMR Leroy, V Van Hoeck, K Lemeire, PEJ Bols, R Cortvrindt, University of Antwerp, Belgium**

Fine tuning of blastocyst development by the insulin-like growth factor system in the rabbit. **Navarrete Santos Anne, Nicole Ramin, René Thieme, Sünje Fischer and Bernd Fischer Martin Luther University, Germany**

Effects of maternal undernutrition on the hypothalamic-pituitary-gonadal axis function in male and female sheep offspring. **Kotsampasi Basiliki, Papadomichelakis G. Balaskas C, Menegatos I and Chadio S. Agricultural University of Athens, Greece**

12.35–13.35: Buffet lunch (plus posters)

13.35–14.30: **Maternal Nutrition Discussion forum:**  
Moderators: **Prof. Tom Fleming**, *University of Southampton, UK* &  
**Dr. Pascale Chavatte**, *INRA, France*

Developmental effects of cholesterol enriched hyperlipidic diets in rabbits **Chavatte-Palmer Pascale**, **P. Laigre**, **M. Dahirel**, **O. Picone**, **L. Lamothe**, *INRA, France*

14.30–14.45: EMBIC: Embryo Implantation Control network”  
**Prof. G. Chaouat**, *Centre de recherche INSERM, France*

14.45-15.10: Experiences of obtaining European frame work programmes”  
**Prof. A. Dinnyes**, *Hungary*

15.10-15.20: Meeting Closure and Concluding Remarks”  
**Dr. Alireza Fazeli**, **GEMINI Chairman**

16.00-18.30: **“MC Meeting”**

19.00: **Dinner**

**Sunday 12<sup>th</sup> October 2008:**

One day Excursion

## **EXPERIMENTAL MODELS**

### **Genes and Pathways Regulating Implantation: Comparative Insights from Sheep**

**Thomas E. Spencer, Greg A. Johnson, Robert C. Burghardt, Guoyao Wu and Fuller W. Bazer**

*Center for Animal Biotechnology and Genomics, Departments of Animal Science and Veterinary Integrative Biosciences, Texas A&M University, College Station*

Implantation in mammals involves shedding of the zona pellucida, followed by orientation, apposition, attachment, adhesion and, in some species, invasion of the blastocyst into the endometrium. Endometrial invasion does not occur in domestic ruminants. Rather, definitive implantation is achieved by adhesion of the mononuclear trophoblast cells to endometrial luminal epithelium (LE) and formation of syncytia by fusion of trophoblast binucleate cells with LE. The noninvasive and protracted nature of implantation in domestic animals provides valuable opportunities to investigate fundamental processes of implantation that are shared among all mammals. After entry into the uterus on Day 4 and formation of a blastocyst by Day 6, ovine blastocysts shed the zona pellucida (Day 8), elongate to a filamentous form (Days 11-16), and adhere to the endometrial LE (Day 16). Recurrent early pregnancy loss in the uterine gland knockout ewe model indicates that secretions of the endometrial epithelia are required for blastocyst elongation and implantation. Interestingly, continuous exposure of the endometrium to progesterone for 8 to 10 days in early pregnancy down-regulates progesterone receptors (PGR) in endometrial epithelia. The loss of epithelial PGR is associated with a reduction in cell-surface mucin (MUC1) and induction of genes in LE and superficial glandular epithelium (sGE) (*CAT2*, *CST3*, *CTSL*, *HIF2A*, *IGFBP1*, *LGALS15*, *SLC2A1*, *SLC5A1*, *SLC5A11*) and then GE (*CST3*, *CTSL*, *GRP*, *SPPI*, *STC1*). These genes encode proteases, protease inhibitors, secreted adhesion molecules, and transporters of amino acids and glucose that modify the uterine milieu to support survival and growth of the conceptus. Interferon tau (IFNT), the pregnancy recognition signal produced by the mononuclear trophoblast cells of the conceptus, acts on the endometrium to induce genes in LE (*WNT7A*) or GE (*GRP*, *PRLR*), stimulate expression of several progesterone-induced genes in LE/sGE, and induce many classical IFN-stimulated genes in GE, stromal cells and resident immune cells. Interestingly, IFNT regulation of most epithelial genes requires progesterone which likely mediates its effects via progesterone receptors (PGR) from PGR-positive stromal cells. Between Days 14 and 16 of pregnancy, the binucleate cells begin to differentiate in the trophoblast and subsequently migrate and fuse with the endometrial LE to form syncytia. In the endometrial glands, *SPPI* and *STC1*, and likely other genes, are further stimulated by CSH1 (placental lactogen) from BNC. Thus, the sequential and combinatorial effects of ovarian progesterone and conceptus hormones (IFNT and CSH1) orchestrate changes in the uterine microenvironment that regulate conceptus development. Emerging evidence indicates that loss of the epithelial PGR and the actions of placental hormones in

sheep and humans modify the local uterine microenvironment to establish uterine receptivity to implantation, as well as ensure secretion of histotroph required for conceptus survival and growth. Further, common gene networks and pathways are being discovered across species during the peri-implantation period. A greater understanding of the cellular and molecular signals that regulate uterine receptivity and implantation can be used to identify causes of recurrent pregnancy loss and to improve pregnancy outcome in domestic animals and humans.

*Supported by grants from the NIH and USDA CSREES.*

## **Which are the functions of the oviduct that we should mimic in vitro?**

**Ann Van Soom**

*Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ugent, Salisburylaan 133, B-9820 Merelbeke, Belgium*

The oviduct or Fallopian tube has many important reproductive functions. When ovulation takes place, it assists at oocyte pick-up and final oocyte maturation, at the same time it provides a sperm reservoir from which only capacitated sperm cells are released and it ensures that fertilization and early embryonic development take place under optimal conditions. The oviduct is interacting with its environment by means of long range signals (e. g. the release of the sperm from the reservoir under the influence of progesterone) and by means of short range signals (e.g. the local secretion of PGE2 by horse embryos which permits their transit from the oviduct to the uterus). The main problem associated with oviduct models in vitro is the dynamic, interactive nature of the changes which happen in the oviduct, during its dialogue with the gametes or embryos. At present, two main approaches can be followed to study these interactions : the first is studying the cross-talk between the embryo and its environment by means of transcriptomics and proteomics based on in vivo specimens, and thus identifying possible trophic factors which can be applied in a in vitro model, or the second is to try and develop a dynamic and responsive in vitro model of the oviduct. Both approaches, with their respective advantages and disadvantages will be discussed in this workshop.

## **An *in vitro* model for transcriptome and proteome studies of oviduct-embryo interactions**

**Wolf Eckhard, Regine Rottmayer, Karina Zitta, Fred Sinowatz, Marc Boelhauve, Stefan Hiendleder**

*Gene Center, LMU Munich, Feodor-Lynen-Str. 25, Germany*

A short-term (24 h) culture system for bovine oviduct epithelial cells (BOEC) was established and evaluated. BOEC were obtained on Day 3.5 of the estrous cycle and processed by mere mechanical means to gain cell aggregates maintaining natural junctions between neighboring cells. Light microscopic examinations showed vigorously beating cilia on the apical side of the BOEC in aggregates. Scanning electron microscopy and transmission electron microscopy confirmed ultrastructural characteristics of BOEC at seeding and after 24 h in culture very similar to the situation *in vivo*. The purity of the epithelial cell culture was > 95 %, as assessed by immunocytochemical methods. For further characterization of cultured BOEC, gene expression patterns were examined after different time spans in culture. Cultured BOEC isolated from ampullae ipsilateral to the ovulation site yielded significantly higher amounts of RNA than their contralateral counterparts ( $2.73 \pm 0.98$  versus  $2.31 \pm 0.14$   $\mu\text{g}$  per  $10^6$  cells). However, quantitative PCR did not detect significant differences in transcript levels between ipsi- and contralateral BOEC for the majority of marker genes (*ESR1*, *ESR2*, *HMGCR*, *OVGP1*, *PGR*, *TRAI1*) throughout the 24 h culture period. For *GPX4*, a gene known as differentially expressed *in vivo*, combined data throughout the culture period showed a significant effect. Marker gene expression of five genes remained stable after 6 h of cell culture, indicating only a short adaptation period after which the culture system provides constant culture conditions. The use of two different sera (estrous cow serum versus cow serum obtained on Day 3.5 of the estrous cycle) in a concentration of 2 % did not affect gene expression patterns, suggesting stable culture conditions. Western blot analysis confirmed ESR1 (estrogen receptor  $\alpha$ ) and PGR (progesterone receptor) protein expression throughout the culture period. In agreement with cyclic differences *in vivo*, stimulation with 10 pg/ml estradiol-17 $\beta$  increased *PGR* transcript abundance in BOEC significantly. A response to the stimulation with 10 ng/ml progesterone was shown as *INOS* (inducible nitric oxide synthase) gene expression increased significantly after steroid treatment. Thus, this culture system provides functional BOEC with an unchanged morphology and maintained functionality compared to cells *in vivo*. It is useful for co-culture experiments with bovine embryos and provides cultured cells in sufficient quantities for holistic transcriptome and proteome studies, thereby helping to decipher early embryo-maternal communication.

**In vivo and in vitro models;  
What we have used? What answers we have got? What we hope to  
have?**

**Alireza Fazeli**

Academic Unit of Reproductive and Developmental Medicine, University of  
Sheffield, Level 4, Jessop Wing, Three Root Walk, Sheffield S10 2SF,  
United Kingdom  
[A.fazeli@sheffield.ac.uk](mailto:A.fazeli@sheffield.ac.uk)

In vivo and in vitro models provide unique opportunities for studying maternal communication with gametes and embryos. Their ease of use, flexibility, cost effectiveness, potential for standardisation are the main characteristics driving their use in our laboratory. In vitro models in comparison with in vivo models, fail to take into account the physiological multiple interactions occurring within the whole organism and therefore they may produce false results or artefacts. But the ease of use of in vitro models, their cost effectiveness and the possibility of providing a controlled and standard environment for experimentation make them ideal choices for systematic approach based investigations.

During the presentation I will exemplify some comparisons between in vivo and in vitro models from data produced in my laboratory. Furthermore I will discuss the potential differences between different in vitro models of female reproductive tract.

A need exists in our field to standardise the in vitro models that we use. Standardization of these models will allow comparison between results obtained in different laboratories. Maybe the first step to achieve this goal is to characterise certain markers that can be used as indications of female reproductive tract environment at the time that particular maternal interactions with gametes and embryos has been measured.

I hope the above would provide some material for the moderated discussion planned during this session.

## **Sperm adhesion and release from oviduct in vitro**

**Gualtieri R. and Talevi R.**

*Dpt. Structural and Functional Biology. University of Naples Federico II, Italy*

The mammalian oviduct is the natural site where crucial reproductive events, i.e., final gamete maturation, fertilization, and early embryo development, naturally take place. In several mammals the interval from the onset of estrus to ovulation may cover several hours or even days. The oviduct provides a suitable environment where spermatozoa transiently adhere to the epithelial cells of isthmus and the motility and capacitation of adhering spermatozoa remain suppressed until ovulation-associated signals induce detachment of selected and stored spermatozoa allowing their migration toward the ampulla for fertilization. Early in vivo oviduct transillumination studies in naturally mated small rodents, such as mice and hamsters, that represent favourable animal models, provided fundamental information about the sperm ascension through the oviduct. However, the development of in vitro sperm oviduct interaction systems has been indispensable to understand the cellular and molecular mechanisms involved. To this end, two oviductal epithelial culture stages have generally been used: explants an early in vitro culture stage and cellular monolayers that form after several days of culture. Although culture of explants until they attach to the substrate and progressively form a monolayer is accompanied by some signs of de-differentiation such as the regression of cilia on the cell apical surfaces and decrease of the cell height, we generally adopted monolayers for ease of handling, fluorescence analysis and bound sperm counting.

The choice to use this culture stage to study bovine sperm-oviduct binding and release in vitro has been substantiated by numerous experiments demonstrating the maintainance of several important features expressed by explants, as well as by the oviduct in vivo. Monolayers retain the ability to bound spermatozoa, to maintain their motility far longer than in unbound spermatozoa, to depress their capacitation, and spermatozoa adhering to monolayers or to explants respond to the treatment with releasing signals in a similar manner. Our studies have been focused on the mechanisms involved in sperm adhesion, selection and release. Main result demonstrate that two different class of molecules, reportedly represented in the oviductal fluid, are capable to induce the sperm release from oviductal monolayers in vitro: sulfated glycoconjugates and disulfide reductants. The possibility to release the sperm sub population selected by in vitro adhesion allowed to demonstrate its superior ability in zona pellucida binding and fertilization. Moreover, we recently showed that disulfide-reductants and sulfated glycosaminoglycans release spermatozoa bound to the fallopian tube epithelium in vitro through the reduction of sperm surface proteins. More interestingly, released spermatozoa promptly recover the ability to adhere after removal of the disulfide-reductant and such a recovery is associated to reoxidation of sperm surface proteins sulphydryls and to reversal of capacitation. These results suggest that the ascension of spermatozoa through the oviduct might be fine tuned by different reversible and irreversible releasing signals.

## **Hormonal and seminal plasma influences on the pig oviduct *in vitro* and *in vivo***

**Adam J. Ziecik and Monika M. Kaczmarek**

*Institute of Animal Reproduction and Food  
Research of Polish Academy of Sciences, Olsztyn, Poland*

Oviduct plays a critical role in reproduction by being the site of fertilization and early embryonic development. We have previously shown that the porcine oviduct possesses immunoreactive and functional LH receptors detected in the epithelium of the tubal mucosa and smooth muscle cells of the pig myosalpinx. Treatment with LH *in vitro* caused relaxation of the oviduct, especially during the peri-ovulatory stage of the estrous cycle. There is also evidence that the combined estradiol and progesterone priming is necessary for LH-induced relaxation of the oviduct in ovariectomized pigs.

The objective of the next studies was to examine the relationship between preovulatory LH surge, estradiol and progesterone levels and EMG activity of the oviduct and uterine horn in gilts using radiotelemetry system. The frequency of EMG bursts in isthmus was significantly reduced by 40-60 percent during period of LH surge in gilts with spontaneously and GnRH-induced preovulatory surge. The decreased frequency and amplitude of bursts was also found in ampulla when compared to period 48-0 h before LH surge. In both groups a rise in progesterone secretion was found 36-40 h after preovulatory LH surge. These results indicate that preovulatory LH surge may play a role in relaxation of oviduct contractility in pigs.

Recent studies have indicated that introduction of semen/plasma (SP) into female reproductive tract orchestrates molecular and cellular changes facilitating embryo development, conception and pregnancy. These changes were also observed in the expression of enzymes of prostaglandin (PG) synthesis pathway. Since the oviduct plays a decisive role in reproduction providing a beneficial environment for gamete maturation, fertilization and early embryonic development, we have investigated whether intrauterine infusion of SP can modulate PG synthesis in the porcine oviduct through the regulation of prostaglandin synthesis enzymes gene expression. We showed for the first time that intrauterine infusion of SP can significantly decrease PGF and PG 9-ketoreductase synthases in oviducts 24 h after SP infusion into the uterine horns when compared to PBS-treated animals both on mRNA and protein levels. In contrast, gene expression of PGHS-2, mPGFS and PGIS was not affected by the SP treatment in all tested periods. Thus, it is possible that SP-induced lower expression of PGFS and 9-KR, which converts PGE<sub>2</sub> to PGF<sub>2α</sub> in the porcine oviduct, may act in favor of PGE<sub>2</sub> action as a mediator of early embryonic development.

The results of these investigations indicate that LH and SP should be added to the list of factors affecting oviduct function.

Poster Session

1.

