Maternal Communication with Gametes and Embryo

Proceeding of the 3rd General Meeting of GEMINI, Soustons, France, 1st - 3rd October 2010

Editors:
Alireza Fazeli and Pascale Chavatte-Palmer
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EUROPEAN CO-OPERATION IN SCIENCE & TECHNOLOGY (COST)

COST - the acronym for European Cooperation in Science and Technology - is the oldest and widest European intergovernmental network for cooperation in research. Established by the Ministerial Conference in November 1971, COST is presently used by the scientific communities of 35 European countries to cooperate in common research projects supported by national funds. The funds provided by COST - less than 1% of the total value of the projects - support the COST cooperation networks (COST Actions) through which, with EUR 30 million per year, more than 30,000 European scientists are involved in research having a total value which exceeds EUR 2 billion per year. This is the financial worth of the European added value which COST achieves. A "bottom up approach" (the initiative of launching a COST Action comes from the European scientists themselves), "à la carte participation" (only countries interested in the Action participate), "equality of access" (participation is open also to the scientific communities of countries not belonging to the European Union) and "flexible structure" (easy implementation and light management of the research initiatives) are the main characteristics of COST. As precursor of advanced multidisciplinary research COST has a very important role for the realisation of the European Research Area (ERA) anticipating and complementing the activities of the Framework Programmes, constituting a "bridge" towards the scientific communities of emerging countries, increasing the mobility of researchers across Europe and fostering the establishment of "Networks of Excellence" in many key scientific domains such as: Biomedicine and Molecular Biosciences; Food and Agriculture; Forests, their Products and Services; Materials, Physical and Nanosciences; Chemistry and Molecular Sciences and Technologies; Earth System Science and Environmental Management; Information and Communication Technologies; Transport and Urban Development; Individuals, Societies, Cultures and Health. It covers basic and more applied research and also addresses issues of pre-normative nature or of societal importance.

Web: www.cost.esf.org
WELCOME TO THE 3rd ANNUAL MEETING OF GEMINI

Message from the Chairman

GEMINI is now over two years old and in February 2011 we will celebrate its third birthday. But we have already approached the third general annual conference of our COST Action. Like the last two meetings, this year GEMINI workshops have attracted a good community of scientists. Not only from Europe, but colleagues as far as New Zealand are joining us in France. Earlier this year we had another very successful meeting. It was the "Systems Biology Research School" in Croatia. It attracted a good crowd of young and enthusiastic scientists in our field. The proceedings of all GEMINI meetings are published as books and recently we have published a special edition of Theriogenology that was dedicated to papers presented in the Lansk meeting. I think all these achievements are the signs of an active and energy full scientific community that is working very hard to understand and explore unknown aspects of maternal communications with gametes and embryo. This was exactly what we wanted to achieve during our COST action and today I am very happy to see it is happening.

I would like to particularly thank the local organising committee of this year’s meeting in France. Organising an international meeting is not an easy job. I am thankful to all the members of local organising committee for volunteering to organise this meeting and being our host.

Mrs Sara Gottliebsen, our administrator, has provided the GEMINI community with an excellent professional service that is exemplary. Sara, thank you very much for your hard work. Here I also need to thank Dr. Laszlo Tecsi that has recently joined our supporting team in GEMINI. I am thankful to Sara and Laszlo for their excellent support.

I want to also to take this opportunity to thank all our invited speakers that have accepted our invitation to come to France. I am very pleased to have them with us in our meeting and I look forward to hearing about the interesting science that they do.

Finally, I want to thank all of you for participating in this meeting. I am grateful to you for your support and dedication to achieve GEMINI’s goals and objectives. It is with your support and contribution to GEMINI programs that we hopefully will build a success story of our COST Action.

Dr Alireza Fazeli
GEMINI Chairman
September 2010
WELCOME TO SOUSTONS

Welcome to France!

We are very glad to welcome you in the Landes region, known for its large pine tree forest, its long sandy beaches with big waves that attract surfers from all over the planet and its food specialties that you will soon discover. Landes are attractively located south of Bordeaux and its famous vineyards, north of the Pyrenees mountains and the Basque region and close to Spain. Basque games have been organized at the end of the meeting so that you get a feeling of regional traditions!

In terms of practical organization, we have had support from the Cost office and from our institution, INRA, Physiology and Breeding systems section. However, without the generous support of General Electrics Healthcare, Pfizer, IMV Technologies and the French union for cooperatives of artificial insemination UNCEIA, we would not have been able organize transportation from the station and the airport and above all to give local grants to poster presenters. The PremUp Foundation for Pregnancy and Prematurity, recognizing the importance of animals as biomedical models and the importance of GEMINI, have been active in helping us with bags and the invitation of a speaker.

Finally, we could not have organized this meeting without the constant support of COST GEMINI and INRA staff, in particular Sara Gottliebsen, who has been answering most of your queries, Sébastien Dime, from our research unit, who has done a fantastic work managing the financial aspects of the conference and Laurent Méniel who has been in charge of the website. Our warmest thanks to all of them!

Now, we hope you will enjoy the exciting scientific programme as well as the nice location and company!

Best wishes

Pascale Chavatte-Palmer, Véronique Duranthon and Olivier Sandra
Local Conference Organisers
3rd annual Gemini Meeting
Soustons, France
1st – 3rd October 2010
Programme

Thursday 30th September 2010
18:00-21:00 Registration Open and Reception
19:00-21:00 Dinner (for registered participants)

Friday 1st October 2010
08:00 Registration open
08:30-08:40 Conference Chairman Opening: Pascale Chavatte-Palmer, INRA, France
08:40-08:50 GEMINI Chairman Opening: Alireza Fazeli, University of Sheffield, UK
08:50-09:25 Plenary Lecture
Tom Fleming, University of Southampton, UK
Why your mother's health and diet around conception will shape your life?

Short Communications WGIII: Maternal Nutrition
09:25-09:45 René Thieme, Martin Luther University, Germany
Mesoderm differentiation is delayed due to type 1 diabetes and induced by insulin in preimplantation rabbit blastocysts
09:45-10:05 Audrey Rosefort, INRA, France
Effect of maternal periconceptional undernutrition on male offspring physiology and testicular development: a sheep model
10:05-10:25 Veerle Van Hoeck, University of Antwerp, Belgium
The effect of high non-esterified fatty acid concentrations during oocyte in vitro maturation on the oxidative metabolism of bovine embryos

10:25-10:55 Coffee Break

Plenary Lectures
10:55-11:30 Tiziana Brevini, University of Milan, Italy
Parthenogenesis in non-rodent species: developmental competence and differentiation plasticity
11:30-12:05 Olivier Morel, PremUp Foundation, France
Importance of domestic animal models for human reproductive medicine
12:05-12:40  Jean-Pierre Ozil, INRA, France
Driving egg metabolism makes it possible to modulate post-natal growth in the mouse

12:40-14:10  Lunch (for registered participants)

Short Communications WGII: Tools, Technologies & Systems Biology

14:10-14:30  Pilar Pallares, INIA, Spain
Phenotypic Characterization by High-Resolution 3D-MRI Evidences Differential Effects of Embryo Genotype on IUGR in Nos3-Deficient Mice

14:30-14:50  Kristy Demmers, AgResearch, New Zealand
Sheep with genetic differences in embryo mortality – a novel resource for investigating embryo-maternal interactions?

14:50-15:10  Susanne Ulbrich, Technical University of Munich, Germany
Methylation analysis of estrogen receptor alpha in bovine endometrium

15:10-15:40  Plenary Lecture
Fulvio Gandolfi, University of Milan, Italy
The Year Past in COST

15:40-16:10  Tea Break

15:40-18:40  Poster Session 1: Informal poster discussion for Working Group II & III

18:40-19:10  Working Group III Discussions
Tom Fleming, University of Southampton, UK

19:10-19:40  Working Group II Discussions
Jean-Pierre Ozil, INRA, France

20:00  Dinner (for registered participants)
Saturday 2\textsuperscript{nd} October 2010

08:30  Registration open

08:30-09:15  Discussion on funding
\textit{Alireza Fazeli, University of Sheffield, UK}

09:15-09:50  \textit{Alireza Fazeli, University of Sheffield, UK}
\textit{Innate immunity and maternal communication with gametes and embryos}

09:50-10:25  \textit{Ann van Soom, Ghent University, Belgium}

Embryo-maternal interactions in the horse: Is it really that important for successful ART?

\textbf{10:25–10:55}  \textit{Coffee break}

10:25-12:30  Poster Session 2: Informal poster discussion for Working Group I

\textbf{12:30–14:00}  \textit{Lunch (for registered participants)}

Short Communications WGI: Experimental Models

14:00-14:20  \textit{Caroline Eozenou, INRA, France}
\textit{Characterisation of the Forkhead box transcription factor FOXL2 in the bovine endometrium during early pregnancy}

14:20-14:40  \textit{Tal Keidar, Hebrew University of Jerusalem, Israel}
\textit{Mating-responsive cytoskeleton proteins are essential for proper egg transport along the oviduct post-mating}

14:40-15:00  \textit{Agnieszka Blitek, Institute of Animal Reproduction and Food Research, Poland}
\textit{Conceptus increases HOXA10 and PTGS2 gene expression, and PGE2 secretion in a co-culture system of luminal epithelial and stromal cells of the porcine endometrium}

15:00-15:30  \textit{Working Group I Discussions}
\textit{Ann van Soom, Ghent University, Belgium}

15:30-15:40  \textit{Conference Close}
\textit{Alireza Fazeli, University of Sheffield, UK}

\textbf{15:40–17:15}  \textit{Tea break}

15:40-17:15  \textit{“MC Meeting”} (MC members only)

17:15-19:30  \textit{Basque games}

\textbf{20:00}  \textit{Gala Dinner}
Sunday 3\textsuperscript{rd} October 2010

One day Excursion (for registered participants)
INVITED

SPEAKER

ABSTRACTS
Why your mother’s health and diet around conception will shape your life?

Our work in recent years has shown that a critical aspect of ‘maternal interactions with gametes and embryos’ concerns developmental plasticity and the setting of offspring phenotype to match the nutritional environment anticipated after birth. This mechanism may confer competitive advantage to the offspring but if set inappropriately can lead to adult disease. Thus, transient maternal protein undernutrition during just the cleavage period in rodents is sufficient to induce cardiovascular, metabolic and behavioural disease in adult offspring fed normal diet. We find a significant early marker of developmental plasticity in this model concerns modulation of nutrient signalling pathways in embryos controlling cellular growth and biosynthesis rates. The development and functional activity of the extra-embryonic lineages responsible for nutrient delivery to the conceptus appear particularly sensitive to early maternal environment, thereby indirectly affecting fetal phenotype. The susceptibility of early embryos to their environment with life-long consequences has broad implications across species including the outcome of clinical assisted conception practice. Moreover, in a new mouse model, we find maternal systemic inflammation and sickness behaviour induced by endotoxin injection just after mating is sufficient to alter blastocyst phenotype in the short-term but lead to metabolic and behavioural effects in the adult long-term, including altered innate immune reactivity. Collectively, the early embryo appears receptive to diverse environmental factors that really do shape our lives; finding ways to control these processes will be an important strategy for health in animals and humans.

Funded by BBSRC, MRC, NICHD (USA)
Parthenogenesis in non-rodent species: developmental competence and differentiation plasticity

Parthenogenesis is the process by which an oocyte can develop without the intervention of the male counterpart. It is common to a variety of lower organisms, while mammals are not spontaneously capable of this form of reproduction. Mammalian oocytes can be activated in vitro, mimicking the intracellular calcium wave induced by the sperm at fertilization, which triggers cleavage divisions and embryonic development. The obtained parthenotes are unable to develop to term and arrest their development at different stages after activation, depending on the species, due to genomic imprinting which causes the repression of certain genes, that are normally expressed by the paternal allele.

Because mammalian parthenotes are inherently unable to form a new individual, human parthenogenetic embryos have recently been proposed as an alternative, less controversial source of embryonic stem cell lines. However many aspects related to the biology of parthenogenetic embryos and parthenogenetic derived cell lines still need to be elucidated. In particular scattered information is available on the consequences of the lack of centrioles and on parthenote ability to assemble a new embryonic centrosome in the absence of the sperm centriole, using the pericentriolar material of maternal origin as a template. Indeed in lower species, successful parthenogenesis largely depends upon the oocyte ability to generate complete and functional centrosomes in the absence of the material supplied by a male gamete, while this control appears to be less stringent in mammalian cells where genomic imprinting is thought to be the main mechanism to ensure bi-parental fertilization. Inability to form a correct centrosome is suggested to lead to aberrant progression of cell cycle and spindle formation. In an attempt to better elucidate some of these aspects, parthenogenetic cell lines, recently derived in our laboratory, have been characterized for their pluripotency. The maternal contribution to differentiation plasticity has been assessed, both in vitro and in vivo, demonstrating the ability of these cells to differentiate into cell types derived from the three germ layers. In our presentation we will discuss the characteristics of these cell lines that are common to bi-parental embryonic stem cells. However we will also describe the presence of an intrinsic deregulation of the mechanisms controlling proliferation versus differentiation and suggest their uni-parental origin as a possible cause.
Friday 1st October 2010

Olivier Morel
PremUP Foundation, France
Olivier Morel (1, 2, 3, 4), Vassilis Tsatsaris (2,3), Pascale Chavatte-Palmer (1,3)

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2. INSERM U767, Paris Descartes University, 4 av. de l’Observatoire, F-75006 Paris, France
3. Premup Foundation (Fondation pour la prévention de la prématurité et la protection du nouveau-né prématuré), 4 av. de l'Observatoire, F-75006 Paris, France
4. Obstetrics and Gynecology unit, Maternité Régionale Universitaire A. Pinard, rue du Dr Heydenreich, 54000 Nancy, France
5. CRII (Centre de Recherche en Imagerie Interventionnelle), INRA, F-78350 Jouy-en-Josas, France

Importance of domestic animal models for human reproductive medicine

Animal models are of critical importance both for Medical doctors, Veterinarian and Scientists. Even if rodents and lagomorphs are certainly the most commonly used species, larger species might be required, especially when surgical approaches or new medical devices have to be evaluated.

Large animal models are widely used in the field of reproductive medicine: for the evaluation and development of new pharmacological treatments, of new invasive procedures in fetuses, of genetic approaches for the understanding of sexual differentiation mechanism, of clones and for the evaluation of the mechanism and consequences of fetal programming. Domestic models were recently used in our group for the evaluation of a new functional imaging approach: the real time quantification of the vascular signal within an organ of interest by 3D Doppler imaging. The pregnant sheep model allowed defining the precise correlation between the 3D Doppler signal and the true blood flow within the volume of interest. The impact of the machine settings and Doppler parameters on the final vascular quantification were also evaluated.

This non-invasive functional imaging approach is of potential great clinical and experimental interest in the field of reproductive medicine. Large animal models were required to evaluate this technique.

Today, the usefulness of this new tool is already tested for the functional evaluation of the ovary, the endometrium, the utero-placental unit and the fetus both in clinical and experimental setups.

Keywords: biomedical research, domestic animal models, reproductive medicine, 3D Doppler
There is compelling evidence that the earliest stages of fertilization in mammalian eggs are sensitive to perturbation arising from in vitro conditions and/or to the nutritional diet of the mother during the peri-conceptional period. Some alterations, such as body size, hypertension or organ:body-weight ratios (1) reflect epigenetical mechanisms that imprinted the genome in the early stages. However the initial physiological parameters that alter egg metabolism and orient the developmental processes are yet to be discovered.

An increasing number of roles for the cellular redox state in general and mitochondria in particular have been found in cell differentiation and death (2). The cell dependence on continual input of energetic substrates and the balance between the oxidized and reduced form of the pyridine adenine dinucleotide co-factors (NADH/NAD⁺) make cell differentiation highly sensitive to environmental variations (3).

In mammals, fertilization increases oocyte metabolism and mitochondrial functioning (2) but its energy production is highly dependent on the carbohydrates present in its environment. It has been clearly shown that the exogenous lactate / pyruvate ratio is the major regulator of the cytosolic redox state (NADH/NAD⁺ ratio) but with pyruvate as the main source that supports the mitochondrial (FAD) oxidative phosphorylation for ATP synthesis. Pyruvate acts as an energetic and redox substrate whereas lactate acts only as a redox substrate during fertilization in the mouse (2). It therefore appears that small changes in the pyruvate/lactate ratio in the culture media can simultaneously alter the input in free energy and the redox potential which both ultimately change the cellular functions. The enslavement of the redox potential, which regulates many transcriptional activators, including the chromatin remodeling machinery (3) within the process of ATP synthesis by the mitochondria, makes it difficult to experimentally discriminate the specific impact of oxidation or reduction on the regulation of early development.

During this presentation I will showcase some recent data from my laboratory, exemplifying how egg environment can manipulate epigenetic memory in the offspring.

The experimental strategy adopted in the present study exploits the fact that the period of zygote activation is not controlled by transcription as transcriptional activities are almost silent at the zygotic stage. Hence, the external media surrounding the zygote appears to be the main driver orienting internal chemical modifications that reshape chromatin structure at a stage where the zygote is not yet functionally organized. We designed four different culture media and concentrated their impact during the pronuclear stage of in-vivo fertilized zygotes.