**Expression of Leukemia inhibitory factor and its receptor during oocyte maturation of adult and pre pubertal sheep.**

**Brevini Tiziana A.L., Kazutsugu Matsukawa, Stefania Antonini, Georgia Pennarossa, Fulvio Gandolfi, Pasqualino Loi and Grazyna Ptak**

*Department of Animal Science, University of Milan,*

*Department of Comparative Biomedical Sciences, University of Teramo, ITALY*

Leukemia inhibitory factor (LIF ) is expressed in the mouse uterus at ovulation and prior to implantation. The presence of LIF and its receptor (LIFR) has also been reported in the ovary in human, mouse and rat at different stages of follicular development. A beneficial influence of this cytokine on pre-implantation embryo development has been shown in several species; however LIF direct effect on the oocyte has not been studied yet.

Aim of the present experiments was to investigate the expression of this cytokine specifically in GV and MII stage sheep oocytes, and their surrounding cumulus cells, both in adult and pre pubertal sheep. For this purpose, RNA was extracted and subsequently reverse transcribed. PCR was carried out with primers that were designed based on bovine and human deposited sequences. GV oocytes were matured: a) in the presence of LIF, b) LIF +anti-LIF antibody c) in control conditions. Following maturation, part of the oocytes was assessed for molecular studies, part for immuno-localization, while the remaining eggs were activated and cultured to the blastocyst stage in standard media. The proportion of developed embryos was evaluated for each group.

PCR studies demonstrated the presence of the LIF/LIFR system in sheep oocytes and in cumulus cells, both at GV stage and at the end of maturation. These results were common to adult and pre-pubertal animals. The system appears to be biologically active and responsive since LIF putative action on cumulus oocyte complexes was inhibited by the antibody against this cytokine. This inhibition involves a reduction of LIFR density on the oocyte membrane, as evidenced by immuno-staining.

Altogether, these findings demonstrate the presence of the LIF/LIFR system in ovine cumulus oocyte complexes and indicate an autocrine/paracrine mechanism of action of this cytokine during the final stages of development of the female gamete.

2.

**Developmental competence of mammalian oocyte is promoted by the presence of Leukaemia Inhibitory Factor (LIF) during transition from germinal vesicle (GV) to metaphase II (MII) *in vitro***

**Ptak Grazyna, Brevini TAL.\*, Gandolfi F.\*, Czernik M., Pizzuto A., Zacchini F., Scapolo PA., Loi P.**

*Department of Comparative Biomedical Sciences, University of Teramo, Italy*

*\*Department of Animal Science, University of Milan, Italy*

Oocyte development is directed by the coordinated expression of cytokines and growth factors. Among them, a functional LIF/LIF receptor system is crucial for various stages of ovarian follicle growth. The indispensability of LIF receptor subunit, GP130 for oocyte ovulation lead us to test the hypothesis that LIF expressed by granulosa cells promotes the maturation of mammalian oocyte. In order to mimic the physiological production of LIF during oocyte transition from GV to MII, mechanically denuded sheep oocytes (i.e. deprived of physiological LIF source) and control cumulus-oocyte-complexes (COCs) were matured *in vitro* (IVM) in medium supplemented with 2000 U/ml of recombinant LIF. Oocytes were fertilized and cultured to blastocyst stage. Blastocyst quality was determined by counting inner cell mass (ICM) cells. LIF significantly enhanced the rate (16% vs. 2%) and quality (ICM: 16 vs. 5) of blastocysts derived from denuded oocytes, whilst the development of blastocysts derived from sheep COCs matured with LIF was not significantly different from controls (36% vs. 33%; ICM: 24 vs.30). In a second series of experiments we tested whether a insufficiency of LIF is involved in determining the low competence of COCs collected from prepubertal sheep. Quantitative analysis by Western blot indicated that LIF/LIF receptors levels in prepubertal sheep granulosa cells are lower than in adults. We observed that the presence of 2000 U/ml of LIF in IVM medium significantly enhanced the rate (28% vs. 8%) and quality (ICM: 22 vs. 12) of blastocysts from prepubertal sheep oocytes. Our results suggest that insufficient LIF level during *in vitro* maturation decreases oocytes developmental competence. Therefore it is advisable to add LIF during IVM, particularly, in situations where granulosa cells, its natural source, are not sufficient or otherwise compromised.

3.

**Functional damage of mammalian blastocyst temporary exposed to polychlorinated biphenyls (PCBs)**

**Zacchini Federica ,Czernik M, Scapolo PA, Loi P, Ptak G.**

*University of Teramo, Department of comparative Biomedical Sciences, Piazza A. Moro, 45; 64100 Teramo, Italy*

It is generally accepted in teratology that the exposition of the preimplantation embryo to the toxic agent causes its imminent death or, if the damage confers only its small proportion, the embryo will recover and continue its development. This is because totipotent embryonic cells, whenever damaged, can be replaced. A number of surveys conducted on populations exposed to environmental contaminants, PCBs, revealed that they represent an important risk factor for the establishment of pregnancy. Whenever and how the short exposition of preimplantation embryos to PCBs is able to influence their development is not known. To study this, sheep blastocysts produced in vitro were incubated for 48 hours with the mixture of 60 PCBs congeners, Aroclor 1254 (A1254) at two different doses, than analysed for 5-bromo-deoxyuridine incorporation (5BrdU) or allowed to restore (48 hours) and analysed for 5BrdU or the ability to outgrowth in vitro. A1254 presented dose-dependent (40 and 20 µg/ml) significant effects vs. control embryos, respectively: inhibition of cell proliferation (33%/40%/60%) which was reversible, as arrested blastocysts resumed a normal growth rate upon A1254 removal; delayed and decreased occurrence of blastocyst's outgrowth (58%/35%/21%); and finally, the arrest of outgrowths following 6 days of culture (0%/14%/20%). This suggests that following exposition to A1254, the embryo is able to restore the original growth rate and even to implant, as demonstrated by their outgrowths in vitro. Notwithstanding, numerous outgrowths derived from treated blastocysts were arrested presumably due to a great extend of ICM damage. Concluding, the exposition of blastocyst to PCBs can initially cause growth restoration followed by its subsequent arrest. It implies that the initial survival of the preimplantation embryo following its exposure to PCBs may not ensure the successful accomplishment of gestation.

4.

**In vitro or in vivo post-fertilization embryo culture affects the gene expression patterns of ovine prepubertal embryos.**

**Bebbere Daniela, Luisa Bogliolo, Stefano Fois, Federica Ariu, Giovanni G. Leoni, Fiammetta Berlinguer, Salvatore Naitana, Sergio Ledda.**

*University of Sassari, via Vienna n°2, 07100 Italy*

Production of embryos in vitro from prepubertal females has enormous potential for research and commercial applications. However, embryos derived from those donors showed reduced in vitro development rate and viability after transfer in recipients. Although the oocyte origin can play a crucial role in determining the developmental competence, the post-fertilization embryo culture environment may affect the blastocyst yield and quality.

Aim of this study was to examine the relative transcript abundance of a panel of developmentally important genes in in vitro and in vivo cultured prepubertal ovine embryos. Cumulus oocyte complexes derived from ovaries of regularly slaughtered prepubertal sheep were matured in vitro in TCM199 with 10% heat-treated OSS, 10 µl/ml of FSH/LH and 100 µM cysteamine, in 5% CO<sub>2</sub> in air at 38.5°C for 24 h. Matured oocytes were fertilized with frozen-thawed ram semen in SOF medium + 2% OSS for 22 h at 38.5°C and 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> atmosphere. Thirty hours post-fertilization, early cleaved embryos (two cell stage) were divided in two groups for culture either in vitro, in SOF + aa + 0.4% BSA in 5% CO<sub>2</sub> and 5% O<sub>2</sub>, or in vivo, in ewe oviduct. Seven days post-fertilization, three groups of 8 blastocysts for each class (3 replicates) were recovered and used for gene expression analysis by reverse transcription followed by Real Time PCR. Higher relative abundance for NaKATPase, IGF2R and Nanog transcripts was observed in in vitro cultured embryos than in in vivo cultured ones (ANOVA; P<0.05), while no differences were detected for OCT4, Aquaporin 3, E-cadherin and IGF2 mRNAs in the two groups. Since embryos belonging to both classes were produced by in vitro fertilization of in vitro matured oocytes, the post-fertilization embryo culture environment itself seems to affect the gene expression pattern of several important genes at the blastocyst stage.

5.

**Preliminary Results: “Can BSA be Used as a Semi-defined Diluent  
in Ram Semen?”**

**Ali Ammar B. T., Pasciu V., Naitana S.**

*University of Sassari v. Vienna 2, 07100 Sassari - Italy*

Semen diluents for most species usually contain fructose or glucose, egg yolk (EY), and glycerol. However, preparation of uniform diluent containing EY is difficult. The objective of this work was to obtain semi-defined ram semen diluents using bovine serum albumin (BSA).

In this study we examined i) the effect of different concentrations of bovine serum albumin (10, 15, and 20% BSA) in semen diluents lacking egg yolk (EY) vs. traditional semen diluent with 20% EY. Frozen-thawed spermatozoa were examined for motility, viability, morphology and sperm acrosome integrity. ii) frozen-thawed spermatozoa prepared with different diluents were used to create IVP embryos. Sheep oocytes were collected from slaughterhouse-derived ovaries and were matured for 24 hr. They were divided into four different groups, fertilized using four frozen-thawed ram semen diluents (single ejaculate), and cultured according to our laboratory protocol (Ledda et al., 1999). The embryos that reached expanded blastocysts stage (Day 6-8) in each group were recorded and vitrified.

The rate of sperm motility after thawing (0 hr) was significantly ( $P<0.05$ ) higher in the 10 and 15% BSA and EY groups vs 20% BSA. The rates of morphological abnormality of sperm in the 10 and 15% BSA and EY groups were significantly ( $P<0.05$ ) lower than in the 20% BSA group at 0 hrs. The rate of sperm viability at 0 hr was significantly ( $P<0.05$ ) lower with 20% BSA vs 10 or 15% BSA or EY.

There was significant difference in cleavage rate( $P<0.05$ ) between 15 vs 20 % BSA groups, and significant difference in blastocyst rates ( $P<0.001$ ) between 20% BSA vs EY groups (Table 1).

In conclusion, the preliminary results support use of 10 or 15% BSA in place of EY as a semi-defined ram semen diluents.

**Table 1:** Rates of cleavage and blastocyst formation rates after fertilization with different frozen-thawed semen diluents.

Diluents Treat.	No. Oocytes	Cleavage Rate*	Blastocyst 6d	Blastocyst 7d	Blastocyst 8d	Total Blastocyst Rate**
20 % EY	44	37/44 (84.0) <b>a</b>	-	8	-	8/37 (21.6) <b>a</b>
10 % BSA	60	48/61 (78.6) <b>a</b>	2	13	3	18/48 (37.5) <b>ab</b>
15 % BSA	49	37/51 (72.5) <b>ab</b>	2	12	-	14/37 (37.8) <b>ab</b>
20 % BSA	58	55/62 (88.7) <b>a</b>	6	21	2	29/55 (52.7) <b>b</b>

\* *a-b* Values in a row of column with different letters differ ( $P<0.05$ ).

\*\* *a-b* Values in a row of column with different letters differ ( $P<0.001$ ).

Ledda et al., 1999. *J. Anim. Sci.* 77: 2234 - 2239.

6.

**Difference in blastocyst developmental rates between embryos derived from sex-sorted and non-sorted ram sperm, both in vivo and in vitro.**

**Beilby Kiri Helene, C Grupen, WMC Maxwell, G Evans**

*The University of Sydney RMC Gunn Bldg (B19), NSW, AUSTRALIA*

Sex-sorted ram sperm produce similar or superior pregnancy rates than non-sorted sperm after laparoscopic insemination [1, 2]. To further assess the fertility, embryo production and developmental rates were examined after the insemination of superovulated ewes with either sex-sorted or non-sorted sperm. Merino ewes (n = 30) were synchronised in oestrus and hormonally stimulated with PMSG (600 i.u.) on sponge removal (SR), FSH (133mg) at decreasing doses every 12 h for 4 d before insemination, and GnRH 24 h before insemination. Each ewe was inseminated in the uterus by laparoscopy 42-44 h after SR with  $15 \times 10^6$  motile sex-sorted or non-sorted spermatozoa. Antegrade flushing of both uterine horns was performed on day 6 after insemination. A parallel study was conducted using semen from the same ejaculates to compare embryonic development resulting from the in vitro insemination of sex-sorted and non-sorted sperm. There was no difference in the overall rate of blastocyst production from sorted or non-sorted sperm in vitro (d6, d7) or in vivo (d6). In vivo embryo development was more advanced after the insemination of non-sorted sperm with a greater proportion of hatched blastocysts recovered on day 6 compared with sorted sperm (p = 0.007). In vitro embryo development was also more progressive for non-sorted treatments with an increased number of hatching blastocysts on day 6 (p = 0.043) and expanded blastocysts on day 7 (p = 0.035). In conclusion, the use of sex-sorted sperm did not affect the efficiency of embryo production. However, the development of embryos derived from sex-sorted sperm was delayed compared with those from non-sorted sperm, both in vitro and in vivo. The application of in vitro embryo production using sorted sperm would appear to accurately represent in vivo events. Furthermore, the slowed development of embryos derived from sex-sorted ram sperm would not appear to negatively impact pregnancy rate.

1. de Graaf et al. 2007. *Reproduction in Domestic Animals* 42, 648-653
2. Beilby et al. 2008. *Theriogenology*, in press.

7.

## **Interaction of semen with the female reproductive tract using the pig as model**

**Waberski Dagmar, H.-J. Schuberth, A. Schnapper, Ä. Honnens, H. Henning, R.H.F. Hunter**

Unit for Reproductive Medicine, University of Veterinary Medicine Hannover,  
Buenteweg 15  
D- 30559 Hannover, Germany

The communication between semen and the female reproductive tract may serve to select a fertilization-competent sperm population, to regulate fertilization-associated events and to create a sensitive microenvironment for embryonic development. A number of in vivo and in vitro models were established to study the interaction between seminal plasma, sperm and cells of the female reproductive tract using the pig as model. Sperm selection is examined using in vitro oviduct binding assays and the in vivo Hannover Gilt model. The characterization of the oviductal sperm population compared to the original sperm population used for insemination gives valuable insight in relevant selection criteria. Analyses of sperm bound to oviductal explants in vitro and accessory sperm in the zona pellucida from in vivo-derived embryos revealed that sperm with an unstable chromatin have a reduced ability to form the oviductal sperm reservoir and consequently participate in the fertilization process only to a low extent. Further in vivo models enable the study of cell-to-cell and endocrine signalling in the female tract before and during fertilization. Of our particular interest is the role of cytokines for regulation of local immune responses affecting the time of ovulation, and possibly fertilization success and embryonic survival. Semen-induced cytokines may undergo counter-current transfer to the ipsilateral ovary and accelerate peri-ovulatory events. In vivo-models used include the cannulation of mesometrial lymphatic vessels, application of cytokines into the ovarian artery and the study of ovarian follicular blood flow by coloured Doppler sonography. The response of uterine lymphatics and their white cell population has been examined after unicornal infusion of semen in oestrous gilts. Immunohistochemical analyses revealed significantly ( $P < 0.05$ ) higher values for MHC class II-positive cells in the semen-treated horns compared to control horns as well as for tissue samples from the utero-tubal junction compared to the uterine wall. Current studies are underway to further elucidate the local response to seminal plasma in the female reproductive tract.

8.