Results reveal that neither the high oxidant nor the high reductant loads during the one cell stage compromise survival to term. However, in zygotes experiencing cytosolic oxidation, animals become smaller when the cellular energy state is increased. In contrast, in zygotes experiencing cytosolic reduction, animals become larger when the cellular energy state is increased. Importantly, the larger or smaller phenotypes persist to the adult age depending on the energy input. It appears that the adult phenotype can be imprinted during fertilization and is redox-regulated and energy-dependent. This effect also appears to be gender-related. Driving the redox potential during fertilization offers a powerful means of dissecting the epigenetic mechanisms that orient the developmental processes.

REFERENCES
Alireza Fazeli
University of Sheffield, United Kingdom
Alireza Fazeli, Wedad Aboussahoud, Reza Aflatoonian, Javier Arturo Sanchez Lopez and Carmen Alminana

Innate immunity and maternal communication with gametes and embryos

Birth of an offspring is the story of a successful collaboration between immune and reproductive system. The whole process of conception, pregnancy and child birth is full of examples of how these two systems successfully manage to work together. Hence, we can find the roots of many causes of infertility in miscommunication between immune and reproductive system.

Immune system can generally be divided to two main systems; innate and adaptive immune system. The adaptive immune system as is apparent in its terminology, is that part of immune system that is developed during our life time and adopts itself with our needs for protection and our environment as we grow old. But we are all born with the innate immune system. It is within us and its job is to protect us from pathogens that we encounter even as early as our embryo is formed.

Despite the importance of the innate immune system and the fact that immunologists from long time ago knew about its existence, our knowledge about it and the molecular mechanisms responsible for mediating innate immunity was very limited. Recently our understanding of innate immune system molecular mechanisms has been expanded, by discovery of a group of molecules known as “Toll Like Receptors (TLRs)”. TLRs are one of the main known groups that can recognise different pathogens and not only are producing a primary line of defence against these pathogens, but also are alerting the adaptive immune system to the presence of pathogens and further initiation of defence mechanisms by immune system against these pathogens.

The history of discovery and characterisation of TLRs in the maternal tract is less than a decade old. Our lab reported one of the initial reports of existence of these molecules in the female reproductive tract and further on described their alteration during the reproductive cycle. These molecules are widespread in different sections and locations of the maternal tract and are temporally regulated during the reproductive cycle. Recent reports continue to expand their role in reproductive system beyond immune responses to pathogens or prevention of reproductive tract diseases. It seems these molecules may be involved in mediation of main stream reproductive events such as gamete interactions and embryonic implantation.

One of the big mysteries in reproduction is the fact that non-self entities such as spermatozoa or even allogeneic embryo (with its own immunogenic signature) that should provoke immune responses are not repelled, but well tolerated and even nourished by the maternal tract. How innate immune system manages to differentiate between different non-self entities and present a harsh reaction to those entities that have the potential to be pathogenic and a welcoming response to those that are responsible for survival of different species on this planet, remains a mystery. One hypothesis can be the existence of molecules produced by embryo or spermatozoa that can dampen the innate immune system ability to recognise them or block their reaction and activation. The other can be the existence of molecules belonging to innate immune system which act as specific molecules to recognise spermatozoa and embryo as good non-self entities. Interestingly from an evolutionary point of view one can postulate that internal fertilization (versus external fertilization) started as soon as living beings on this planet learned how to differentiate between good and bad non-self entities.

Studying the role of innate immune system in maternal interaction with gametes and embryo will have a tremendous impact on our understanding of mechanisms responsible for infertility. It may provide an opportunity for application of new therapies for treatment of infertility, as well as understanding how immune system modulates its responses. Application of such knowledge will be beyond the treatment of the reproductive system disorders and may potentially help us in providing treatments for auto-immune diseases and growing incidence of allergies.
Embryo-maternal interactions in the horse: Is it really that important for successful ART?

Early embryonic development in the horse is characterized by a number of peculiarities. After an unusually long stay in the oviduct (~6 days), the equine embryo passes through the utero-tubal papilla. Unfertilized eggs on the other hand are retained in the oviduct. This selective oviductal transport is linked to the stage specific production of prostaglandin E2 by the equine conceptus, an extremely early example of embryo-maternal interaction. Another enigmatic feature exemplifying this early interaction in the horse is the formation of an acellular glycoprotein capsule very soon after the arrival of the horse embryo in the uterus. When equine embryos are produced in vitro, and therefore in the absence of the maternal tract, they differ markedly from their in vivo counterparts in terms of the kinetics of development, incidence of apoptotic cells, inner cell mass morphology, capsule formation and gene expression patterns. Nevertheless equine blastocysts produced in vitro or in vivo are equally able to establish pregnancy after intra-uterine transfer to a recipient mare. We will cover the unique aspects of the interaction between the early horse embryo and the oviduct or uterus, as well as the consequences of the absence of these interactions when the embryos are produced in vitro. To illustrate species-specificity, comparisons are made with the situation in human and bovine.
ORAL PRESENTATIONS FROM ABSTRACTS
**Mesoderm differentiation is delayed due to type 1 diabetes and induced by insulin in preimplantation rabbit blastocysts**

The preimplantation period is a very vulnerable period in embryo development. During preimplantation development the embryo is closely dependent from maternal nutrition and hormones. Metabolic disorders such as diabetes mellitus or obesity negatively influence embryo development. Infants from diabetic mothers, for example, have a 2.5fold higher risk for congenital heart defects. We used an experimentally induced type 1 diabetes model to closer investigate embryo development and gastrulation under diabetic conditions.

Blastocysts grown in diabetic mothers or cultured in vitro without insulin/IGF1 were analysed for mesoderm formation by morphological characterisation of the embryonic disc (development of the anterior marginal crescent = stage 1, the posterior gastrula extension (PGE) = stage 2, primitive streak/node = stage 3) and gene expression pattern of the mesoderm specific marker *Brachyury*. Blastocysts from mothers with type 1 diabetes were developmentally delayed and blastocysts cultured without insulin or IGF1 did not gastrulate, were arrested in early gastrulation (stage 1) or died. The mesoderm inducing molecules Wnt3a and Wnt4 were investigated. Wnt3a levels in blastocysts from diabetic mothers were decreased while Wnt4 was not affected. In vitro supplementation of insulin showed a positive effect on Wnt3a and Wnt4 expression levels. The effect of insulin on Wnt3a was diminished by specific inhibition of MAPK-signalling with PD098059.

Insulin facilitated Wnt3a, Wnt4 and *Brachyury* expression. The temporally and stage-dependent induction of *Brachyury* expression by insulin is mediated via regulation of the Wnts.

Taking together we conclude a close relationship between insulin/IGF1 and the mesoderm formation network in the rabbit blastocyst. Diabetogenous dysregulations as soon as during gastrulation could be related with congenital heart defects seen in infants from diabetic mothers.

*Supported by DFG NA 418/4-2 and the Wilhelm Roux Programme of the MLU, Faculty of Medicine*
Effect of maternal periconceptional undernutrition on male offspring physiology and testicular development: a sheep model

Many studies have shown that maternal undernutrition during gestation can condition offspring adult health. The aim of this project was to evaluate the long term effects of maternal periconceptional undernutrition on offspring physiology and reproductive function. Post-natal development of male offspring is presented here.

52 control females of Merinos d’Arles breed (R100) were fed a normal diet adapted to their needs and 64 restricted females (R50) received 50% of their dietary needs from 15 days prior to 30 days after synchronized breeding. Thereafter, both groups were fed to meet their nutritional requirements. Male offspring were weighed at birth and every week. Plasma leptin and cortisol concentrations were monitored monthly. Organ weights were recorded at commercial slaughter and histological analysis was performed on testicles and adrenals.

22 live R100 and 34 live R50 male lambs were obtained. Gestation was significantly longer in the restricted group (p<0.01). Plasma leptin concentrations were significantly lower at birth (p<0.001) and tended to be higher at 4 months of age (p=0.09) in R50. There was a significant interaction between group, age and litter size for basal cortisol concentrations: in singletons, cortisol was significantly lower in R50 at 3 mo of age (p<0.05) and tended to be higher at 4 mo whereas in twins, cortisol was significantly lower at birth (p<0.05) but not thereafter. Carcass to live weight ratio and perirenal fat to live weight ratio were increased in all R50 (p<0.05). Adrenal to live weight ratio tended to be increased in R50 lambs (p=0.06) but adrenal corticomedullary ratio was not different between groups. There was no significant difference for the analysis of testicular tissue. The expression of genes involved in testicular differentiation is currently being studied by quantitative PCR.

These results confirm the effects of periconceptional undernutrition and litter size on metabolic function but not on testicular histology.
The effect of high non-esterified fatty acid concentrations during oocyte in vitro maturation on the oxidative metabolism of bovine embryos

Previous research has shown that exposure to elevated non-esterified fatty acid (NEFA) concentrations during in vitro maturation induces aberrant oocyte developmental competence, a finding recently associated with a reduction in embryo quality and viability. There is a need to investigate the mechanisms by which exposure to high NEFA concentrations impairs fertility. In the present study we hypothesized that mitochondria are important targets for NEFA induced toxicity in the oocyte. This study aimed to evaluate the gross oxygen consumption, an indicator of overall energy production, of individual zygotes and blastocysts derived from oocytes matured under elevated NEFA conditions.

During serum-free maturation, bovine COCs were matured in 1) physiological NEFA = CONTROL (150 µM of total NEFA, i.e. oleic, stearic & palmitic acid), 2) elevated stearic acid = HIGHSA (75 µM SA) and 3) elevated NEFA = HIGHCOMBI (425 µM total NEFA). Following IVF, resulting zygotes were cultured in SOF + 5% FCS. The oxidative metabolism was measured using a Nanorespirometer (Unisense DK), a modified Clark-type electrode which enables non-invasive measurement of oxygen consumption.

There was no statistically relevant difference in zygote (day 1) oxygen consumption between groups. However, HIGHCOMBI embryos (day 7) consumed significantly less oxygen (factor 0.59) compared to CONTROL embryos (P<0.02) and HIGHSA embryos tend to consume less oxygen (factor 0.77) compared to CONTROL embryos (P<0.2).

We can conclude that the metabolism of embryos, originating from NEFA exposed oocytes, is altered and the reduction in oxygen consumption of these embryos might suggest that mitochondrial dysfunction is involved in the earlier observed reduction of embryo quality.
Pilar Pallares  
Head of Animal Unit  
INIA, Spain

| Phenotypic Characterization by High-Resolution 3D-MRI | Evidences Differential Effects of Embryo Genotype on IUGR in Nos3-Deficient Mice |

The Nos3-knockout mice, deficient for endothelial Nitric Oxide Synthase (NOS3), are affected by reductions in number and weight of the embryos and constitute a good model for some features of preeclampsia and intrauterine growth retardation (IUGR). Deficiencies in conceptus growth and survival may result from factors inherent to the embryo itself or from deficiencies in uterine function. In current study, we aimed to discriminate the effects of embryonic genotype, independently of maternal genotype that can affect uterine environment. Therefore, by using Magnetic Resonance Imaging (MRI), we characterized the phenotype of mouse foetuses defective for NOS3 (NOS3−/−; n=6), normal wild-type (NOS3+/+; n=5) and heterozygous (NOS3+/−; n=16). All of them were littermates obtained by breeding heterozygous mice (NOS3+/−); therefore, the maternal genotype was the same for all the foetuses. At Day 13.5 (i.e.: Theiler stage TS 21-22), females were anesthetized and scanned with 3D-MRI. There were not found significant differences between NOS3+/+ and NOS3+/− in any of the different measurements of the embryos and the gestational annexes, although there was a trend for larger sizes in NOS3+/+. On the other hand, all of the measurements in both NOS3+/+ and NOS3+/− were significantly larger than in NOS3−/− (P<0.05), except for the difference between the area of the vesicle and the embryo. The reduction in the crown-rump length of NOS3−/− reached 12% when compared to NOS3+/+ (P<0.05); the effect was higher for head measurements (16% for occipito-snout length and biparietal diameter, P<0.05 for both) and trunk diameter (17%, P<0.05). Overall, the maximum area of foetuses in longitudinal planes decreased a 27% (P<0.05) when comparing NOS3+/+ to wild-type NOS3+/−. It is possible to hypothesize that IUGR in NOS3−/− may be related to a reduction of 29% found in the maximum thickness of the placenta, which would compromise nutritional delivery to the foetus.
**Sheep with genetic differences in embryo mortality – a novel resource for investigating embryo-maternal interactions?**

Embryo mortality is a major factor limiting reproductive efficiency in sheep, with between 20-30% of potential lambs lost between ovulation and birth. Most losses have been reported to occur in the first month of pregnancy, but the underlying factors are not well understood. A better understanding of embryo mortality, including the pathways involved, is necessary to improve embryo survival in sheep. The identification of a sheep line with potential genetic differences in embryo survival provides a unique resource for investigating embryo-maternal interactions.

Eleven rams for progeny testing were selected from a commercial flock in which previous studies had revealed a pattern of high embryo survival in certain family lines. Embryo mortality was measured in 11 to 38 daughters per sire at 1.5 and 2.5 years of age, by comparing the number of eggs ovulated with offspring born to that oestrous cycle. On average, 30% of ovulated eggs did not result in live offspring, with mortality increasing as ovulation rate increased (P<0.01). This is consistent with previous studies and confirms that uterine capacity is an important limitation in sheep, with the chance of an individual oocyte becoming a lamb declining as ovulation rate increases.

The sire of the ewe significantly affected embryo mortality (P<0.05), with the average number of ovulated eggs lost ranging from 12 to 42% in different sire groups over the two years (average ovulation rate ranged from 2.0 to 2.3). An exciting observation was that enhanced embryo survival was noted in daughters of one ram, but not in daughters from his full-sibling (P<0.05), which suggests a single gene could underlie enhanced embryo survival in this line of sheep. Our long-term aim is to identify this putative embryo survival gene, which will involve obtaining embryo mortality data on the daughters from the different sires over repeated breeding seasons.

In order to investigate the underlying embryo-maternal interactions, future studies will first determine the timing of embryo mortality differences and this period will be the focus of future research.
Next to the setting of critical epigenetic marks during embryonic development, DNA methylation is increasingly discussed as dynamic mechanism involved in transient regulation of transcription. We questioned whether differential methylation of an intragenic region of ESR1 would take part in transcriptional regulation of ESR1 during the estrous cycle and early pregnancy in bovine endometrium. Therefore, endometrial samples were collected from three groups of heifers (n=6 each) slaughtered at estrus (P4<0.1 ng/mL) or 18 days after estrus (day 18 control) (P4>3.2 ng/mL), or during early pregnancy (day 18 pregnant) (P4>3.3 ng/mL). In addition, blood leucocytes from cyclic heifers (n=6) extracted following alkaline lysis of erythrocytes were analyzed. Total DNA was extracted and bisulphite-converted. Specific fragments of the intron 1 region (+812 to +1393 bp downstream of the bovine ESR1 promotor) comprising 16 CpG sites were amplified using high resolution melt (HRM) analysis and subsequently analyzed on a single CpG-site resolution by pyrosequencing. In addition, global DNA methylation was analyzed by luminometric methylation assay (LUMA). Neither HRM nor pyrosequencing of individual CpG sites downstream of the ESR1 promoter revealed significant differences between the analyzed groups of endometrial samples, which were only weakly methylated (6 % average methylation). However, leucocytes expressing only minute amounts of ESR1 transcripts not only displayed a higher degree of global methylation (83 % versus 76 % average methylation in endometrium, respectively), but also locally showed a distinctly different HRM shape and were significantly more methylated over the analyzed CpG sites (31% average methylation). Thus, although necessary for appropriate tissue-specific expression, DNA methylation of the analyzed region of ESR1 does not seem to be involved in transcriptional regulation of the endometrium neither during the cycle nor during establishment of pregnancy.
Saturday 2\textsuperscript{nd} October 2010
14:00-14:20

Caroline Eozenou
Carvalho A.\textsuperscript{1}, Gall L.\textsuperscript{1}, Forde N.\textsuperscript{2}, Giraud-Delville C.\textsuperscript{1}, Pannetier M.\textsuperscript{1}, Auguste A.\textsuperscript{1}, Tarrade A.\textsuperscript{1}, Charpigny G.\textsuperscript{1}, Roche JF.\textsuperscript{2},
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Characterisation of the Forkhead box transcription factor FOXL2 in the bovine endometrium during early pregnancy