**Immortalization and characterization of porcine endometrial cell lines, a new approach to *in vitro* model of implantation.**

**Bodek Gabriel, Agnieszka Blitek., Jolanta Kiewisz, Adam J. Ziecik.**

*Institute of Animal Reproduction and Food Research of Polish Academy of Sciences,  
Tuwima 10, 10-747 Olsztyn, Poland*

To establish endometrium *in vitro* model we have generated the permanent cell lines from porcine uterine somatic cells. Endometrial cells, including luminal epithelium (LE), glandular epithelium (GE) and stroma (ST) cells, were enzymatically isolated from the uterus of a day 3 of estrous cycle. After removing fibroblasts and red blood cells, the primary cultures were immortalized by transfection with a Simian virus 40 T-antigen (SV40 T-ag). Fugene 6 was used as a transfection agent for next 4-6 hours in the culture medium. The fast proliferating cells were selected from cultures and initially scanned for presence of transfected vector the SV40 T-ag. All isolated colonies expressed transfected vector. Expression of SV40 T-ag gene was confirmed by RT-PCR. Phase contrast microscopy revealed that LE and GE cells exhibit cobblestone morphology, whereas ST cells exhibit spindle-shaped morphology cells. Immunostaining showed LE and GE cells positive for vimentin and cytokeratin, while ST cells were positive for vimentin only. Real time PCR indicated that all cell lines expressed both estrogen and progesterone receptors. Western blotting showed expression of PGE<sub>2</sub> (PBES<sub>1</sub>) isoform in epithelial cells. Stimulation of glandular cells by estradiol (1-100nM) and progesterone (10-1000nM) showed tendency to the increase in proliferation rate. However, lower doses of pLH (luteinizing hormone, 10-100pM) also stimulated the proliferation of glandular cells, whereas the highest dose (1000pM) caused the inhibition of proliferation. Taking together immortalized cell lines can serve as a perfect *in vitro* model for studying interactions between endometrial cells and embryos in the pig.

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9.

**The rabbit embryo as an alternative model to re-visit in-vitro-culture-induced alterations of embryonic gene expression.**

**Leandri Roger Dominique, Archilla, C.; Vignal, C.; Peynot, N. ; Renard, J.P. and Duranthon, V.**

*INRA Jouy en Josas, UMR BDR 78352 Jouy en Josas France*

In vitro culture (IVC) has now been shown to affect gene expression in early embryos. Large scale analyses have been mainly assessed using the mouse embryo. This embryo displays however specific features during early development, and this is the reason why we aimed at studying the IVC effects using the rabbit embryo as an alternative and more accurate model for mammalian embryo.

A nylon microarray (2000 unique genes) dedicated to rabbit embryo EGA (embryonic genome activation) and first differentiation was constructed. At two developmental key stages, EGA and blastocyst stage, we compared gene expression patterns of embryos obtained in four conditions : B2 medium, B2 plus 2,5 % fetal calf serum (B2S), a sequential human medium (ISM1/ISM2) and in vivo. Genes with a significant culture condition effect were selected by ANOVA and multiple correction testing (Benjamini-Hochberg procedure (FDR)). Expression of these genes between two conditions was further compared using paired T-test.

111 and 87 genes displayed a significant culture condition effect at the early morula (EGA) and blastocyst stage respectively. These two sets of genes very partially overlap. Whatever the stage, the number of genes showing a differential expression between in vitro and in vivo conditions was similar for B2, B2S and ISM1/ISM2.

At EGA, the number of genes commonly affected by the three in vitro conditions suggests a common “in vitro condition” effect on gene expression which is no more the case at the blastocyst stage. Several genes involved in mitochondrial function are under-expressed whatever the in vitro condition.

At the blastocyst stage, the effect of IVC highly depends on the medium and a large variety of gene functions is affected. Among the genes commonly affected by in the 3 in vitro conditions, important genes involved in energetic metabolism, cell differentiation and adaptative response to external stimulus are under expressed.

Further experiments are now required to analyze post-implantation consequences of these in vitro culture conditions.

10.

**Can the female reproductive tract and the zona pelucida select for dna-unfragmented spermatozoa?**

**Hourcade Juan de Dios, M. Pérez-Crespo, B. Pintado\* and A. Gutiérrez-Adán**

*National Institute of Agricultural Research and Technologies; \* National Center of Biotechnology, Crtra de la Coruña, Km 5.9 Madrid SPAIN*

The aim of this work was to determine if DNA integrity is one of the keys of spermatozoa selection in female track. DNA damage of spermatozoa was artificially induced by scrotal heat treatment (HT) (42°C, 30 min). Twenty-one days after HT, spermatozoa were recovered from the epididymis caudae of CD1 mice and from the uterine horns near the cervix (Uc), from the uterine horns near the oviducts (Uo), and from the oviducts (Ov) of CD1 females 1-2 h after mating with HT and control males. In each region we determined numbers of spermatozoa, motility and sperm DNA integrity by COMET. Females naturally mated with HT or control males were sacrificed at Day-14 of pregnancy, and number of foetuses/resorptions was recorded. Additionally, IVF was performed with epididymal sperm from HT or control males. Two hours after IVF attached and un-attached spermatozoa to the ZP were recovered and samples were evaluated for sperm motility (CASA), sperm zona-binding, and sperm DNA fragmentation (COMET). Also cleavage rate of fertilized oocytes with sperm from HT or control males were analyzed. One way ANOVA was used to compare the results form each group. Epididymal sperm count ( $12 \cdot 10^6$  and  $4.4 \cdot 10^6$  control vs HT), sperm motility (75% and 21% respectively) and testis weight (133.90 mg and 68.76 mg, respectively) were significantly reduced after heat treatment ( $P < 0.001$ ). For the heat treatment, COMET values decreased significantly during the transit from Uc to Uo and from Uo to Ov (COMET Moment: 12.5, 8.5, and 2 respectively,  $p < 0.001$ ). Heat treatment reduced numbers of foetuses ( $7 \pm 0.5$  vs  $5 \pm 0.49$ , control vs HT) but number of resorptions was not altered. Spermatozoa bound per ZP in IVF experiments ( $55 \pm 7$  and  $13 \pm 6$ , control vs HT) and cleavage rate ( $61 \pm 1$  vs  $15 \pm 6$ , control and HT respectively) were significantly reduced in the HT group. Two hours after IVF, Spermatozoa attached to the ZP in HT group showed a significant decrease in COMET parameters compared with unattached spermatozoa. Our results indicate that DNA integrity sperm selection mechanisms are present in both, the female tract and the ZP.

11.

## **Using Embryonic Stem (ES) Cells To Model Early Development: An Initial Comparison of DNA Methylation Status in ES and Differentiated Cells between Species**

**Lucas Emma \*, Smith, N.M., Denning, C.D. and Young, L.E.**

*Wolfson Centre for Stem cells, Tissue Engineering and Modelling (STEM), University of Nottingham, Centre for Biomolecular Sciences, University Park, Nottingham NG7 2RD, UK*

*(\*Present address: School of Biological Sciences, Building 62 Boldrewood Campus, University of Southampton, Southampton, SO16 7PX)*

During mammalian preimplantation development, DNA methylation has been shown to undergo genome-wide and sequence specific remodelling in a species-specific manner. Alterations in maternal nutrition and *in vitro* culture have been shown to adversely influence this remodelling, leading to later inappropriate gene expression and phenotypic manifestations, e.g. large offspring syndrome. However, changes in post-implantation DNA methylation have been less well examined.

Embryonic stem (ES) cells, derived from the blastocyst inner cell mass (ICM) present an attractive opportunity to study DNA methylation changes during differentiation *in vitro* as these cells progress from a pluripotent status to one of lineage commitment, and to model the effects of altered maternal environment on specific differentiation pathways. We aimed to provide an initial characterisation of the global DNA cytosine methylation (5-methylcytosine) status of ES cells from both mouse and human, in relation to differentiated fibroblasts from the same species. Relative undermethylation of ES cells was observed in both mouse and human in comparison to fibroblasts. Additionally, differential organisation of the 5-methylcytosine signal was observed in interphase nuclei from ES cells and fibroblasts. Expression analyses revealed that the DNA methyltransferase enzymes, responsible for establishment and maintenance of cytosine methylation, were differentially expressed in correspondence with their differing developmental roles. Expression of the *de novo* methyltransferases, *DNMT3A* and *DNMT3B*, was higher in ES cells than fibroblasts, in both mouse and human. In mouse, nuclear localisation of *Dnmt1* protein was observed in both cell types, as well as in human fibroblasts. Surprisingly, in human ES cells nuclear localisation of *DNMT1* protein was not detected. Species differences in the expression of DNA methyltransferases in ES cells may reflect differences in the developmental regulation of DNA methylation, and suggest caution should be applied to the extrapolation of findings between species.

12.

**Oviductal Microsomal Epoxide Hydrolase (mEH/Ephx1) Reduces Reactive Oxygen Species (ROS) Level and Enhances Pre-implantation Mouse Embryo Development**

**Lee Kai-Fai, Ana W.Y. Cheong, Yin-Lau Lee, Wei-Min Liu, William S.B. Yeung**

*The University of Hong Kong L7-47, Lab Blk, FOMB, 21 Sassoon, Pokfulam, Hong Kong*

Somatic cell-embryo coculture enhances embryo development in vitro by producing embryotrophic factor(s) and/or removing harmful substances from the culture environment. Yet, the underlying molecular mechanisms on how somatic cells remove toxicants from the culture medium remain largely unknown. By using suppression subtractive hybridization, we identified a number of mouse oviductal genes that were up-regulated when developing preimplantation embryos were present in the oviduct. Microsomal epoxide hydrolase (mEH or Ephx1) was one of these genes. Ephx1 detoxifies genotoxic compounds and participates in the removal of reactive oxygen species (ROS). The transcript of Ephx1 increases in the oviductal epithelium at the oestrus stage and on Day 3 of pregnancy, as well as in the uterus of ovariectomized mice injected with oestrogen or progesterone. The immortalized human oviductal epithelial cells (OE-E6/E7) express Ephx1 and improve mouse embryo development in vitro. Addition of cyclohexene oxide (CHO), a competitive Ephx1 inhibitor, to the culture medium increased intracellular and extracellular ROS levels of OE-E6/E7 cells and suppressed the beneficial effect of the cells on embryo development; while CHO at these concentrations had no adverse effect on the growth of OE-E6/E7 cells and embryo development in vitro. Taken together, Ephx1 in oviductal cells may enhance the development of cocultured embryos via protecting them from oxidative stress. Our result supports the notion that somatic cell coculture may enhance embryo development via removal of deleterious substances in the culture medium.

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13.

**Expression of *Hox* genes in bovine oocytes and preimplantation embryos: preliminary results**

**Paul Delphine, R. Rezsöhazi and I. Donnay**

*Institut des Sciences de la Vie, Unité des Sciences vétérinaires, Place Croix du Sud 5  
box 10, B-1348 Louvain-la-Neuve (Belgium)*

Homeotic (*Hox*) genes encode transcription factors that play an important role in patterning the main body axis from the gastrulation stage on. A few studies have provided evidence that some *Hox* genes might be expressed before implantation in mammalian embryos. The objective of this study is to establish the expression pattern of various *Hox* genes during oocyte maturation and preimplantation development.

Bovine oocytes and in vitro produced embryos were collected at different stages. RNA was reverse-transcribed using hexamers. Nested PCR using specific intron spanning primers were performed to detect transcripts corresponding to 7 *Hox* genes: *Hoxd1*, *Hoxa3*, *Hoxd4*, *Hoxb7*, *Hoxb9* and *Hoxc9*. Quantitative real-time PCR (qPCR) is currently in progress.

*Hoxd1* transcripts were detected up to the 5-8 cell stage only, this was confirmed using qPCR. *Hoxb7* transcripts were detected at all stages except at the 9-16 cell stage. This pattern is likely to be due to maternal mRNA degradation before the major onset of embryonic transcription (MET) during the 4<sup>th</sup> embryonic cell cycle, followed by new expression from the embryonic genome. *Hoxb9* transcripts were detected at all stages. This could result from a delay in maternal mRNA degradation and/or to an early embryonic transcription. qPCR results show that relative amount of *Hoxb9* mRNA increases between 5-8 cell and morula stage. *Hoxa3* mRNA have been detected at all stages using nested PCR although preliminary qPCR results indicate that relative amount of *Hoxa3* mRNA is decreasing after the 5-8 cell stage. *Hoxc9* transcripts have been detected in all samples until the 5-8 cell stage, detection was inconstant at later stages, preliminary real-time PCR results are confirming this pattern. Finally, *Hoxd4* mRNA have been detected in all samples from the 9-16 cell stage on, detection was inconstant at earlier stages.

*This work was funded by the Fonds National pour la Recherche Scientifique de Belgique.*

Reference :

1 Ponsuksili S et al. Mol Reprod Dev. 2001 60:297-301.

14.

**Lysophosphatic acid modulates prostaglandin secretion in the bovine uterus**

**Woclawek-Potocka Izabela , Junichi Komiyama, Jean Sebastian Saulnier-Blache, Edyta Brzezicka, Mamadou Moussa Bah, Kiyoshi Okuda, Dariusz J. Skarzynski**

Tuwima 10 St. 10-747 Olsztyn, Poland

Lysophosphatidic acid (LPA) modulates prostaglandin (PG) synthesis via LPA receptor type 3 (LPA3) in the murine endometrium. The lack of functional LPA3 in mice may lead to embryo mortality. In the present study, we examined the role of LPA in the bovine uterus. We confirmed that LPA is locally produced and released from the bovine endometrium. Moreover, there are enzymes involved in LPA synthesis (*phospholipase (PL) D<sub>2</sub>* and *PLA<sub>2</sub>*) in the bovine endometrium during estrous cycle and early pregnancy. LPA given into the aorta abdominalis induced P4 and PGE<sub>2</sub> secretion and prolonged the life span of bovine corpus luteum (CL) *in vivo*. These actions were inhibited by LPA1 antagonist (Ki16425). Expression of *LPA1* was positively correlated with the expression of *PGE<sub>2</sub> synthase (PGES)* and negatively correlated with the expression of *PGF<sub>2α</sub> synthase* (aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity - *PGFS*) during early pregnancy. The overall results indicate that LPA is locally produced and released from the bovine endometrium. Moreover, in the endometrium in cattle unlike in rats and pigs there is only LPA1 receptor. The obtained results also suggest that LPA may serve as a luteotropic factor in cattle stimulating P4 and PGE<sub>2</sub> secretion. Positive correlation between the mRNA expressions of *LPA1* and *PGES* during the estrous cycle and early pregnancy can explain how LPA contributes to luteotropic PGE<sub>2</sub> synthesis and augmenting P4 in cow. Although LPA may be one of the important contributors at the estrous cycle and early pregnancy in cow, the exact LPA's roles in embryo – maternal interactions and supporting CL action needs further investigation.

15.

**Concentrations of active metabolites of phytoestrogens and prostaglandins in the blood of early pregnant and cyclic cows.**

**Bah Mamadou Moussa, Izabela Woclawek-Potocka, Dariusz J. Skarzynski**

Tuwima 10 St. 10-747 Olsztyn Poland

Phytoestrogens acting as endocrine disruptors may induce pathologies in the female reproduction. In the study we determined whether phytoestrogens present in the soy bean and their metabolites are detectable in plasma of cows fed diet rich in soy and compared the changes in their concentrations in blood plasma of cyclic and pregnant heifers after feeding with soy bean. We also examined whether these phytoestrogens influence reproductive efficiency and prostaglandin (PG) synthesis during the estrus cycle and early pregnancy.

We found daidzein and genistein in fodder and their metabolites in serum and urine. In the blood plasma of early- and late-pregnant heifers, we found lower concentrations and time-dependent decreases in daidzein and genistein in comparison to the cyclic heifers. Moreover, we noticed significant increases of phytoestrogen metabolites in the blood plasma of early-pregnant heifers. The mean number of artificial insemination and pregnancy rate in experimental (cows fed with soy-bean 2.5 kg/day) herds were almost double in the soy-diet herd in comparison to control animals (standard diet). The concentrations of  $\text{PGF}_{2\alpha}$  metabolite (PGFM) were significantly higher in the blood plasma of experimental heifers than those in control heifers throughout the first 21 days after ovulation and AI. The higher levels of PGFM were positively correlated with equol and p-ethyl phenol concentrations in the blood.