In mammals, implantation is critical for the outcome of pregnancy and involves a tightly regulated communication between the endometrium and the embryo. Our recent transcriptome analyses using bovine endometrium have revealed the expression of several families of transcription factors, including several Forkhead box (FOX) transcription factors during early pregnancy. Among them, the expression of a member of the FOXL sub-class - FOXL2- has been detected. FOXL2 is one of the most crucial genes involved in ovarian differentiation and until now its expression has appeared to be restricted to pituitary, ovary and foetal eyelid. To determine how FOXL2 contributes to endometrial physiology, we characterized the expression, regulation and cellular localisation of FOXL2 using real-time PCR, western blot and immunohistochemistry analyses under a number of physiological and experimental parameters. The expression of FOXL2 was confirmed at both transcriptional and protein levels in the cyclic and pregnant bovine endometrium. FOXL2 transcript was expressed from day 5 to day 20 post-oestrus and the expression level was independent of systemic progesterone concentrations during the pre-implantation period. Implantation (20 days post oestrus) was associated with a significant decrease of FOXL2 transcript and protein expression in the pregnant endometrium. Interferon-tau, the major signal of pregnancy recognition in ruminants, partially accounted for the reduction in FOXL2 expression. At the cellular level, FOXL2 was found to be localised in the luminal epithelium, the stroma and the glandular cells of the bovine endometrium. Altogether, our results suggest FOXL2 as a major factor associated with the female reproductive axis in mammals. The uterine expression of FOXL2 deserves further investigation to better understand the biological functions of this transcription factor during pregnancy.
Mating-responsive cytoskeleton proteins are essential for proper egg transport along the oviduct post-mating

In the fruit fly, *Drosophila melanogaster*, mating triggers the entry of the female reproductive tract into a new developmental stage, where an optimal environment is created for ovulation and fertilization. During mating, the male transfers to the female sperm and seminal fluid molecules that trigger behavioural and physiological changes in the female. Mating induces extensive remodelling of the oviduct, accompanied by a change in gene expression, specifically up-regulation of proteins with a known role in epithelial cell polarity and cellular junctions. This raises the hypothesis that these mating-responsive cytoskeleton proteins are involved in the tissue remodelling that take place in the oviduct epithelium post-mating.

To examine the role of mating-responsive cytoskeleton proteins in the oviduct epithelium post-mating, RNAi was employed to knock down protein level in the lateral oviducts of females and their reproductive success was subsequently examined. To further characterize the effect of mating on oviduct epithelium, we also examined the spatial localization of the mating-responsive cytoskeleton proteins in the oviduct pre- and post-mating.

Results indicated that females with reduced levels of mating-responsive cytoskeleton proteins in the lateral oviducts, laid fewer eggs during the first 6 hours post-mating and some interestingly displayed an increase in egg-laying during the subsequent days. During this time, a change in spatial localization of cytoskeleton proteins in the oviduct was observed in conjunction with an increase in protein abundance. We further showed that the oviduct is made up of distinct regions, differing from each other in the response to mating and in cellular junction architecture.

This work yields valuable understanding to the changes occurring in the female oviduct post-mating and how these changes are involved in the intricate process of egg-laying. As a model organism, understanding the mechanisms involved in reproductive success in *Drosophila* can help unravel reproductive systems of more evolved organisms.
Pregnancy establishment requires development of the embryo and an intricate program of uterine preparation. Synchronous development of the endometrium is highly dependent on ovarian steroids, but the embryo further modulates the endometrial milieu making it competent for implantation. Search for markers of uterine receptivity revealed that *HOXA10* expression is essential for uterus development and successful implantation in humans, primates and mice. *HOXA10* expression was also demonstrated in adult porcine uteri, however there is little information concerning the regulation of this gene expression. Therefore, the present *in vitro* studies were conducted to examine: 1) the effect of estradiol (E\(_2\)) and progesterone (P\(_4\)) on *HOXA10* gene expression in porcine endometrial luminal epithelial (LE) and stromal (ST) cells, and 2) the effect of conceptus-exposed medium on *HOXA10* and prostaglandin endoperoxide synthase-2 (*PTGS2*) expression and PGE\(_2\) secretion from LE and ST cells in a co-culture model. Treatment of ST cells with E\(_2\) and P\(_4\) alone or both steroids added simultaneously resulted in increased *HOXA10* expression (P<0.05). E\(_2\) had no effect on *HOXA10* mRNA level in LE cells after either 24 or 48 h of culture. Addition of conceptus-exposed medium to the apical compartment of the co-culture system resulted in elevated expression of *HOXA10* and *PTGS2* in LE cells cultured in collagen-coated inserts (P<0.01), but also in ST cells cultured in basal compartments (P<0.05), and was accompanied by increased concentrations of PGE\(_2\) in medium.

Summarizing, steroids are important modulators of *HOXA10* expression in ST cells of the porcine endometrium. *HOXA10* and *PTGS2* gene expression and PGE\(_2\) secretion may be regulated by conceptus-derived factors, what indicates that conceptus may increase its survival by enhancing endometrial preparation for implantation. Additionally, the co-culture system of LE and ST cells used in the present experiment is a promising model for the study of endometrial response to conceptus products.
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<td>Maternal metabolism and the influence on fertility: a study of the follicular microenvironment.</td>
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<td>Simone Gebhardt</td>
<td>PhD Student</td>
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<td>Dynamic Changes of Messenger RNA Profiles of Equine Endometrium during the Oestrous Cycle</td>
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POSTER

PRESENTATIONS

FROM ABSTRACTS
Maternal communication with gametes and embryo is crucial for maintaining embryo development and establishment of pregnancy. Several studies have indicated that local responses are generated by female reproductive tract towards gametes and embryo. However, no defined in vitro model currently exists to allow detailed and systemic investigation of maternal communications with gametes and embryo. Therefore, we aimed to establish and characterise an in vitro model based on boar sperm interaction with a porcine immortalised oviductal epithelial cell line (TERT-OVEC) to understand the nature of this process and evaluate different factors that may affect this model.

A simple in vitro model system of spermatozoa co-incubation with TERT-OVEC cells was established. The factors tested to influence the model were categorised into sperm factors and cell factors. Sperm factors included sperm presence, viability (live versus dead), concentration and source of spermatozoa. Cell factors included cell passage and cell type. We also assessed the response of oviductal cells to inert substances. After 24 hours of sperm-oviductal cells co-incubation, RNA was extracted, purified and used to synthesise cDNA utilised for quantitative real-time PCR. Alteration of genes such as heat shock 70 kDa (HSPA8), Adrenomedulin (ADM) and prostaglandin E Synthase (PGES) was considered as the end point of the assay.

The results showed an alteration in gene expression in response to presence of live spermatozoa. Dead spermatozoa were not as effective as live spermatozoa in induction of oviductal transcriptome alterations. Different boar semen showed varied responses in inducing gene expression alterations. Gene expression was down-regulated when the cell passage increased. Reproductive cells revealed a higher value of gene expression alteration comparing to kidney epithelial cells. Inert substance did not induce a significant response in oviductal cells. These experiments demonstrated the diversity of different factors that can affect outcomes generated by in vitro sperm oviduct interaction models.
Effect of superovulation on prostaglandins synthesis in the porcine oviduct

The oviduct plays a crucial role in reproduction providing a beneficial environment for gamete maturation, transport and fertilization. Prostaglandins are among the central mediators of ovulation, fertilization and maintenance of pregnancy. Synchronization of estrus and superovulation are commonly used to obtain large numbers of embryos. The aim of the studies was to investigate the enzymes of prostaglandins biosynthesis in the porcine oviducts from gilts on day 3 of the estrous cycle or from gilts inseminated after double synchronization or superovulation. For superovulation, gilts were injected with 1500 IU PMSG and 1000 IU hCG 72 h later. The double synchronized gilts were treated with 750 IU PMSG and 500 IU hCG 72 h later and after 17 days challenged with the same gonadotropins. Three days after insemination oviducts were collected and divided into ampulla and isthmus. Western blot was used for analysis protein expression of COX-2 and PGFS (AKR1C3) and immunofluorescence for cellular localization. COX-2 was expressed exclusively in epithelial cells. In isthmus, COX-2 was mainly localized in apical pool of epithelial cells. In ampulla, COX-2 was observed in apical and basal sites of epithelial cells. Generally, the immunofluorescence in epithelial cells was higher in ampulla compared with isthmus. However, COX-2 immunostaining was higher in oviduct of double synchronized and superovulated animals than in normal cycling or inseminated gilts. Western blot analysis confirmed the presence of COX-2 and AKR1C3 protein in the porcine oviduct. The preliminary analysis revealed increase (p<0.05) of AKR1C3 protein expression in isthmus of superovulated animals when compared with the cycling and double synchronized gilts. Differential expression of COX-2 and AKR1C3 in ampulla and isthmus and increase of AKR1C3 in superovulated animals suggest that PMSG/hCG stimulation may result in change of prostaglandin synthesis, which in turn may affect gamete/embryo quality and transportation.
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Production of recombinant bovine oviductal glycoprotein-1 (OVGP1)

The OVGP-1 is the major non-serum protein present in the oviductal fluid produced by secretory epithelium cells. This glycoprotein has been related with different processes during the fertilization. Thus, bovine OVGP1 facilitates sperm capacitation and significantly increases the in vitro fertilization. The aim of this study was the production of bovine OVGP-1 by means of recombinant DNA technology for its use in assisted reproductive techniques. Total RNA was isolated from bovine oviduct and cDNA was synthesized with oligo-dT as primer. The complete open reading frame of OVGP1 was amplified by PCR and cloned into pCAGGs-6xHIS expression vector by means of the Cla I and Kpn I restriction sites by standards procedures. OVGP-1 construct was transiently expressed in human embryonic kidney cell line HEK 293T cells. The production of OVGP-1 protein was analyzed by western-blotting in the cell lysates and in the conditioned media using an anti-histidine polyclonal antibody. Western blot analysis demonstrated the existence of a band in cell lysates and media with an apparent molecular weight of approximately 75 kDa. The recombinant protein showed a lower molecular weight compared to that native bovine OVGP1 (95 kDa). This result suggests that HEK cells produce a recombinant protein with a lower glycosylation compare to the OVGP1 produced by the bovine oviduct. The protein expression follows a time-dependent kinetics, with the maximal level of expression observed at 48 hours post-transfection. In conclusion, bovine OVGP1 was successfully expressed in HEK 293T cells and is secreted to cell culture medium. Recombinant OVGP1 has probably a different glycosylation pattern compare to the native OVGP1. Future experiments will be necessary to analyze the biological role of the recombinant protein and its different glycosylation. This study was supported by MICINN (AGL2009-12512-C02-01-02).
The extracellular heat shock protein HSPA8 increases the development of the mouse zygote cultured in absence of carbohydrates

HSPA8 is the most abundant heat shock protein in the oviduct but its physiological function is unknown. The aim of the current study was to understand the function of the HSPA8 when one cell stage zygotes are cultured in absence of carbohydrates for 15 hours.

Methods: Fertilized mouse zygotes at the pronuclear stage were cultured in vitro at 37°C in M16 media depleted of carbohydrates for 15 hours. Three doses of HSPA8 were tested, i) 5µg ml⁻¹; ii) 10µg ml⁻¹; and iii) 15µg ml⁻¹. A total of 520 zygotes were used. The zygotes that went to two-cell were cultured in vitro for 5 days and the numbers of blastocyst were recorded.

Results: When zygotes are cultured in absence of carbohydrates, only 44% (8/18) went to two-cell and 6% of them (1/18) reached the blastocyst stage. In contrast when zygote are cultured in presence of HSPA8, the survival rate increases; i) for 5µg ml⁻¹, 46% (12/26) went to two-cell and 8% (2/26) of blastocyst were obtained; ii) for 10µg ml⁻¹, 44% (8/18) went to two-cell and 33% (6/18) of blastocyst were obtained; with 15µg ml⁻¹, 50% (9/18) went to two-cell and 33% (6/18) of blastocyst were obtained.

Conclusion: HSPA8 has a positive effect on the development of zygotes cultured in absence of exogenous supply of carbohydrates. This model system which exploits the natural process of autophagy can be used for investigating the potential effect of different oviductal protein factors on a short period of zygotic development i.e. one cell stage zygote to two cell stage embryo. Further modifications and development of this method can help to unravel epigenetic effects that oviductal proteins have on future development and health of offspring.
Transcriptome studies during early pregnancy and oestrous cycle in cattle, swine and horse as model for the analysis of common and species-specific mechanisms of establishment of pregnancy

Reproductive biology is diverse between different mammalian groups, particularly during the pre-implantation phase. To identify common and species-specific mechanisms related to establishment and maintenance of pregnancy we are analysing transcriptome changes in the endometrium at different time points of early pregnancy and during the sexual cycle in cattle, swine and horse. Transcriptome analyses are performed by the use of DNA microarrays and next-generation sequencing technologies (Illumina RNAseq). Especially RNAseq will be tested for across-species analysis of gene expression. So far a number of studies during early pregnancy and the oestrous cycle were done in bovine, porcine and equine endometrium and revealed thousands of differentially expressed genes. Furthermore, analyses of different time points of pregnancy defined the time when the first responses of the endometrial transcriptome to the presence of an embryo(s) can be observed. For an initial comparison of endometrial gene expression changes between species, microarray data obtained from equine endometrium at days 12 and 16 of pregnancy a Gene Set Enrichment analysis (GSEA) was performed. The results showed considerable overlaps with genes up-regulated during the window of implantation in human endometrium, with genes up-regulated in porcine endometrium at day 14 of pregnancy, and with genes up-regulated in bovine endometrium at day 18 of pregnancy and revealed a number of genes that probably have conserved roles in mammals. The comparison of the datasets showed also the importance of interferons during early pregnancy beyond pregnancy recognition in ruminants as indicated by the up-regulation of interferon-induced genes in all three species. Finally analysis of first RNAseq data obtained from bovine, porcine and equine endometrial samples is in progress to prove applicability of RNAseq for across-species transcriptome analyses.
Developmental competence and expression of TP-1 in parthenogenetic or IVF ovine embryos

The aim of this study was to evaluate the *in vitro* and *in vivo* developmental competence of parthenogenetically activated ovine oocytes. *In vitro* pre-implantation development, relative expression of interferon-tau (TP-1) and *in vivo* development after embryo transfer were evaluated.

Cumulus oocyte complexes derived from ovaries of slaughtered sheep were matured *in vitro* in standard conditions. MII oocytes were fertilized with frozen-thawed ram semen (IVF) or activated with ionomycin (5µM, 5 min.) and 6-DMAP (2mM, 3h) (PA). Zygotes were cultured in SOF+ aa + 0.4% BSA in 5% CO₂ and 5% O₂ up to blastocyst stage. TP-1 expression was analyzed by reverse transcription followed by Real Time PCR in PA and IVF embryos at the blastocyst stage.

Fifty two PA and 50 IVF blastocysts were transferred into 30 pre-synchronized recipient ewes (3/4 embryos per animal) by laparotomic technique. Pregnancies were recorded from day 20 on and embryo development was monitored by transabdominal probe (Philips HD 11, 5-8 MHz microconvex).

Similar cleavage rates were observed in the two classes, while IVF embryos displayed higher *in vitro* development to blastocyst stage compared to PA embryos (62.2% vs 56.3%, P<0.05). A higher TP-1 transcript abundance was observed in PA blastocysts compared to IVF (ANOVA; P<0.001). Nine out of 15 ewes were pregnant with parthenotes (60%) and 8 with IVF embryos (53%). On days 24 and 26, eleven PA embryos were recovered, while pregnancies with IVF embryos were carried to term.

In conclusion, until day 24 ovine PA embryos did not show any loss of *in vivo* developmental competence, nor differences detectable by ultrasonographic monitoring, compared to pregnancies with IVF embryos. The higher abundance of TP-1 in parthenogenetic blastocysts may explain the unexpectedly high rate of development showed by parthenotes after transfer into recipients. TP-1 is in fact the primary signal necessary for the establishment of pregnancy in ruminant species [Bazer et al, 1997].

References
Sperm penetration of cumulus oophorus, zona pellucida and oocyte membrane are thought to occur by means of enzymatic hydrolysis and mechanical force. Metalloproteases have been associated with membrane alterations prior to gamete fusion. Furthermore, a family of zinc-containing metalloproteases, the matrix metalloproteases (MMPs), might play a role during fertilization since they are involved in degradation of extracellular matrix. This study was conducted to evaluate the effect of metalloprotease inhibition on fertilization parameters during in vitro fertilization in pigs. We used phosphoramidon for inhibition of zinc-independent metalloproteases and 1,10-phenanthroline, a high affinity zinc-chelator, for inhibition of MMPs.

In vitro matured oocytes were transferred to fertilization medium with phosphoramidon (10 and 100 µM), 1,10-phenanthroline (5 and 20 µM) or without inhibitor and frozen-thawed epididymal semen was added giving a final ratio of 600 sperm per oocyte. After 2 or 6 h of co-incubation, oocytes were vortexed in HEPES-buffered medium to remove the protease inhibitor and cultured for 22 h. Presumed zygotes (n=1425) were stained with 10 µg/ml bis-benzimide to assess fertilization rate, polyspermy and sperm penetration index (mean number of penetrated spermatozoa per fertilized oocyte).

Presence of 10 or 100 µM phosphoramidon did not affect fertilization parameters. After 2 h of co-incubation, 5 and 20 µM 1,10-phenanthroline significantly reduced the fertilization rate with more than 50%. After 6 h of co-incubation, the control group and 5 µM 1,10-phenanthroline showed similar fertilization and polyspermy rates. However, in the 20 µM group, normal and polyspermic fertilization were inhibited more than 80% and 90%, respectively. The sperm penetration index was 1.07, compared to 2.36 in the control group.