In conclusion, physiological status (cyclicality or pregnancy) of the females influence phytoestrogens metabolism and changes concentrations of isoflavone metabolites in the blood plasma. We surmise that early-pregnant heifers are more sensitive to active isoflavone metabolite actions than cyclic and late-pregnant heifers. Soy-derived phytoestrogens and their metabolites disrupt reproductive efficiency and uterus function by modulating the ratio of  $\text{PGF}_{2\alpha}/\text{PGE}_2$ , which leads to high, non physiological production of luteolytic  $\text{PGF}_{2\alpha}$  in cattle during estrous cycle and early pregnancy.

16.

**A bovine endometrium co-culture model for the investigation of early embryo-maternal crosstalk**

**Karina Zitta, Susanne Ulbrich, Fred Sinowatz, Marc Boelhauve, Stefan Hiendleder, Eckhard Wolf**

*Biotechnology, LMU Munich, Hackerstrasse 27, Germany*

The coordinated production of prostaglandins (PGs) by endometrial epithelial (EC) and stromal cells (SC) is a key component in maternal recognition of pregnancy in ruminants. However, it is unknown whether and how EC and SC interact to produce PGs under different stimuli. To address this question, we compared the morphology, expression of candidate genes, and PG secretion profile of EC mono-cultures with EC maintained in co-culture with SC. The morphology of EC grown in co-culture was more similar to fresh ex vivo-derived EC. RT-qPCR analyses revealed that oxytocin receptor (*OXTR*) and interferon tau (IFNT) receptor (*IFNAR*) transcripts were expressed by cultured EC, both in the absence and presence of SC. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and F<sub>2α</sub> (PGF<sub>2α</sub>) concentrations in media from mono- and co-cultures were measured by specific EIAs. Stimulation with 100 nM oxytocin decreased the PGE<sub>2</sub>/PGF<sub>2α</sub> ratio in media from co-cultured EC+SC cells (0.6±0.2 vs. 1.5±0.6 in non-stimulated controls; p<0.05). IFNT (100 ng/ml) shifted PG secretion of EC+SC co-cultures towards the pregnancy protective PGE<sub>2</sub> (2.36±0.96 vs. 0.99±0.44 in the absence of IFNT; p<0.05), but this effect was not seen in EC mono-cultures. The presence of embryos significantly enhanced *IFNAR* transcript levels in EC co-cultured with SC (2.6±0.7-fold vs. control without embryos, p<0.05), but not in EC mono-cultures. Our findings indicate that interactions between endometrial EC and SC markedly influence the responsiveness of EC to oxytocin, IFNT, and other embryonic signals. This requires consideration in attempts to establish *in vitro* systems for studying maternal interactions with gametes and embryos.

17.

### **Effects of guaiazulene on in vitro maturation of bovine zygotes, and on mRNA transcripts related to embryo quality**

**Dovolou Eleni, M. Clemente, G.S. Amiridis, I. Messinis, A. Kalitsaris, A. Gutierrez-Adan, D. Rizos**

*Faculty of Medicine, Univ. of Thessaly, Clinic of Obstetrics & Gynaecology, University Hospital, Larissa, Greece*

It is believed that Reactive Oxygen Species (ROS) production contribute to reduced in vitro bovine embryo production as they can cause meiotic arrest of oocytes, embryonic block and cell death. We examined whether guaiazulene (G) an exogenous antioxidant added in maturation medium would affect embryo development and the quality of the produced blastocysts. Bovine cumulus oocyte complexes (COC's) were aspirated from ovaries of slaughtered cows, and divided into 4 groups. COC's were matured in TCM199 with FCS and EGF (control<sup>-</sup>, n=459), supplemented with 0.1mM of G (n=497) or 0.01mM G (n=468) or 0.05%DMSO-the G diluent (Control<sup>+</sup>, n=467) at 39°C under an atmosphere of 5% CO<sub>2</sub> in air with maximum humidity. After 24h oocytes were inseminated and co-incubated for further 24h. Zygotes were cultured in groups of 25 in 25 µl of SOF with 5% FCS at 39 °C under an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> in air with maximum humidity. Blastocyst yield was recorded on days 6, 7, 8 and 9. All day 7 blastocysts from each group were snap frozen and stored for mRNA extraction. Quantification of transcripts for mRNA of genes related to metabolism (AKR1B1, PGHS-2 – COX-2, GADPH, GLUT-5) and to implantation (GPX1, G6PD, PLAC8) was carried out by real time quantitative RT-PCR. Data for embryo development and on transcript abundance were analyzed by  $\chi^2$  and ANOVA respectively. No differences were found between groups (cleavage: Control<sup>-</sup>: 74.20%; Control<sup>+</sup>:74.58%; 0.1mM: 71.63%; 0.01mM: 71.61%; d9 blastocyst yield: Control<sup>-</sup>: 28.26%; Control<sup>+</sup>:25.80%; 0.1mM: 25.25%; 0.01mM: 25.86%). The relative abundance of genes studied varied between groups but these differences were not significant.

Our results imply that guaiazulene has no a direct effect on early embryo development or on embryo quality at least on the mRNA transcripts studied. Further studies using the same antioxidant in post fertilization period are in progress.

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18.

**Plasminogen activator (PA) activity in the uterine luminal fluid of superovulated cows**

**Rekkas Constantinos, G.S. Amiridis<sup>2</sup>, A. Gutierrez - Adan<sup>3</sup>, D. Rizos<sup>3</sup> and Th. Tsiligianni<sup>1,2</sup>**

<sup>1</sup>*NAGREF-VRI -Thessaloniki*, <sup>2</sup>*Faculty of Veterinary Medicine - Karditsa, GREECE*,  
<sup>3</sup>*INIA-Madrid ,SPAIN*

Plasminogen Activators are associated with a variety of reproductive processes including spermatogenesis, ovulation and embryo implantation. They are produced by many cell types, including endothelial cells of oviduct and uterus, and cumulus cells. In this study we examined the activity of Plasminogen Activator (PA) in the uterine luminal fluid of superovulated cows and its possible associations with the superovulatory response. Embryos and a sample of uterine fluid flushed from each horn were collected on day 7 (estrus day 0) from 20 cows superovulated with porcine FSH in an 8 decreasing dose schedule (p-FSH, 400 mg, Folltropin, Canada). The activity of PA was determined spectrophotometrically. The cows were classified according to superovulatory response on the basis of corpora lutea (CL) per ovary (CL1, 1 to 3; CL2, 4-5 and CL3, >5) and according to the total number of embryos (TE) collected per horn (TE1, 0; TE2, 1 to 3; TE3, >3). The mean activity of PA was significantly lower ( $p < 0.05$ ) in CL3 compared to CL1 group ( $126.68 \pm 13.77$  and  $183.50 \pm 19.09$ , respectively). It was also significantly lower in T3 compared to T1 ( $94.80 \pm 2.52$  and  $161.69 \pm 12.62$ , respectively). There was a negative relationship between the activity of PA and the number of corpora lutea formed, as well as between the activity of PA and the number of embryos collected. We conclude that PA activity of the uterine luminal fluid on day 7 is related negatively to superovulatory response and embryos produced and/or being available for collection in the bovine.

*Collaboration via Joint R&T Programs between Greece and Spain*

19.

**Hydrogen peroxide supplementation before fertilization of bovine oocytes has no effect on in vitro fertilization or glutathione content.**

**Vandaele Leen, Thys Mirjan, Bijttebier Jo, Van Soom Ann**

*Reproductive Biology Unit, Faculty of Veterinary Medicine, Ghent University  
Salisburylaan 133, 9820 MERELBEKE, Belgium*

Previous studies have shown that a short pulse of 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> to cumulus oocyte complexes (COCs) before fertilization increased blastocyst rates and diminished apoptotic cell ratio (ACR) in blastocysts (Vandaele et al., 2007). The aim of the present study was to investigate if this positive effect on the long term (7 days) was caused by increased fertilization rates and/or glutathione content of the treated oocytes.

After in vitro maturation, 1160 mature bovine COCs (3 replicates) were exposed to six H<sub>2</sub>O<sub>2</sub> concentrations (0.01 $\mu$ M; 0.1 $\mu$ M; 1 $\mu$ M; 10 $\mu$ M; 100 $\mu$ M; 1mM) for 1 hour before fertilization. Meanwhile two control groups were kept in maturation medium without pyruvate and maturation medium with pyruvate, respectively. In each replicate 30 oocytes per group were denuded and frozen until they were assayed for glutathione content, while the remaining oocytes were fertilized in vitro. For the determination of penetration and fertilization rate, 60 zygotes per group were denuded, fixed and stained with Hoechst at 21-22 hpi. Logistic regression compared penetration and fertilization between groups and non-parametric Kruskal-Wallis analyzed differences in glutathione content between groups.

Penetration and fertilization rates were significantly reduced after incubation in extreme high concentration of H<sub>2</sub>O<sub>2</sub> (1mM, 34.4 and 22.1% respectively), but did not differ between lower concentration groups and/or control groups. Penetration rates in other groups ranged between 70.7 and 74.5%. Fertilization rates ranged between 51.0 and 56.6%. Glutathione content varied between 3.5 and 4.2pmol/oocyte and did not differ significantly between groups.

In conclusion, higher blastocyst rates and lower ACRs after incubation of mature COCs with 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> cannot be attributed to improved fertilization rates or increased glutathione content. This might suggest that a short stress pulse (100 $\mu$ M H<sub>2</sub>O<sub>2</sub> for 1 hour) is able to change gene/protein expression of e.g. Hsp70 which may favour embryos in their interaction with their environment and in their development.

### **Expression of progesterone receptors on bovine preimplantation embryos**

**M. Clemente<sup>1</sup>, J. de La Fuente<sup>1</sup>, T. Fair<sup>2</sup>, P. Lonergan<sup>2</sup>, A. Gutierrez-Adan<sup>1</sup>, D. Rizo<sup>1</sup>**

*<sup>1</sup>Dept de Reproduction Animal, INIA, Madrid, Spain. <sup>2</sup>School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland*

Physiological effects of progesterone are mediated by interaction of the hormone with specific intracellular progesterone receptors (PRs) and by binding to membrane receptors PGRMC1 and PGRMC2. The objective of this study was to determine the mRNA abundance of three progesterone receptors on Day 7 and 13 bovine embryos derived in vitro or in vivo. In vitro embryos were produced by standard oocyte maturation, fertilization and culture procedures. In vivo blastocysts were collected from superovulated heifers on Day 7 by uterine flushing or Day 13 at slaughter. Quantification of transcripts for PGR, PGRMC1 and PGRMC2 was performed on groups of 10 Day 7 and individual Day 13 embryos by real-time quantitative RT-PCR. Progesterone receptor mRNA copy number was determined using standard curves for cDNA standards generated by PCR. PGR receptor was expressed only in in vitro blastocyst Day 7 with a small number of mRNA copies per embryo ( $10 \pm 2.7$ ) and in in vivo Day 13 embryos again with a small number of mRNA copies per embryo ( $104 \pm 36.7$ ). The mRNA abundance for PGRMC1 was significantly higher in in vitro Day 7 blastocysts, with more mRNA copies per embryo ( $10111 \pm 2548$  vs  $2230 \pm 318$ ), compared to in vivo. For Day 13 embryos no difference was found either for mRNA abundance or mRNA copies per embryo for in vivo and in vitro ( $38317 \pm 8515$  vs  $3946 \pm 389$ ). The mRNA abundance or the number of mRNA copies per embryo for PGRMC2 was similar between in vivo and in vitro Day 7 blastocysts ( $4383 \pm 820$  vs  $1366 \pm 160$ ) and Day 13 embryos ( $15095 \pm 3487$  vs  $2716 \pm 857$ ). In conclusion, the present study demonstrates changes in the abundance of three progesterone receptors mRNA in in vivo and in vitro bovine derived embryos on Day 7 and Day 13 raising the possibility of a direct effect of progesterone on the embryo.

21.

**Oxidative status at mating and pregnancy outcome in dairy cows**

**Celi Pietro, Merlo Mariacristina, Barbato Olimpia and Gabai Gianfranco**

*University of Sydney, PMB 3, Camden NSW, 2570 AUSTRALIA*

Recently, a potential role for oxidative stress (OS) as a trigger for cell death during luteolysis has been identified. Excessive free radical generation damages luteal cell membranes and affects progesterone (P4) production (Kato et al., 1997. Rev Reprod 2: 81), potentially leading to a failure in embryo development. It is possible that the corpus luteum is highly susceptible to OS and free radicals may contribute to the low fertility of dairy cows. This evaluated whether OS may affect the establishment of pregnancy in dairy cows. The study used 40 pasture fed Friesian cows. On the day of artificial insemination (AI; Day 0), and on Days 30 and 42, fresh blood samples were collected for determination of reactive oxygen metabolites (ROM) and biological antioxidant potential (BAP) concentrations. Plasma from the blood samples was assayed for concentrations of P4, pregnancy associated glycoprotein (PAG), advanced oxidation protein products (AOPP) and glutathione (GSH). Plasma PAG concentrations at days 30 and 42 were used for pregnancy diagnosis. Statistical analysis included between-subjects main effect of AI outcome (AI resulted in pregnancy, AI+; AI not resulted in pregnancy, AI- and embryo mortality, EM), within-subjects main effect of postpartum period (Period 1: < 80 day; Period 2: 81-120 days; Period 3: >120 days) and their interaction. Plasma ROM and BAP concentrations were not related to AI outcome. Plasma ROM concentrations increased ( $P<0.01$ ) while BAP decreased ( $P<0.05$ ) during the postpartum period, possibly as a consequence of the parallel increase in ROMs; antioxidant depletion is considered the consequence and not the cause of oxidative stress. GSH and AOPP concentrations were higher in EM than in AI+ and AI- ( $P<0.05$ ). However, because of the low number of EM cases, this finding needs to be confirmed by further studies. Considering that the maintenance of redox homeostasis is quite complex, further studies are required to clarify the role of OS on the fertility of dairy cows.

22.

**SOCS (Suppressor of Cytokine Signaling) are differentially affected by the biotechnologies of the embryo and interferon-tau (IFNtau) in the bovine endometrium at implantation.**

**Sandra Olivier, Cagnard A; Mansouri N, Giraud-Delville C, Rodde N, Chaouat G & Reinaud P**

*INRA, UMR 1198 Biologie du Développement et Reproduction; ENVA, UMR 1198 Biologie du Développement et Reproduction; CNRS, FRE 2857, F-78350 Jouy en Josas, France*

In mammals, the biotechnologies of the embryo (e.g. IVF) affect the transcriptome of the embryo and are associated with subsequent pathological pregnancies and adult diseases. The altered profiles of the embryo-secreted factors may therefore modify the conceptus-endometrium cross-talk, including the SOCS proteins that could be considered as major integrators and intracellular sensors of the cytokine signalling activity. Using cattle as an animal model, we investigated the impact of AI or IVF-produced embryos as well as IFNtau -the embryo signal for pregnancy recognition in ruminants- on the regulation of CIS, SOCS1, SOCS2, SOCS3 and SOCS6 in the endometrium at implantation.