Results point to a possible involvement of zinc-dependent metalloproteases in fertilization of pig oocytes. Further experiments will focus on zinc-containing metalloproteases present in the gamete membrane and/or the surrounding matrix.

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**The effect of cytokines on peroxisome proliferator-activated receptors (PPARs) mRNA expression in porcine endometrium during periimplantation period**

Peroxisome proliferator-activated receptors (PPARs) are transcriptional factors, which belong to the family of nuclear receptors. There are three known isoforms of the receptor, PPARα, PPARβ/δ and PPARγ in which PPARα is significantly expressed in the liver, heart and skeletal muscles. PPARγ mRNA is present mainly in fat tissue, while PPARβ/δ is the most widespread isoform of PPAR. Recently, the role of PPARs are studied in the reproductive processes.

The aim of the present study was to verify the influence of cytokines involved in the implantation in pigs, interleukin 6 (IL-6) and gamma interferon (INFγ), on PPARs mRNA expression in porcine endometrium slices at 14th day of pregnancy (the periimplantation period) after 6h and 12h of incubation. Gilts (n=4) with an average body weight of 100 kg and 7 months of age were hormonally stimulated and synchronized by single intramuscular injection of PMSG followed by hCG. The animals were inseminated twice, 24h and 36h after hCG treatment and were slaughtered at 14th day after the first insemination. The mRNA expression of PPARs was analyzed by quantitative real-time RT-PCRs.

After 6h of incubation the rise of PPARα and PPARβ/δ mRNA expression level was found in endometrial slices treated with 50ng/ml INFγ. The decline of PPARγ mRNA expression level after 6h of incubation was significant in the presence of 5ng/ml INFγ and 10ng/ml IL-6. After 12h of incubation, the rise of PPARγ mRNA expression was observed in endometrial explants treated with 5ng/ml INFγ and 10ng/ml IL-6.

In conclusion, the changes in PPARs genes expression interact with INFγ and IL-6, which suggest that the receptors are involved in the implantation process in pigs.

This research was supported by the Ministry of Science and Higher Education (Project N N311 360235).
Embryo spacing correlates with embryo mortality or abnormal prenatal and postnatal muscle development. Previously we postulated that lysophosphatidic acid receptor 3 participated in proper embryo development and adequate embryo spacing in pig uterus. We showed that LPA3 is higher expressed in endometrium of pigs on days 6-7, 8-9, 11-12 and 13-14 of pregnancy compared to corresponding days of cyclic pigs. Recently, we determined that in unilateral pregnant pigs the significantly higher content of lysophosphatidic acid (LPA) was measured by HPLC in uterine horn with developing embryos compared to non gravid horn. There was no difference in the endometrial LPA content in the horns from non pregnant pigs. Using unilateral pregnant pigs where only one uterine horn had a contact with developing embryos, on day 12 we found significantly negative correlation between LPA3 mRNA levels and mRNA of prostaglandin E2 synthase (PGES) prostaglandin F2α synthase (PGFS) and between PGF2α content and receptor LPA3 expression gene only in the gravid uterine horn. It was suggested that embryonic signal may affect PGF2α and LPA3 mRNA expression. Therefore according to the profile of embryo secretion and to endometrial receptivity we treated endometrium explants in vitro with PGE2, E2 and INFγ and IL6. The results of the incubation showed that IL6 treatment significantly increased LPA3 mRNA levels in endometrial explants from both cyclic and pregnant gilts, whereas incubation with PGE2, E2 and INFγ significantly increased LPA3 gene expression in endometrium of cyclic gilts. To study the effect of selective agonist of LPA3 and LPA1/LPA2 receptors on the production of PGE2 in endometrium of cyclic and pregnant pigs from day 14 of pregnancy/cycle we incubated endometrial slices with different doses of OMPT (selective agonist of LPA3 receptor) and L-α-LPA (selective agonist of LPA1/LPA2 receptor). Result indicated that only one dose of OMPT (68 nM) and only in endometrial explants from pregnant gilts decreased the production of PGF2α after 6 h of incubation. OMPT (340nM) in dose highest used dose increased the PGE2 synthesis after 6 h of incubation both in explants from pregnant and cyclic gilts. Concluding our results we can sustain our postulate that LPA through its receptor, LPA3 modulates prostaglandin secretion in the pigs uterus and makes suitable environment in the uterus for proper implantation affecting embryo development.
Characterization of immortalized endothelial cells derived from porcine uterus

To determine the ability of porcine uterine endothelial cells (EC) for in vitro studies we have generated the immortalized cell line EnUt–3 from porcine uterine microvessels. EC were enzymatically isolated from the uterus on a day 3 of estrous cycle. The cells were purified by nano-beads coated with lectin. The primary cultures were immortalized by transfection with vector carrying Simian Virus 40 T antigen (SV40 T-ag). Lipofectamine LTX was used as a transfection agent. Expression of SV40 T-ag was examined by RT-PCR. Phase contrast microscopy showed characteristic cobblestone shape of immortalized cells. EnUt–3 cells exhibited positive VE–cadherin and von Willebrand factor (vWF) – a major endothelial cell markers. The expression of these markers appeared a specific pattern for different compartments of cell. Immunostaining for VE–cadherin was localized in cell membrane, while vWF was observed in cytoplasm around nucleus. We analyzed the ability of EnUt–3 cells to form capillary like structures on Matrigel. Cells demonstrated extensive tube formation ability in three – dimensional environment between 6-8 hours. Cell proliferation assay was performed based on chemical reduction of growth indicator AlamarBlue. Different concentrations of P4, E2 and recombinant hCG were examined. After 48 hours of stimulation all tested hormones significantly increased proliferation rate of EnUt-3 cells. Summarizing, the functional and structural profile of immortalized EnUt–3 cells reflect characteristic features of the primary culture cells, which makes them useful model for studies on the angiogenesis in vitro.

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Development of a hypoprogesteronemic sheep model in early pregnancy

Overfeeding adolescent ewes throughout pregnancy promotes maternal growth at the expense of placenta development resulting in a foetal intrauterine growth restriction (IUGR) associated with low plasma progesterone concentrations. Uterine secretions in response to progesterone are important for conceptus growth, particularly during early pregnancy. In order to investigate the role of progesterone in the establishment of IUGR phenotype, we have experimentally reduced circulating progesterone in pregnant ewes during the peri-implantation period using trilostane, a 3beta-hydroxysteroid dehydrogenase inhibitor, but without alteration of nutrition.

Primiparous pregnant ewes were injected subcutaneously with trilostane (T, 30 mg/day in DMSO) or DMSO (control; D) either every 8 hours for 5 days (days 11-16) in experiment 1 (D: n=6, T: n=6), or every 12 hours for 10 days (days 6-16) in experiment 2 (D: n=7, T: n=10) until slaughter at day 16 post-oestrus. No pregnancy loss occurred during either protocol. The average number and the morphology of the concepti recovered from T- or D-treated ewes did not differ. Upon trilostane treatment, progesterone concentrations were significantly reduced in experiment 1 (day 16: -36.5% of pretreatment levels, ranging from 25.2-54.9%) and this reduction tended to be higher (P<0.077) in ewes with lower total luteal weight. In contrast, in experiment 2 the concentrations remained constantly low from day 6 to day 16, whereas they increased significantly in the control group. In T-treated ewes in experiment 1, total luteal weight and plasma progesterone concentrations were strongly correlated at day 16 (R²=0.91, P<0.003). In experiment 2, luteal tissue weight was more correlated with progesterone concentrations on day 5 than on day 16 (R²=0.72, P<0.002), likely because the trilostane treatment was initiated before the complete development of corpora lutea. The endometrial (caruncular and intercaruncular) expression of galectin-15, serpin and osteopontin are currently analyzed by RT-qPCR in this hypoprogesteronemic sheep model with sustained pregnancy.
Altered embryo-maternal interactions affect placental development with a subsequent impact on foetal growth, pregnancy outcome and postnatal health. Recent publications have revealed major perturbations of endometrial physiology in early pregnant cows carrying embryos produced by somatic cell nuclear transfer (SCNT). To gain new insights on the molecular mechanisms taking place in the endometrium during normal and perturbed implantation, we characterized the gene expression and regulation of selected STAT/SOCS signalling factors, a major transduction pathway for implantation in the mammalian uterus. In the pregnant endometrium collected from artificially inseminated (AI, day 20 post-oestrus) cows and compared to cyclic cows (i) transcript expression, protein amount and phosphorylation level of STAT1 were significantly up-regulated (6.07, 3.3 and 10.7-fold respectively) (ii) CIS, SOCS1 and SOCS3 expression was not significantly affected whereas SOCS6 was up-regulated (2.12-fold) and SOCS2 was down-regulated (0.6-fold) (iii) SOCS expressing cell types were different including a high staining intensity for SOCS6 in the luminal epithelium and a strong decrease for SOCS2 staining in the stroma cells. Infusion of recombinant interferon-tau (IFNT) in bovine cyclic uteri in vivo (day 14 post-oestrus, 100 ng/ml, 2 hours) led to the induction of STAT1 phosphorylation as well as the up-regulation of STAT1, SOCS1 and to a lesser extent SOCS3 expression. In the endometrium of SCNT pregnant cows compared to AI pregnant cows, STAT1 expression (transcript or protein) was not affected whereas SOCS2 expression was significantly down-regulated ($P < 0.05$). Collectively, our results show 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. In SCNT pregnancy, the altered endometrial expression of SOCS2 at implantation may affect placental then foetal development therefore contributing to the final outcome of pregnancy. Additional studies are necessary to clarify this aspect.
Early signals of pregnancy in mouse peripheral immune cells