At day 20 of pregnancy, only SOCS6 transcripts appeared to be significantly regulated in the bovine caruncles (cycle vs pregnancy:  $0.85 \pm 0.46$  vs  $4.16 \pm 0.51$ ;  $P < 0.001$ ). Nevertheless, in pregnant animals, the immunocytochemical analyses showed modifications of the endometrial localisation for several SOCS proteins (SOCS1 and SOCS6 in the luminal epithelium, SOCS2 in the stroma). In primary endometrial bovine cells incubated with recombinant ovine IFNtau (100 or 1000 ng/ml), the expression of SOCS1, SOCS3 and to a lesser extent CIS transcripts were stimulated (15-fold and 5-fold induction respectively) after 30 minutes or 2 hours whereas SOCS2 and SOCS6 were not affected by IFNtau, even after a 24h treatment. The recipient cows for IVF-produced embryos displayed a reduced endometrial expression of SOCS1 and SOCS2 when compared to the AI pregnant cows ( $P < 0.05$ ). Collectively, our results have shown SOCS1 and SOCS3 as IFNt-target genes in cattle. The cellular localisation of the SOCS proteins suggests their potential involvement in the regulation of the adhesion/early placentation process. Upon IVF, the modification of the endometrial SOCS expression at implantation may affect the placental then the foetal development therefore contributing to the final outcome of pregnancy. Additional studies are necessary to clarify this aspect.

23.

**Changes in mRNA level of several factors involved in steroidogenesis, metabolism and apoptosis in cumulus cells during in vitro maturation of bovine oocytes.**

**Mohamad Salhab, Christine Perreau, Pascal Mermillod and Svetlana Uzbekova**

*Institut National de la Recherche Agronomique, UMR85 Physiologie de la Reproduction et des Comportements (INRA – CNRS – Université de Tours – Haras Nationaux), PRC INRA, 37380 Nouzilly, France*

Cumulus cells (CC) are specialized granulosa cells tightly connected to the oocyte through a gap junctions network. They provide metabolic support to the oocyte, which is essential during its differentiation toward full developmental competence. The presence of CC (as cumulus oocyte complexes or COC) is also essential for complete oocyte maturation, including meiotic evolution to metaphase II and several cytoplasmic aspects. Cattle oocytes can resume meiosis and progress to metaphase-II during 24h of in vitro maturation (IVM). However, their developmental competence is highly compromised as compared to in vivo matured oocytes. We were looking at the evolution of the concentration of several mRNA, involved in steroidogenesis, energetic metabolism and apoptosis, in CC of bovine COC subjected to IVM. Bovine COC were subjected to IVM in serum-free 199 medium complemented with hormones and growth factors. Samples of oocytes were collected after 3h (oocyte GV stage, compact cumulus), 6h (GV breakdown), 10h (metaphase-I, expanded cumulus) or 24h (metaphase-II, highly expanded cumulus, rupture of gap-junction). Relative mRNA expression in CC at these different time-points was analyzed by real-time RT-PCR. During IVM, CC were producing increasing amount of progesterone and expanded significantly from 10h of IVM as compared with immature ones. Expression of *PTGS2* (prostaglandin synthase 2) and *STAR* (Steroidogenic Acute Regulatory protein) genes progressively increased during culture. In contrast, *CYP19A1* (aromatase) mRNA level decreased significantly during IVM. Transcripts levels of *BAX* (Bcl2-associated X protein), *ERS2* (estrogen receptor-2beta) and *TGFB* (transforming growth factor- $\beta$ ) were relatively stable between different IVM time-points. Gene expression of *PGR* (progesterone receptor), *CLU* (clusterin) and *SCD* (stearoyl-CoA desaturase) increased up to 10h of IVM and then dropped to initial levels at 24h.

These different profiles confirmed the involvement of CC in prostaglandins and progesterone pathways and highlight the oocyte-cumulus interactions during IVM.

24.

## **Role of oviductal cells in fertilization in the horse**

**Sylvie Mugnier, Morgane Kervella, Ghylène Goudet**

*INRA, UMR85 Physiologie de la Reproduction et des Comportements, CNRS,  
Université de Tours, Haras Nationaux 37380, Nouzilly, France*

Despite numerous studies, the mechanism of fertilization in mammals remains puzzling. The secretory cells of the oviduct synthesize proteins that have been shown to interact with gametes. Moreover, oviductal epithelial cell (OEC) coculture promotes *in vitro* fertilization (IVF) in human, bovine and porcine. In the equine species, numerous attempts to establish an efficient IVF technique during last decades remained unsuccessful, and no repeatable IVF technique is available yet. However, the coculture of equine spermatozoa and oocytes with OEC has never been investigated. The aim of our study was to test the influence of coculture with OEC on equine IVF, and further to clarify the role of oviductal cells in the mechanism of sperm-egg interaction.

Equine oocytes were collected from slaughtered mare ovaries and *in vitro* matured for 30 hours. Porcine oviducts were collected from slaughtered gilts 6 hours after ovulation and OEC were cultured for 24 hours. Equine sperm was collected from one of our experimental stallions, diluted and incubated with calcium ionophore. Equine oocytes were cocultured with OEC during 2 hours, and then spermatozoa were added. After 24 hours, the number of fertilized oocytes was evaluated.

When equine spermatozoa and oocytes were incubated without OEC, no fertilization was observed (0/16) whereas 4/15 oocytes (26%) were fertilized (2 pronuclei) after coculture with OEC.

In conclusion, equine IVF rate is improved by OEC coculture. The oviductal cells may be of particular importance for fertilization in this species, and the horse may be an interesting model to study the role of oviduct in regulating sperm-egg interaction.

25.

**The *bucky ball* gene organizes germ plasm assembly in the zebrafish oocyte**

**Frank Bontems, Amandine Stein, Florence Marlow, Jacqueline Lyautey, Mary C. Mullins, Roland Dosch**

*Université de Genève, Département de Zoologie et Biologie Animale  
Sciences III, 30, quai Ernest-Ansermet, CH-1211 Genève 4, SWITZERLAND*

In many organisms the formation of gametes during embryogenesis is specified by maternal cytoplasmic determinants termed germ plasm. Germ plasm assembles during oogenesis forming a distinct cellular structure such as pole plasm in *Drosophila* or the Balbiani body in several organisms. However, in vertebrates the key regulators of Balbiani body assembly are unknown.

We show that in *bucky ball* (*buc*) mutant zebrafish the formation of the Balbiani body is disrupted identifying *buc* as a key regulator of germ plasm assembly in vertebrates. Moreover, we molecularly identify the *buc* gene, which is exclusively expressed in the ovary displaying a novel, dynamic mRNA localization pattern initially detectable within the Balbiani body. Interestingly, overexpression of *buc* generates additional germ cells in the zebrafish embryo providing evidence that *buc* is the first vertebrate gene sufficient to recruit germ plasm components. We discovered *buc* homologs in many vertebrate genomes including mammals suggesting that it may be a common regulator of germ plasm formation in vertebrates and thus provide an entry point to molecularly dissect the oocyte Balbiani body structure.

## **TOOLS, TECHNOLOGIES & SYSTEMS BIOLOGY**

### **Principles of systematic sampling and stereological investigation of complex tissues**

**Ruediger Wanke**

*Institute of Veterinary Pathology University of Munich Veterinaerstr. 13 D-80539  
Munich Germany E-mail: [wanke@patho.vetmed.uni-muenchen.de](mailto:wanke@patho.vetmed.uni-muenchen.de)*

The traditional approach to morphological analyses is descriptive and, given that the morphological changes are mainly qualitative, this approach is fairly accurate. However, subjective assessment of primarily quantitative morphological changes is often poorly reproducible. Histological descriptions using terms like 'few' or 'many' or subjective judgements like 'mild / moderate / severe' are useful in the initial stages of many scientific studies, but answering more penetrating questions commonly requires quantitative data.

The terms 'morphometry' and 'stereology' are frequently used interchangeably to designate quantitative morphological analyses. Stereology may briefly be defined as a methodology to spatially interpret sections, while the term morphometry refers to the measurement of morphological structures. Thus, morphometric analyses are always quantitative, but not necessarily three-dimensional analyses, whereas stereological analyses are always 3D-analyses, but may be either quantitative or qualitative (e.g., reconstruction of 3D-objects from serial sections) analyses.

Stereology is fundamentally statistical in its nature and, thus, requires randomness. In contrast to qualitative investigations, where "choosing" of samples is the usual way to proceed, random and systematic random sampling is essential for quantitative stereological investigations and should be applied at every level of the sampling hierarchy.

The strength of stereological methods is that they provide data for 3D-structures in 3D-quantities. A first set of stereological principles aims at the measurement of structural parameters such as volume density, surface density or length density of a component in the reference space. A second set of stereological principles considers individual objects. Traditional stereological methods for counting and sizing of particles require a priori assumptions on their shape and variation in size in order to apply appropriate unfolding algorithms. These model-based methods are commonly said to be biased because the assumptions are difficult to prove. Starting during the 1980s, a new generation of stereological methods such as the disector, the selector, the fractionator or the nucleator has been developed, which allow counting and sizing of particles without bias caused by assumptions of size and shape. These stereological methods that rely only on sampling designs for their unbiasedness have become known as 'design-based' or 'unbiased' stereology. Methods like the vertical section procedure, the orientator or the isector provide solutions to the problem of measuring structures with preferred orientation (anisotropy). Frequently, stereological studies may only be biologically meaningful if the parameters are expressed in absolute terms (total quantities) and not only as ratios (densities). If only ratios are considered it is not possible to know if the amount of the structure / particles of interest or the

reference space volume is varying (or both), and there are remarkable examples in the literature where investigators have fallen into this so called reference trap. To avoid the reference trap the volume of the reference space has to be determined taking processing-induced volume changes of the tissue into account. Determination of the reference space volume can easily be achieved with the Cavalieri method, which allows the efficient and unbiased estimation of the volume of any object.

In conclusion, numerous studies have impressively demonstrated the utility of quantitative stereology in biomedical research. The use of quantitative techniques imposes a greater degree of objectivity and reproducibility in the assessment of morphological features. Quantitative morphological data provide a counterpart for quantitative data common to many biomedical disciplines, thereby allowing studies of structural and functional relationships. Further, detection of subtle morphological changes that escape subjective judgement is possible by quantitation.

## **Holistic proteome studies of minimal samples: immature versus *in vitro* matured oocytes**

**Georg J. Arnold**

*Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, Ludwig-Maximilians-University Munich*

A number of biological processes is dependant on or regulated by biochemical events on the protein level, e.g., protein activation by phosphorylation or by enzymatic processing of inactive precursors, or protein release from specific depositories. These molecular events are analytically addressable exclusively on the protein level and can not be deduced from, e.g., transcriptome studies. As a consequence, proteome analysis has become an indispensable tool to study a broad variety of biological phenomena. Two major strategies, two-dimensional gel electrophoresis or liquid chromatography of tryptic protein hydrolysates followed by tandem mass spectrometry (LC-MS/MS) are currently applied for holistic proteome analysis. The protein amount necessary is usually in the of 20 to 100  $\mu\text{g}$  range, making the generation of the biological probe extremely laborious, if not impossible, in many cases.

Only recently, a highly sensitive method referred to as 2D DIGE saturation labeling was introduced, facilitating the quantitative analysis of very limited amounts of biological samples. We used this method for a proteomic study of the *in vitro* maturation process of bovine oocytes, a complex and critical step in assisted reproduction techniques in humans and animals. By optimizing the detection and quantification step of fluorescent dye labeled proteins, we were able to run analytical two dimensional gels with only 250 ng total sample protein per gel, and could identify ten proteins with significant alterations in abundance from a preparative gel. Identified protein species include the translationally-controlled tumor protein,

enzymes of the Krebs and pentose phosphate cycles, clusterin, 14-3-3  $\epsilon$ , elongation factor-1 gamma, and redox enzymes such as polymorphic forms of glutathione S-transferase Mu 5.

In a qualitative approach based on nano-LC-MS/MS, we have started to identify the most prominent compounds of the entire oocyte proteome. The highly sensitive methods for proteome analysis will be applied to studies of early stages of embryogenesis and embryo development.

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## **Mathematical modelling and control of physiological systems: a Systems Physiology approach of the gonadotrope axis**

**Frédérique Clément**

INRIA Paris-Rocquencourt Research Centre  
78153 Le Chesnay Cedex, France

After positioning the role of mathematical modelling within the field of Integrative Biology, we will present an approach of multi-scale modelling and control of the gonadotrope axis. This approach lies at the boarder between Applied Mathematics and Control theory on one hand, Physiology and Cellular Biology on the other hand. Our long term goal is to dispose of interconnected, multi-scale models of the different organs building –up the gonadotrope axis and to be able to use them to take up physiological and clinical questions. Our work is based on the coupling between different mathematical (mainly: conservation laws and dynamical systems) with biological knowledge and data. We have been especially interested in the FSH-dependent selection process from a control viewpoint naturally leads to a multi-scale model, that takes into account the cellular, tissular and organic levels, as well as endocrine feedback between the organs. This model further articulates with a bottom-up Systems Biology approach focused on the biochemical basis of FSH control, that consists in organising and formalising the biochemical network involved in FSH signal transduction.

## **Procedural Reconstruction of a Three Dimensional Graphical Model of an Oviduct**

**Burkitt Mark, Daniela M. Romano, Dawn Walker and A. Fazeli**

*University of Sheffield, Department of Computer Science, 211 Portobello, Sheffield, S1 4DP, United Kingdom*

The mammalian oviduct has a complex structure consisting of interweaving folds of soft tissue. Due to the small scales involved, visualising interactions between gametes, embryo and the oviduct epithelium in a three dimensional (3D) context can be difficult. The aim of this investigation was to create a 3D model of the oviduct in porcine species.

Several techniques for generating the model were investigated. Firstly, a micro Computerised Tomography (CT) scanner and histological slides were used to obtain structural data and accurate measurements. Several procedural methods, including reconstruction from structural data, fractal based mesh deformation and cloth modelling techniques were then investigated to automate construction of a 3D model. The best graphical results were obtained from an approach derived from cloth modelling. The inside and outside oviduct epithelia were each represented using a single thread. As the model is constructed, the threads change shape in response to pre-defined 'physical forces', which are based on measurements taken from the histological slides. This provides a realistic representation of the change in folds from the isthmus to the ampulla. The state of each thread is captured at fixed intervals, and the recorded data is used to construct a complete 3D model.

In conclusion, we have developed a tool for constructing 3D graphical representations for oviducts of different lengths, scales and complexity using a force based procedural method. This tool allows models of oviducts from different species at different stages of the reproductive cycle to be generated and viewed in three dimensions. This method will be the basis of future research, and will be extended to allow the visualisation of interactions between gametes, embryo and the oviduct epithelia.

### **Embryo-Maternal communication in bovine oviduct: A transcriptomic approach**

**Schmaltz Barbara, P. Debey, Y. Locatelli, S. Uzbekova, C. Perreau and P. Mermillod**

*INRA – PRC F-37380 Nouzilly, France*

In mammals, the oviduct provides optimal environment for transport and survival of spermatozoa, fertilization process and early embryonic development. During the preimplantation period, the beneficial effects on development are suspected to be due to embryotrophic factors secreted by oviductal epithelial cells. The aim of our study was to establish an in vitro co-culture system in which bovine oviductal epithelial cells (BOEC) promote bovine embryonic development in order to study the maternal control of early embryo development.

Mucosae were mechanically expelled from bovine slaughterhouse oviducts. BOEC were cultured in TCM-199. The medium was replaced by TCM-199 or SOF when cells reached confluence. Bovine zygotes were obtained by in vitro maturation and fertilisation of oocytes recovered from slaughterhouse ovaries. Presumptive zygotes were randomly cultured in the presence or absence of BOEC monolayer in SOF (385 and 378 respectively) or TCM-199 (389 and 387 respectively). Cleavage rates were determined on day 2 post insemination (dpi) and blastocysts rate were observed on 8 dpi. RT-PCR were performed on a set of specific oviduct marker genes.

The presence of BOEC in SOF culture did not affect the cleavage rate (79% vs 72%,  $p > 0.05$ ) but significantly increased blastocyst rate (37% vs 27%,  $p < 0.05$ ). In TCM-

199, the presence of BOEC increased blastocyst formation (26% vs. 10%,  $p < 0.05$ ) but not the cleavage rate (76 % vs 73%,  $p > 0.05$ ). All Transcripts of specific marker genes were detected during all the culture period.

Our results indicate that cultured BOEC still expressed oviduct specific marker genes and improved embryo development. In conclusion, this culture system seems to be adapted to study the effect of oviduct on early embryo development. Further studies will be required to assess embryo viability and to identify other oviduct marker genes (cDNA array hybridation).

### Poster Session

26.

#### **The effect of oestradiol on expression of prostaglandin synthesis pathway enzymes and PGE2 receptors in the pig endometrium**

**Agnieszka Waclawik<sup>1</sup>, Agnieszka Blitek<sup>1</sup>, Henry N. Jabbour<sup>2</sup>, Adam J. Ziecik<sup>1</sup>**

<sup>1</sup>*Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, 10-747 Olsztyn, Poland;* <sup>2</sup>*MRC Human Reproductive Science Unit, The Queens Medical Research Institute, Edinburgh, EH16 4TJ, UK*

Before implantation, endometrium and trophoblast synthesize elevated amount of prostaglandin E2 (PGE2). Up-regulation of PGE synthase (mPGES-1), a key enzyme in PGE2 synthesis, in porcine endometrium and trophoblast during this period was recently demonstrated (Waclawik et al., 2006, *Endocrinology* 147:210-221). This study was designed to establish whether porcine embryo signal, oestradiol (E2) regulates expression of prostaglandin synthesis enzymes and PGE2 receptors (PTGER2 and PTGER4) and to determine the site of action of PGE2 in porcine endometrium.

Endometrial tissue explants were obtained from gilts (n=6) on days 10-13 of the oestrous cycle and incubated for 24h with vehicle only (control) or E2 (1, 10 or 100 nM). After incubation, medium was collected for PGE2 and PGF2 $\alpha$  measurement. Effect of E2 on expression of the prostaglandin synthesis enzymes and PTGER in endometrial explants was examined by Western blot and real-time RT-PCR. Localization of protein expression of PTGER2 and PTGER4 was analyzed in uterus of cyclic (n=12) and pregnant gilts (n=12) by immunohistochemistry.

E2 increased mPGES-1 mRNA content and protein expression of PTGS2, mPGES-1 and PTGER2 ( $p < 0.05$ ), but down-regulated PGFS and CBR1 protein content ( $p < 0.05$ ) and had no effect on PTGER4 expression. E2 induced dose-dependent increase of PGE2 secretion which at the highest dose, reached  $133.4 \pm 6.5\%$  of control ( $p < 0.01$ ). However, there was no effect of E2 treatment on PGF2 $\alpha$  secretion. PTGER2 and PTGER4 proteins were highly expressed in luminal and glandular epithelium of endometrium and blood vessels. These studies suggest E2 regulates the protein expression of endometrial prostaglandin synthesis enzymes in such way that favours production of PGE2. PGE2 could exhibit a luteoprotective effect and may act locally through endometrial PTGER4, and especially PTGER2 receptors. Moreover, endometrial PTGER2 may be involved in positive feedback loop action of PGE2 during its increased synthesis in early pregnancy in porcine uterus.

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27.

### **L-selectin mediated adhesion of the porcine embryo**

**Østrup Esben, Gunnar Borreard, and Poul Maddox-Hyttel**

*University of Copenhagen, Groennegaardsvej 7, DK-1870 Frederiksberg C  
Denmark*

In man, L-selectin expression in the early embryo is involved in trophoblast adhesion to the uterine mucosa. The aim of this study was to investigate the potential role of the L-selectin adhesion-system in the adhesion and placentation of porcine embryos. A total of 20 gilts were slaughtered in groups of five at Days 10, 13, 15 and 18 post insemination (p.i.). The recovered uteri were flushed with 1% serum/PBS to recover embryos. Endometrial samples and embryos were fixed in either 4% PFA/PBS for immunohistochemistry or RNAlater for expression analysis. Quantitative RT-PCR was used to investigate expression of L-selectin and its ligands. The protein localization of L-selectin and the L-selectin ligand epitopes MECA-79 and PEN5 was investigated by immunohistochemistry. The mRNA expression of L-selectin was approximately 16 fold up-regulated in the endometrium at Day 13, 49 fold up-regulated at Day 15, and 36 fold up-regulated at Day 18 compared to the expression at Day 10. Expression of the potential L-selectin ligands, CSPG-2 and podocalyxin, in embryos from Day 10 and Day 15 were shown by RT-PCR. Staining for L-selectin protein was mainly observed in the epithelial cells of the endometrium, whereas staining for MECA-79 and PEN5 was observed on trophoblast cells of selectively Day 15 embryos. Apparently, the staining was confined to certain regions of the trophoblast. Our results for the first time demonstrate that key components of the L-selectin adhesion system are present in the porcine uterus and embryo around the time of initial placentation. Interestingly, the components are expressed in a pattern opposite to that found in man: In the pig, L-selectin is expressed in the endometrium and the ligands in the trophoblast with the reverse being true in man. In fact, this is the first time L-selectin ligands have been described to be present on the surface of embryos.

28.

**Coupling experimental and modeling approaches for driving the dynamic of  $\text{Ca}^{2+}$  signal during egg activation**

**Andrey Philippe, Banrezes, B. and Ozil, J.-P.**

*NOPA, BDR INRA, Domaine de Vilvert, 78352 Jouy-en-Josas, France*

**Introduction.** At fertilization, intracellular calcium oscillations provide an important initial cellular signal which drives the completion of meiosis and initiates development. Experimental modification of the  $\text{Ca}^{2+}$  signal has shown that the egg is very sensitive to minute changes and that the integration of this signal can impact the developmental process until term. To better understand the long-term effects of the  $\text{Ca}^{2+}$  signal, it is important to identify the critical parameters underlying the cyclical release and re-uptake of intracellularly stored calcium and to be able to precisely drive this dynamic. We here describe the joint experimental and modeling approach we are developing to these ends.

**Methods.** *Experimental approach:* freshly fertilized eggs taken before the formation of pronuclei were subjected to a series of transmembrane  $\text{Ca}^{2+}$  influx by electroporation in a special chamber. The dynamics of  $\text{Ca}^{2+}$  oscillations in response to the overabundant injection of  $\text{Ca}^{2+}$  into the cytosol were recorded by  $\text{Ca}^{2+}$  imaging. *Modeling approach:* eggs were represented using two compartments (cytosol and endoplasmic reticulum).  $\text{Ca}^{2+}$  dynamics were generated by numerically solving systems of ordinary differential equations describing the activity of  $\text{Ca}^{2+}$  channels (Ins3P-receptor) and pumps (SERCA) that allow membrane fluxes between these two compartments. Model parameters were adjusted to qualitatively reproduce the features of the experimentally observed patterns.

**Results.** The experimental response of fertilized eggs overloaded with  $\text{Ca}^{2+}$  takes the form of a series of rapid oscillations the frequency of which is slowed down as the duration of the transient increases. The observed patterns are highly reproducible. The initial model exhibits a rich spectrum of  $\text{Ca}^{2+}$  dynamics and can account for the characteristics of experimental observations.

**Conclusion**

Coupling experimental and modeling approaches to study  $\text{Ca}^{2+}$  signaling during egg activation will make it possible to efficiently identify critical parameters. By correlating the future embryo phenotypic variations with minute changes in the model parameters that shape signaling dynamics, we will gain new opportunities of intervening during the early steps of development.

29.

**Innovative instrument-based solutions to optimize ex vivo development in the reproductive field.**

**Avazeri-Sideris\* Nathalie, Daniel N.<sup>o</sup>, Duranthon V.<sup>o</sup>, Renard JP.<sup>o</sup>, and Ozil JP.<sup>o</sup>.**

*\*Bracer Biotech. Domaine de Vilvert Bat 232*

*<sup>o</sup> UMR Biologie du développement et de la Reproduction. INRA 78352 Jouy en Josas Cedex, France*

Reproducing the natural environment which impacts embryo development represents a major scientific and medical challenge. In this context a new approach has been developed based on technology of microfluidics. The objective of this microtechnology is to mimic the dynamics physiological environment.

The Dynxcell system is composed of a fully integrated control unit that makes it possible to dispatch up to 8 different media with a wide range of flow rate, softwares developed to fully monitor system integration and a microfluidic chamber where the embryos (up to 100 in several groups) are placed, and maintained in controlled conditions of gas and temperature.

The preliminary results presented here aimed at analyzing the effects of long term culture (three days) in this dynamic system by comparing rabbit embryo development obtained either in this microfluidic system or in static condition.

For practical purpose the culture medium retained for this comparison was M199 with fetal calf serum.

Embryos were in vivo fertilized and recovered from the rabbit females at the two cell stage, 24 hours post-coitum. They were then dispatched into two batches: half were cultured in static conditions whereas the other half were cultured in Dynxcell system with a flow rate of 1.6 $\mu$ L/sec.

The kinetics of preimplantation development was recorded during five experiments, involving a total of 177 and 182 embryos in dynamic and static culture respectively.

Our results evidenced a slightly faster development of the embryos in the DynxCell system: after a 72h culture period, 49.6% of the embryos cultured in the Dynxcell system had reached the blastocyst stage, versus 20.9% for the embryos cultured in the same medium in static conditions.

Ongoing experiments showed that such a three days culture in Dynxcell is compatible with normal full term development of rabbits after transfer to foster mothers.

## **Alterations in gene expression patterns of oocytes/embryos of different origin**

**Wrenzycki Christine**

*University of Veterinary Medicine Hannover, Clinic for Cattle, Reproductive  
Medicine Unit, Bischofsholer Damm 15, 30173 Hannover, Germany*

The preimplantation bovine embryo is initially under the control of maternal genomic information that is accumulated during oogenesis. Soon, the genetic program of development becomes dependent upon new transcripts derived from activation of the embryonic genome. The early steps in development including maturation, fertilization, timing of first cleavage, activation of the embryonic genome, compaction, and blastocyst formation can be affected by the environment (in vivo/in vitro) as well as the production procedure itself. These perturbations can possibly result in a dramatic decrease of the quality of the resulting blastocysts, and may even affect the viability of offspring born after transfer.

The underlying mechanisms are widely unknown at present, but epigenetic modifications of embryonic and fetal gene expression patterns, primarily caused by alterations in DNA methylation are thought to be involved. Appropriate DNA methylation is essential for regular transcription during mammalian development and differentiation. Studies to unravel mRNA expression patterns from embryos of different origin have revealed numerous aberrations ranging from suppression of expression to de novo over-expression or more frequently to a significant up-regulation or down-regulation of a specific gene. However, as there is some evidence that mRNA and protein levels are not always related, studies are necessary to characterize both mRNA and protein levels of specific genes including their localization during early preimplantation development in single embryos. Unravelling the underlying molecular mechanisms will contribute to the production of viable embryos and aid to improve biotechnologies applied to early mammalian embryos.

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**Changes in uterine and luteal blood flow during early pregnancy in cattle**

**Honnens Aenne, Claudia Voss, Kathrin Herzog, Detlef Rath, Heinrich Bollwein**

*Clinic for Cattle, University of veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany*

The objective of this study was to characterize changes in uterine and luteal blood flow during the first three weeks of pregnancy in dairy cows. Transrectal colour Doppler examinations of the luteal glands (CL) and the uterine arteries were carried out in 40 multiparous lactating German Holstein cows on Days 3, 6, 9, 11, 13, 15, 18 and 21 after insemination (=Day 0). On Day 25, pregnancy diagnosis was performed. Uterine blood flow resistance was reflected by the pulsatility index (PI) in the uterine arteries ipsilateral to the CL. Luteal blood flow was characterized by the maximum coloured area within a vertical plane of the CL ( $A_{CL}$ ). Eighteen cows were diagnosed as pregnant and 22 cows as non-pregnant. Four non-pregnant cows showed no oestrus signs until Day 25 and were excluded from further analyses. Pregnant cows showed a transient decrease ( $P<0.05$ ) in PI values between Days 3 and 15, reaching minimum values on Day 11. Between Days 15 and 18 PI values increased ( $P<0.05$ ) by 26%. On Day 18, pregnant cows had 40 % higher ( $P<0.05$ ) PI values compared to non-pregnant cows. On Day 21, values had declined ( $P<0.05$ ) again. These changes did not occur in non-pregnant cows ( $P>0.05$ ). Until Day 13, pregnant and non-pregnant cows showed similar changes ( $P>0.05$ ) in luteal blood flow. Between Days 13 and 15  $A_{CL}$  increased ( $P<0.05$ ) in pregnant cows, whereas in non-pregnant cows values decreased ( $P<0.05$ ) between Days 13 and 18 ( $P<0.05$ ). From Day 15 onwards pregnant cows had higher ( $P<0.05$ )  $A_{CL}$  values than non-pregnant cows. Results indicate that pregnant cows have an early increase in uterine blood flow during the first to second week of pregnancy, which is followed by an increase in luteal blood flow in the end of the second week.

32.

## **The Functional Differentiation of Ovarian Follicular Granulosa Cells; the Importance of Transcriptome Studies**

**Kõks Sulev<sup>1</sup>, A. Velthut<sup>3</sup>, A. Sarapik<sup>2,4</sup>, E. Reinmaa<sup>5</sup>, U. Soomets<sup>6</sup>, Ü. Jaakma<sup>2</sup>,  
A. Salumets<sup>3,4,5</sup>**

*<sup>1</sup>Institute of Physiology, University of Tartu, <sup>2</sup>Department of Reproductive Biology, Estonian University of Life Sciences, <sup>3</sup>Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Tallinn, <sup>4</sup>Department of Obstetrics and Gynaecology, University of Tartu, <sup>5</sup>Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, <sup>6</sup>Institute of Biochemistry, University of Tartu, Estonia*

Ovarian folliculogenesis is a complex and long-lasting process continuing from the initiation of the development of primordial follicles until the pre-ovulatory antral follicles stage about a year. In antral follicles two functional granulosa cell types can be recognised: mural (enveloping the whole antrum, MG) and cumulus (surrounding the oocyte, CG) granulosa cells. In infertility treatment using in vitro fertilization, ovaries are stimulated with exogenously administered follicle stimulating hormone (FSH). Oocyte picked up in IVF offer an excellent opportunity to analyse the functional properties of granulosa cells. The aim of this study was to compare the transcriptomes of MG and CG cells. The study group consisted of 20 infertile women undergoing ovarian FSH stimulation and IVF, with MG cells obtained from follicular fluid and CG cells acquired after oocyte denudation for micromanipulation. The gene expression analysis was conducted using Affymetrix GeneChip technology that provides genome-wide transcription profiling of 33 000 transcripts. The transcriptomes of MG and CG cell populations varied significantly; we found 19 and 22 cell-specific transcripts, respectively, differing in their expression levels at least 2 times ( $P < 0.0001$ ). Ontology analysis of the transcripts showed higher expression of genes involved in immune regulation (interleukins IL1B and IL18) in MG cells. In CG cells, pathways participating in cell-to-cell interactions, tissue remodelling and protein degradation were more clearly distinguished (tenascin-C). The results of the current study help to enhance the understanding about the basic biological processes of ovarian folliculogenesis and to identify the ways of improving the effectiveness of ovarian stimulation in IVF.

## **MATERNAL NUTRITION**

### **Preimplantation environment and postnatal cardiovascular and behavioural consequences.**

**Adam J. Watkins<sup>\*</sup>, Christopher Torrens<sup>†</sup>, Jane K. Cleal<sup>†</sup>, Clive Osmond<sup>‡</sup>, Judith J. Eckert<sup>\*\*†</sup>, William P. Gray<sup>#</sup>, Mark A. Hanson<sup>†</sup> and Tom P. Fleming<sup>\*</sup>**

*<sup>\*</sup> School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK.*

*<sup>‡</sup> MRC Epidemiology Resource Centre, Southampton General Hospital, Southampton SO16 6YD, UK.*

*<sup>†</sup> Institute of Developmental Sciences, Developmental Origins of Health and Disease (DOHaD) Division, School of Medicine, University of Southampton, Southampton General Hospital, Southampton SO16 6YD, UK.*

*<sup>#</sup> Division of Clinical Neurosciences, School of Medicine, University of Southampton, Southampton General Hospital, Southampton SO16 6YD, UK.*

The sensitivity of the mammalian preimplantation embryo to its immediate environment has been demonstrated both in vitro and in vivo and in different species. Here, we investigated the effects of a maternal low protein diet (LPD) given exclusively during preimplantation development in the mouse on cardiovascular health, behaviour and physiology of the offspring.

Virgin female MF-1 mice (7-8.5 weeks) were mated with MF-1 studs and randomly assigned to either normal protein diet (18% casein; NPD) or LPD (9% casein) throughout gestation or LPD exclusively during preimplantation development (3.5 days; termed Emb-LPD) before being returned to NPD for the remainder of gestation. At birth all litters were adjusted to a mean of 6 (3 males and females). At birth, Emb-LPD offspring were significantly heavier than NPD offspring ( $P > 0.05$ ), and Emb-LPD females remained heavier for up to 6 months. Offspring behavioural patterns were assessed using burrowing, open field and nesting tests as measures of affective and anxiety behaviour during several repeated assays. Emb-LPD females showed significantly elevated open field activities (distance travelled and vertical counts), and significantly decreased time spent resting, when compared to controls ( $p \leq 0.05$ ). At 15 and 21 weeks, LPD and Emb-LPD offspring displayed significantly elevated systolic blood pressure, as measured by tail cuff, when compared to NPD offspring ( $P < 0.05$ ). At 22 weeks of age, isolated mesenteric arteries from LPD and Emb-LPD males displayed attenuated responsiveness to the endothelial-independent vasodilator isoprenaline ( $P < 0.04$ ) and a trend towards an impaired responsiveness to the nitric oxide donor sodium nitroprusside ( $P < 0.01$ ) when compared to NPD arteries using fine wire myography. At 28 weeks of age, female left kidneys were processed in

glycol methacrylate, sectioned and, using stereological techniques, glomerular number was estimated using the physical fractionator principle. No difference was observed in mean female left kidney glomerular number between any of the treatment groups. LPD females displayed a significantly elevated serum angiotensin converting enzyme (ACE) activity ( $P = 0.05$ ), while Emb-LPD males and females had a significantly elevated lung ACE activity when compared to NPD offspring ( $P < 0.05$ ).

These data demonstrated the sensitivity of the mouse preimplantation embryo to maternal undernutrition, the subsequent long-term changes to offspring health, and identify potential mechanisms underlying the phenotypic changes induced.

### **Periconception maternal low protein diet: embryo responses and long-term consequences**

**J.J. Eckert<sup>A,B</sup>, S. Brooks<sup>B</sup>, E. Burt<sup>A</sup>, R. Sihota<sup>B</sup>, R. Porter<sup>B</sup>, H.J. Leese<sup>C</sup>, T.P. Fleming<sup>B</sup>**

<sup>A</sup>*DOHaD, School of Medicine,* <sup>B</sup>*School of Biological Sciences, University of Southampton, Southampton, UK;* <sup>C</sup>*Department of Biology, University of York, York, UK*

#### **Embryo responses**

Conditions experienced during the peri-conceptual period including culture in vitro and maternal diet composition can profoundly alter the development of the embryo/foetus and can result in adult health problems such as obesity, hypertension and cardiovascular diseases (see Watkins presentation). This induction or ‘programming’ occurs during the pre-implantation period. In our in vivo mouse model, embryonic changes appear to be mediated by altered maternal serum and reproductive tract composition which, in turn, impact on blastocyst phenotype. For example, we have found reduced protein levels and growth factor expression and changes in amino acid composition in maternal serum or uterine fluid as a consequence of low-protein maternal diet fed exclusively during the preimplantation period (Emb-LPD). Importantly, amino acid levels in blastocysts derived from Emb-LPD mothers were also altered, mostly distinct from maternal changes. This coincided with a compensatory increase in trophectoderm cell numbers evident just prior to implantation. A similar morphological response, with contribution of PKC-mediated signalling, can be observed in an in vitro culture model. Manipulation of specific amino acid levels in the culture medium similar to those found in maternal uterine fluid after Emb-LPD also led to trophectoderm increase when exposed from the 2-cell stage onwards. However, exposure to the same altered amino acid levels in vitro from the 8-cell stage onwards, well after activation of the embryonic genome in the mouse, induced an overall reduction of cell numbers. Collectively, these data suggest that very narrow critical windows of sensitivity may determine the nature of the compensatory changes whereby the early embryo senses and adapts to prevailing conditions.

The biological relevance of early adaptations seen at the blastocyst stage is under constant debate. We now present mechanistic insight that such adaptations could, indeed, have long-lasting consequences. Blastocysts derived from Emb-LPD mothers behaved very differently over at least 3-4 consecutive days compared to those from controls in an *in vitro* outgrowth model even when exposed to the same control culture conditions. For example, we saw an increased spreading activity alongside a decrease in cell density in Emb-LPD embryos. Sensitivity to an inhibitor of mTOR, a critical signalling pathway in coordinating cellular growth according to nutrient availability, remained reduced in Emb-LPD embryos in this model. This suggests that homeostatic settings can be altered longer-term and is one example how physiological adaptations may affect the process of implantation. Another extra-embryonic lineage forming the primitive-endoderm derived yolk sac, showed a compensatory increase in nutrient transport. Changes in the implantation process could provide a route whereby foetal growth and differentiation can be affected longer-term by altered nutrient availability for the developing conceptus. Such developmental plasticity would make biological sense in adapting to prevailing conditions to enhance developmental health. Thus, it seems plausible to suggest that changes in nutrient availability *after* immediate responses have already been induced, ie predictions become inappropriate, could entail profound long-lasting consequences for offspring health.

### **Dietary and environmental factors: effects on embryos and long-term development.**

**Karen L Kind, Cheryl J Schelbach and Jeremy G Thompson.**

*Research Centre for Reproductive Health, Discipline of Agricultural and Animal Science and Discipline of Obstetrics and Gynaecology, University of Adelaide, Adelaide, South Australia, 5005.*

Increasing evidence indicates that perturbing the environment of the developing oocyte or embryo not only impacts on blastocyst development and implantation success, but can also have adverse consequences for fetal, placental and postnatal outcomes. Long-term effects following *in vitro* embryo culture have been described in rodent and livestock species. Similarly, an altered *in vivo* environment, associated with variation in maternal diet or metabolic state during the periconceptional period, can affect embryonic, fetal and adult development. The cumulus-oocyte complex (COC) and embryo respond to changes in their environment with adaptations including alterations in gene expression, metabolism and cell number. While these adaptive responses may act to maintain cellular and metabolic function of the COC or embryo they can have adverse effects on subsequent events, such as implantation, placentation and fetal development, and associated consequences for long-term health. Our studies have focused on metabolic and signalling pathways that may contribute to such responses in the COC and embryo, and the outcomes of altering such pathways. For example, we have investigated the effects of exposing COCs and embryos to elevated concentrations of glucosamine, a popular dietary supplement. Glucosamine is metabolised through the hexosamine biosynthesis pathway (HBP), an alternative pathway for glucose metabolism. Elevated glucosamine levels upregulate the activity of this pathway. Glucosamine addition during *in vitro* oocyte maturation,

in the presence of adequate glucose levels, has negative effects on murine and bovine blastocyst development. Similarly, *in vivo* injection of glucosamine to young mice for 3-6 days before and 1 day after mating reduces implantation rate and litter size. In older mice, periconceptual glucosamine treatment did not alter litter size, but fetal weight was reduced and the incidence of fetal malformations was increased. Maternal obesity is known to be associated with altered glycaemic control. Therefore, we also assessed the effects of glucosamine in mice fed a high fat (22%) diet. Maternal high fat feeding reduced fetal weight and increased the incidence of fetal malformations, compared to control fed (7%) mice, resembling the outcomes observed with periconceptual glucosamine treatment. No further effects, other than those induced by diet alone, were observed following glucosamine treatment of obese mice. Thus, perturbation of a glucose metabolic pathway during the periconceptual period alters embryonic and fetal development. Products of the HBP influence cell signalling and transcription factor activity. Therefore, altered activity of this pathway provides an example of how variation in substrate supply within the environment of the COC and early embryo can influence metabolic, and possibly other cellular functions, with consequences for subsequent development.

### **High non-esterified fatty acid concentrations during murine follicle culture are detrimental for the oocyte's developmental competence**

**JLMR Leroy<sup>1</sup>, V Van Hoeck<sup>1</sup>, K Lemeire<sup>2</sup>, PEJ Bols<sup>1</sup>, R Cortvrindt<sup>1</sup>**

<sup>1</sup>*University of Antwerp, Gamete Research Center, Laboratory of Veterinary Physiology, Wilrijk, Belgium*

<sup>2</sup>*EggCentris N.V., Zellik, Belgium*

High non-esterified fatty acid (NEFA) concentrations, associated with negative energy balance, diabetes Type II and obesitas, may hamper normal cell function. Elevated NEFA concentrations are reflected in the follicular fluid of dairy cows during severe negative energy balance. Bovine oocytes exposed to such NEFA levels during *in vitro* maturation displayed a significantly reduced meiotic competence. To investigate the effect of a long-term exposure of NEFAs on folliculogenesis and oogenesis, a murine follicle bio-assay was used.

Pre-antral mouse ovarian follicles (n= 6x10/condition) were cultured for 12 days in the presence of 50  $\mu$ M palmitic, 40  $\mu$ M stearic or 100  $\mu$ M oleic acid. Every 4 days, follicle development was morphologically graded. After 12 days, cumulus enclosed oocytes were isolated and *in vitro* matured for 18h. Cumulus cell expansion was evaluated and part of the COCs were denuded for oocyte nuclear maturity assessment (n= 2x10/condition) and the remainder were subjected to IVF (n= 4x10/condition).

Neither follicle survival or differentiation nor cumulus expansion was affected. However, the nuclear maturation rate ( $\pm$ SD) significantly dropped after follicle culture in the presence of palmitic (71% $\pm$ 13), stearic acid (73% $\pm$ 11) or oleic acid (74% $\pm$ 14) compared to the control group (91% $\pm$ 10) ( $P$ <0.05). Cleavage rate ( $\pm$ SD) significantly dropped (58% $\pm$ 34; 60% $\pm$ 9; 50% $\pm$ 24 for palmitic, stearic and oleic acid, respectively) compared to the control group (82% $\pm$ 16) ( $P$ <0.05). In conclusion, maternal metabolic conditions leading to increased lipolysis and long-term high

NEFA concentrations during the oocyte's growth and maturation phase can hamper fertility through a reduced oocyte's developmental competence.

## **Fine tuning of blastocyst development by the insulin-like growth factor system in the rabbit**

**Navarrete Santos Anne, Nicole Ramin, René Thieme, Sünje Fischer and Bernd Fischer**

*Department of Anatomy and Cell Biology, Martin Luther University, Große Steinstraße 52, Germany*

The effects of insulin and insulin-like growth factors (IGF) on embryo development during early pregnancy are important for the understanding of metabolic disorders associated with endocrine diseases.

Rabbit blastocysts express the insulin (IR) and insulin-like growth factor receptors (IGFR) in a specific arrangement in the embryoblast (Em) and trophoblast (Tr). The expression pattern depends on the cell lineage and the developmental stage. This differential receptor expression pattern leads to divergent effects of the ligands in respect to downstream signalling and target gene activation.

We have analysed the main pathways of the IR/IGFR system, MAPK and PI3-K, and the downstream target genes c-fos (for mitogenesis), hexokinase (HK, key enzyme for glycolysis), phosphoenolpyruvate carboxykinase (PEPCK, key enzyme for gluconeogenesis) and Bcl-x(L) (for anti-apoptosis), employing different blastocyst stages. IGF1 acts primarily mitogenic and anti-apoptotic in the Em, whereas insulin is the growth factor of the Tr stimulating both, mitogenesis and metabolic genes. IGF2 activates both signalling pathways in the Em. The influence of hyperglycaemic conditions on the IGFR system and its metabolic target genes was investigated during in vitro culture. Six day old rabbit blastocysts were cultured for 1 to 4 hours in 1mM, 10mM and 25mM glucose-containing synthetic media, respectively, and analysed for mRNA expression of IR, IGF1R, HK and PEPCK. The glycolytic enzyme HK was up regulated, whereas the gluconeogenetic enzyme PEPCK was down regulated by increased glucose concentrations. Both the IR and IGF1R were significantly down regulated by 25mM glucose. An additional insulin treatment did not further change the transcript numbers of these genes, indicating a loss of insulin sensitivity of the blastocyst under hyperglycaemic conditions.

Taken together we show that the rabbit blastocyst sensitively reacts to ambient maternal growth factors. Hyperglycaemia leads to decreased insulin sensitivity, indicating a critical role of glucose for blastocyst development.

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## **Effects of maternal undernutrition on the hypothalamic-pituitary-gonadal axis function in male and female sheep offspring**

**Kotsampasi Basiliki, Papadomichelakis G. Balaskas C, Menegatos I and Chadio S.**

*Agricultural University of Athens, Dept. of Animal Science ,75 Iera odos 11855  
Athens Greece*

A number of studies have established that nutrition as early as in embryonic life can affect reproductive potential in adulthood. The aim of the present study was to examine the effects of maternal undernutrition on the hypothalamo-pituitary-gonadal axis function in sheep offspring, at different ages postnatal.

Pregnant ewes were fed to 100% throughout pregnancy (Control) or to 50% from 0-30 (R1) or from 31-100 day of gestation (R2). After birth lambs were weighed and fed *ad libitum* up to the end of the experiment. At 2, 5.5 and 10 months of age a GnRH challenge was conducted to evaluate pituitary responsiveness. At slaughter (10 months) ovaries and testes were removed and examined both macroscopically and histologically. The LH and FSH response to GnRH challenge did not differ between groups at 2 and 5.5 months of age in both sexes, but at 10 months of age a higher ( $P<0.05$ ) FSH response was recorded for males of R2 and for females of R1 group. A lower ( $P<0.01$ ) Sertoli cell number and a smaller ( $P<0.05$ ) seminiferous tubule diameter was detected in testis of R2 males, accompanied by a higher number of apoptotic Sertoli cells ( $P<0.05$ ). Total number of visible follicles and corpora lutea did not differ between groups, but a significant higher ( $P<0.05$ ) number of small follicles was found in ovaries of R1 group and a lower number ( $P<0.05$ ) of large corpora lutea in the ovaries of R2 lambs. Mean proliferative index of granulosa cells was lower in mature follicles in ovaries of R2 group ( $P<0.01$ ).

In conclusion, the results of the present study showed that maternal undernutrition influence pituitary sensitivity in adult animals underfed *in utero* and also provide clear evidence for a direct effect of nutrient restriction during pregnancy on male and female gonads in adulthood.

## **Developmental effects of cholesterol enriched hyperlipidic diets in rabbits**

**Chavatte-Palmer Pascale, P. Laigre, M. Dahirel, O. Picone, L. Lamothe**

*INRA, UMR 1198 Biologie du Développement et Reproduction; ENVA, UMR 1198  
Biologie du Développement et Reproduction; CNRS, FRE 2857, France*

We have previously shown that administration of a hypercholesterolemic hyperlipidic diet to rabbit does throughout puberty and pregnancy significantly restricted foetal growth of the offspring. The aim of this study was to determine the relative role of cholesterol and fatty acids in this model.

Twenty-five does were fed from age 10 weeks with one of 5 diets: S and SC (Soy oil, 8% lipids with or without 0.1% Cholesterol), R and RC (Rapeseed oil, 8% lipids, with or without 0.1% Cholesterol) and Co (Control, 2% lipids). They were bred at 18 weeks. Foetal growth was monitored by weekly ultrasound. Pups were weighed at birth. Additionally, foetuses and placentas from a further 2 to 4 does per group were collected at 15 days (half pregnancy).

Does spontaneously adjusted their caloric intake: body weight were not statistically different between groups. Mean litter size did not differ either. Conceptus length was significantly reduced on d9 in SC (-14%) and RC (-5%) vs C ( $p<0.05$ ). Foetal length was statistically reduced at d15 in groups RC and S (-9%,  $p<0.05$ ) but not in SC. Foetal weight did not differ at d15, but placentas were significantly heavier in S compared to other groups ( $P<0.05$ ). Foetal abdominal perimeter was significantly smaller in SC, RC and R at d22 and in SC at d28 ( $p<0.05$ ). Birth weight was significantly decreased in all groups (-10%, -18%, -21% and -7% in SC, S, RC and R, respectively,  $P<0.05$ ).

In conclusion, cholesterol and lipid enriched diets induced IUGR with variable effects according to the N3/N6 fatty acid ratio and cholesterol content, with more significant effects observed in the cholesterol enriched diets. These effects are observed very shortly after implantation, indicating that oocyte and/or embryonic development may be affected. Further work is in progress to evaluate these early effects.

#### Poster Session

33.

#### **Impact of maternal undernutrition on the hypothalamic-pituitary-adrenal axis function in sheep offspring at different ages postnatal**

**Chadio Stella, Papadomichelakis G, Kotsampasi B, Deligeorgis S, Zervas G, Menegatos I.**

*Agricultural University of Athens, Dept. of Animal Science, 75 Iera odos, 11855 Athens, Greece*

Epidemiological and experimental data support the “fetal programming” hypothesis, which proposes that alterations in fetal nutrition and endocrine status leads to permanent adaptations in fetal homeostatic mechanisms, producing long term changes in physiology and determine susceptibility to later disease. Altered hypothalamic-pituitary-adrenal (HPA) axis function has been proposed to play an important role in programming of disease risk. The aim of the study was to examine the effect of maternal nutrient restriction on the HPA axis function in sheep offspring. Pregnant ewes were fed to 100% throughout pregnancy (Control) or to 50% from 0-30 (R1) or from 31-100 day of gestation (R2). Maternal blood samples were collected weekly from day 70 of gestation to term, for hormones determination. After birth lambs were weighed and fed *ad libitum* up to the end of the experiment. Three CRH challenges were conducted at 2, 5.5 and 10 months of age and ACTH and cortisol levels were determined. Maternal  $T_3$ ,  $T_4$  and insulin levels were lower ( $P<0.05$ ) in group R2,

whereas cortisol levels were higher ( $P < 0.05$ ) in group R1, compared to other groups. The area under the curve (AUC) for ACTH and cortisol response to CRH challenge was greater ( $P < 0.05$ ) in lambs of group R1. Pituitary-adrenal axis response to CRH in terms of AUC did not differ between groups at 5.5 months of age, but basal plasma cortisol concentration as well as concentration at 120 min after CRH administration were elevated in group R1. No significant differences in response to CRH administration were detected at the age of 10 months. It is concluded that the HPA axis is programmable by altered nutrition *in utero*, whereby the sensitivity of the axis to exogenous stimulation is enhanced during early postnatal life and attenuated during ageing, suggesting a role for postnatal influences in resetting of the HPA axis.

34.

### **Insulin and IGF1 promote mesodermal differentiation in rabbit blastocysts**

**Thieme René, Nicole Ramin, Sünje Fischer, Bernd Fischer and Anne Navarrete Santos**

*Department Anatomy and Cell Biology, Martin Luther University, Faculty of Medicine, Grosse Steinstrasse 52 D-06018 Halle (Saale), Germany*

Maternal hormones and growth factors regulate embryo metabolism and govern preimplantation embryo development. The molecular and cellular mechanisms of developmentally highly relevant hormones of the insulin family (insulin and IGF1) during early pregnancy, however, are largely unknown. We have investigated the influence of insulin (17nM) and IGF1 (1.3nM) on mesoderm formation in gastrulating rabbit blastocysts during *in vitro* culture. Gastrulation stages were characterized morphologically (development of the anterior marginal crescent = stage 1, the posterior gastrula extension (PGE) = stage 2, primitive streak/node = stage 3). Blastocysts which were cultured without insulin or IGF1 did not gastrulate, were arrested in early gastrulation stage 1 or died. The mesodermal marker Brachyury and the signalling factor Wnt4 were quantified and localised in gastrulating blastocysts *in vivo* and *in vitro*. The expression of Brachyury was temporally controlled and correlated closely with mesoderm differentiation. During gastrulation Brachyury was expressed solely in the PGE, whereas Wnt4 was distributed over the whole embryonic disc and to a lower amount in the trophoblast. Wnt4 expression peaked with mesoderm induction, suggesting a potential function as developmental timer during gastrulation. Insulin and IGF1 induced Wnt4 and Brachyury expression in a temporally and developmentally stage-dependent manner, indicating that the effects of insulin and IGF1 on Brachyury are mediated via regulation of Wnt4 expression. Our findings point to Wnt4 as one of the first trigger molecules in the mesoderm formation network.

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35.

### **Adiponectin signalling in the pre- and periimplantation rabbit blastocyst**

**Fischer Sünje, René Thieme, Nicole Ramin, Anne Navarrete Santos and Bernd Fischer**

*Department Anatomy and Cell Biology, Martin Luther University, Faculty of Medicine Grosse Steinstrasse 52, D-06018 Halle (Saale), Germany*

Adipokines are hormones secreted by the adipose tissue. They have various functions in lipid and carbohydrate metabolism. Recent studies point to a role of adipokines in reproduction. Although some data have been collected for leptin, the role of adiponectin in reproduction, embryo metabolism and the crosstalk between embryo and maternal tissue during implantation has not been studied so far.

Rabbit blastocysts express adiponectin and the adiponectin receptors 1 and 2 (adipoR1 and R2). On day 6 and 7 *p.c.*, we found adiponectin in the glandular epithelium of the endometrium and in the trophoblast and embryonic disc of the blastocyst. AdipoR1 and R2 were expressed in both cell lineages, too. In the gastrulating blastocyst, the embryonic disc showed a stronger staining for adipoR1 in the anterior marginal crescent (ACM). A gradual expression pattern was found for adipoR2 with decreasing staining from anterior to posterior. A stimulation of day 6 *p.c.* blastocysts with adiponectin (1mM) *in vitro* enhanced 5'AMP-activated protein kinase (AMPK) phosphorylation and decreased the transcript numbers of the metabolic target gene phosphoenolpyruvate carboxykinase (PEPCK), demonstrating the functionality of the adiponectin receptor(s) downstream signalling. PEPCK is the key enzyme of gluconeogenesis, indicating a potential role of adiponectin in embryonic glucose metabolism.

In conclusion, during the pre- and periimplantation period, members of the adiponectin pathway are expressed in the endometrium and blastocyst. In the gastrulating blastocyst, the embryonic disc is characterised by a specific adiponectin receptor expression pattern. Our findings point to a role of the adiponectin pathway in blastocyst differentiation and embryo-maternal interactions during the pre- and periimplantation period.

36.

### **Developmental effects of cholesterol enriched hyperlipidic diets in rabbits**

**Picone Olivier, Archilla, C., Peynot, N., Dahirel, M., Gall, L., Chavatte-Palmer, P. and Duranthon, V.**

Epidemiological studies in humans and animals have demonstrated that the incidence of metabolic diseases is markedly increased when maternal nutrition is altered at critical periods of oocyte, embryo or foetal development. We previously showed that the administration of a hypercholesterolemic (0.2%) hyperlipidic (8% fatty acids) HH diet to rabbit does throughout puberty and pregnancy significantly restricted foetal growth of the offspring. The objective of this work is to evaluate the effects of the HH diet on genomic expression in pre-implantation rabbit embryo.

Female rabbits were fed with a control (2% fatty acids) or HH diet from 10 weeks of age and mated 8 weeks later. 16-24 cells embryos (embryonic genome activation stage) were collected 48h after mating. RNA were extracted from 4 independent pools of 20 embryos from each diet. Each RNA sample was subsequently amplified by two independent procedures (global RT-PCR or aRNA in vitro transcription), resulting in 16 hybridizations with a dedicated, custom-made micro-array. Results of hybridization were analysed by Anova (GENANOVA software).

Twenty-four genes were differentially expressed with the HH diet ( $P < 0.05$ ). The main differentially expressed genes were Adipophilin; Regucalcin; FGF Beta 1; MAPK 7; PAPOA; MGN. Work is currently on-going to confirm these results by qRT-PCR.

This work demonstrates that gene expression at the stage of embryonic activation may be modified by maternal a hypercholesterolemic hyperlipidic diet. The consequences of other diets (with or without cholesterol and with various saturated or unsaturated fatty acids) are also currently being analysed.

37.

**Fertilized mouse eggs can use glucose, lactate or pyruvate as a sole source of energy during the first cell cycle and give healthy offspring.**

**Banrezes Bernadette, Eugénie Canon, Thierry Sainte-Beuve, José Cancela\*  
Jean-Pierre Ozil**

*INRA Jouy en Josas 78352 France*

It is now readily accepted that early events during pre-implantation development can have long-term consequences (Ducibella et al. 2006). Among the complex mechanisms through which early events can affect embryo survival and the health of offspring, we here examine the long term influence of the source of energy inside the culture media during a period of 15 hours starting from the time of pronuclei formation and lasting until the first cell division.

**Methods.** Fertilized eggs at the PN stage were exposed in-vitro at 37°C for 15 hours to 5 different formulas of M16; i) M16 free of energy; ii) M16 with 0,33mM Pyruvate only, iii) M16 with 23mM Lactate only; iv) M16 with 5mM Glucose only. v) standard M16 with 0.33 mM pyruvate, 23mM lactate and 5 mM glucose. For each treatment we monitored the intracellular level of the pyridine nucleotide (NAD(P)H and FAD) which contributes to regulating the REDOX potential (Dumollard et al

2007) by autofluorescence. Experimental embryos at 2-cell stages were transferred to pseudo-pregnant recipients and the rates of living offspring were recorded.

**Results.** i) Eggs cultured in M16 free of energy displayed a low level of NAD(P)H and high level of FAD fluorescence, showing that the redox potential was relatively high and the mitochondrial metabolism was inhibited. In this case only 1% of eggs reached the 2-cell stage. ii) Eggs cultured in M16 pyruvate, also displayed a low NAD(P)H fluorescence but with almost no increase in FAD fluorescence. All of them cleaved and 69% gave birth to living offspring; iii) Eggs cultured in M16 lactate displayed a high level of NAD(P)H with almost no change in FAD. 89% went to 2 cells and 58% of them gave birth to offspring; iv) Eggs exposed to M16 glucose displayed a low level of NAD(P)H and high level of FAD. 29% of them went to 2 cells, but 48% of them gave birth to living offspring; v) Eggs cultured in standard M16 displayed an intermediary level of NAD(P)H and FAD. 100% went to 2 cells and 68% of them gave birth to living offspring.

**Conclusion.** The results show that the substantial stores of amino acid, glycogen or lipids contained in the egg cytoplasm cannot meet the energetic needs beyond 15 hours when eggs are cultured in M16 free of energy. However, the fact that any additional energetic source including glucose, lactate or pyruvate added to M16 devoid of energy during this period of time can rescue the eggs shows that the eggs are to some extent capable of metabolizing glucose and lactate for survival during the first cell cycle. In consequence, the high or low redox potential that varies according to the source of energy does not seem to alter survival to term. Hence, this method makes it possible to evaluate the long term epigenetical changes linked to the change in redox potential during the first cell cycle.

Ref: Ducibella T, Schultz RM, & Ozil JP (2006). *Semin Cell Dev Biol* **17**, 324-332  
Dumollard R, Ward Z, Carroll J, & Duchon MR (2007) *Development* **134**, 455-465.

38.

**Mouse egg does not require an external source of energy during the period of egg activation to activate and form pronuclei.**

**Banrezes Bernadette Sainte-Beuve, T; Canon, E; and Ozil J.-P.**

INRA Jouy en Josas UMR BDR 78352 Jouy en Josas France

**Introduction,** Egg activation is triggered by a series of intracellular  $Ca^{2+}$  oscillations that last a few hours before the formation of the pronuclei. It has been shown that the range of  $Ca^{2+}$  signaling following fertilization has long term effects on both gene expression and development to term (Ozil *et al.*, 2006). The effects may be either intrinsic to the embryo (downstream events of  $Ca^{2+}$  signaling) or extrinsic via the composition of the culture media or the oviduct fluid which provide material and sources of energy. Regarding the energy which is mandatory for the viability and the development of mammalian embryos (Campbell & Swann, 2006), it is not clear how efficiently the egg can rely on its own internal source of energy during activation compared to the additional energy brought into play by the culture media. Here we

examined both the pattern of the  $\text{Ca}^{2+}$  signal and the rate of parthenogenetic activation of mouse oocytes submitted to a series of  $\text{Ca}^{2+}$  transients while oocytes were cultured in M16 media with various energetic substrata.

**Methods:** In a microfluidic chamber freshly ovulated oocytes at MII stage were submitted to a series of 24 transmembrane influxes of  $\text{InsP}_3$  (one every 8 minutes for 3 hours) which induced  $\text{Ca}^{2+}$  release from the endoplasmic reticulum. Four formulas of M16 were used: i) free of energy; ii) with 0,33mM pyruvate only; iii) with 23mM lactate only; and iv) with complete M16. After treatment, experimental eggs were replaced in an incubator and cultured in-vitro with complete M16 to record their developmental potential.

**Results:** In all experimental groups, irrespective of the formula of the culture media during treatment, all eggs were capable of forming pronuclei and proceeding up to cell cleavage. They displayed similar patterns of  $\text{Ca}^{2+}$  transients, except for eggs activated in M16 free of energy which displayed more spontaneous oscillations following  $\text{InsP}_3$  stimulation. In that case, only 48% of eggs were capable of forming blastocysts in-vitro. When only pyruvate or lactate were present in the culture media, 81% and 67% of blastocysts were obtained respectively. With complete M16 100% of the eggs were capable of forming blastocysts.

**Conclusion:** These results clearly show that in absence of any energy supply from the environment during the period of egg activation eggs are still capable of releasing  $\text{Ca}^{2+}$  from ER, activating and developing to the blastocyst stage. However, developmental potential up to the blastocyst stage appears to be correlated with the energy supplied by the oocyte environment during the period of egg activation. Lactate or pyruvate as sole sources of energy can be metabolized during the period of egg activation. The possibility of varying the composition of the culture media and the intermediary metabolism which set the redox potential (Dumolard et al. 2007) during the period of egg activation makes it possible to better understand the physiological parameters that are critical for long term developmental potential of the embryos.

**Ref:** Ozil JP, Banrezes B, Toth S, Pan H, & Schultz RM (2006). *Dev Biol* **300**, 534-544.

Dumollard R, Ward Z, Carroll J, & Duchen MR (2007). *Development* **134**, 455-465.

## **Experiences of obtaining European frame work programmes**

**Dr Andras Dinnyes**

*Biotalentum Ltd, Aulich L. u. 26, Godollo, Hungary & Szent Istvan University, Godollo,  
Hungary [andrasdinnyes@yahoo.com](mailto:andrasdinnyes@yahoo.com)*

This lecture will present the processes in EU FP7 for selecting topics for calls, the type of calls and the proposal evaluation system and most important steps to maximize the scores. Analyses of strategies and possibilities on promoting the topics of the „Gemini” project for future calls, networking among COST members and improving the joint scientific potential of the COST participants will be presented.

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