Immune tolerance protecting the embryo and fetus from attack of mother immune cells is shaped from the day of fertilization till parturition. It seems that not only local but also systemic immune compartment is engaged in the establishment of immunological balance in pregnant female. Regulation of specific immune response against allogenic antigens is dependent on antigen recognition and presentation through ligands–receptors interaction between antigen presenting cells (APC) and lymphocytes. The aim of this study was to assesses the expression of co-stimulatory molecules on spleen APCs in mouse during preimplantation period of pregnancy. Female C57Bl/6J (H2b) mice were mated with DBA (H2d) and BALB (H2d) males. Control animals were brought into pseudopregnancy by cervical stimulation. Half (0,5) and 3,5 days after mating or stimulation, the number of cells and the expression of CD80, CD86, CD40, MHC II costimulatory molecules were determined by flow cytometry, in subpopulations of APCs (dendritic cells CD11c+, macrophages F4/80+, B lymphocytes and cells with CD11blow or CD11bhigh expression. At 0,5 day after mating a few of costimulatory molecules expression on APCs was significantly changed. In contrary, on 3,5 day after mating all examined APC showed significantly higher expression of CD86 molecule and CD11b(high) and F4/80+ cells showed increased level of CD40 antigen in compare with pseudopregnant mice independently on male strain used for mating. However, the level of expression of MHCII and CD80 in some populations of the cells was strain dependent. Moreover, the number of CD11c+ CD80+ was lower in mice mated with BALB and DBA males on 3,5 day and on 0,5 day mated with BALB males in compare with control. In conclusion during preimplantation period of pregnancy the costimulatory phenotype of peripheral APCs is changed. Increased expression of CD86 on all examined APCs and decreased number of dendritic cells with CD80 molecules expression suggests broad involvement of activating costimulatory pathway dependent on CD28 molecule presented on lymphocytes.

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Adiponectin, a pleiotropic hormone secreted from adipose tissue, can mediate some negative effects of obesity on female health, and can participate in the impaired reproductive performance of obese women. Using a mouse model, we examined effects of different adiponectin isoforms on development of preimplantation embryos in vitro. We found expression of adiponectin receptors at all stages of mouse preimplantation development. We applied full-length adiponectin, and compared its effects with two other protein isoforms – truncated globular adiponectin and mutated full-length adiponectin which can only form trimer, but not hexamer or high molecular weight forms. Administration of full-length adiponectin significantly changed the embryo distribution, increasing the proportion of embryos with high cell numbers and decreasing the proportion of embryos with lower cell numbers. In contrast, administration of the trimeric adiponectin decreased the proportion of embryos with higher cell numbers and increased the proportion of embryos with lower cell numbers. The analysis of cell number in embryos that reached the blastocyst stage confirmed the opposite effects of the two adiponectin isoforms. Incubation of embryos with the full-length adiponectin led to significant increase in the embryo cell number, whereas incubation of embryos with the trimeric adiponectin led to significant reduction of the cell number in blastocysts. Moreover, the administration of trimeric adiponectin significantly increased the proportion of dead cells in the blastocysts. On the other hand, we found no significant effect of globular adiponectin on the examined parameters. In summary, our results indicate that adiponectin can directly influence development of the preimplantation embryo, and the effects of adiponectin on the embryo are isoform dependent. These results suggest a possible way how maternal obesity could affect the embryo in very early pregnancy.

This work was supported by the Slovak Academy of Sciences under contract VEGA 2/0041/09 and Slovak Research and Development Agency under contract APVV-0620-07.
The fate of embryo after selective inhibition of cell cycle regulators during the first mitosis

Preimplantation embryo expresses a number of receptors important for the initiation of phosphatidylinositol 3-kinase pathway (PI3K). The PI3K - Akt/PKB - CDK1 cascade is implicated as a key regulator of various cellular functions, including the cell cycle control. The aim of this study was to evaluate the effect of selective inhibition of Akt/PKB (with SH6) and CDK1 (with Roscovitine) during the first mitosis of zygote. Mouse one-cell stage embryos derived from *in vivo* fertilized oocytes were cultured *in vitro* for 12 h with or without the presence of inhibitor. Selective inhibition of Akt/PKB or CDK1 caused the arrest of embryo development. SH6-treated zygotes (arrested at the pronuclear stage) showed significantly higher occurrence of various apoptotic features (nuclear fragmentation, positive TUNEL-labeling, the presence of active caspase-3 in both nucleoplasm and cytoplasm). Although Roscovitine-treated embryos similarly did not enter into the first mitosis, the typical apoptotic cell death features were not observed in them. Thus, Akt/PKB seems to be the primary target responsible for mediating an anti-apoptotic signal in zygote.

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Effect of IL1β on PGF2α synthesis, secretion and metabolism in porcine uterus during early pregnancy and luteolysis

Early pregnancy is a critical period for embryo-maternal interactions and embryonic survival in pigs. We suggest that interleukin 1β (IL1β) is involved in synthesis, secretion and metabolism of luteolytic PGF2α in porcine uterus during early pregnancy. Individual endometrial and myometrial slices (200-210 mg) harvested on days 10 to 11, 12 to13 and 15 to 16 of pregnancy (n = 5, each group) or the estrous cycle (n = 5, each group) were pre-incubated in 2 ml of M199 medium for 18h in atmosphere of 95%CO₂ and 5%CO₂ in 37°C and incubated for 12 h with control medium or IL1β (10ng/ml). Expression of PGFS mRNA in pregnant endometrium and myometrium was decreased in response to IL1β during days 12 to 13 and 10 to 11, respectively. In cyclic pigs IL1β increased abundance of PGFS mRNA in the endometrium harvested on days 12 to 13 and 15 to 16. Secretion of PGF2α in response to IL1β was increased during days 10 to 11 in endometrium and 12 to 13 in myometrium during pregnancy, respectively. In cyclic pigs this stimulatory effect was observed in the endometrium and the myometrium during days 10 to 11 and 15 to 16. Endometrial secretion of PGFM in response to IL1β was increased during days 12 to 13 and 15 to 16 of pregnancy and on days 10 to 11 of the estrous cycle in both tissues. The presence of 15-PGDH in porcine uterus during early pregnancy and luteolysis confirms local tissue metabolism of PGF2α. In conclusion: 1) IL1β enhances endometrial synthesis of PGF2α in cyclic pigs around time of luteolysis and decreases PGF2α synthesis during maternal recognition of pregnancy; 2) IL1β increases metabolite of PGF2α (PGFM) in pregnant endometrium around time of maternal recognition of pregnancy; 3) 15-PGDH is active in porcine uterine tissues.

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Recovery of early embryos and protein from the bovine uterus for proteomic assays (DiGE)

We recovered uterine fluid (UF) from heifers (n=16) carrying Day-4 (n=30-140) or Day-5 (n=30-50) IVP embryos transferred. On Day-8, uterine horns were flushed and responses compared to those in cycles with the same recipients being sham transferred. Blood P4, recoverable protein and embryos were analyzed. Flushing’s were performed with either 30- or 45-mL PBS+protease inhibitor. Recipients transferred with embryos were extensively flushed with PBS+PVP. Embryos were searched through stereomicroscope, and initial flushes containing UF were centrifuged at 4°C, aliquoted and stored at -150°C. Responses were 1/ Live embryos; 2/ Only degenerated or no embryos; and 3/ Sham. Protein was quantified by a Bradford assay in 43 out of 44 flushes (range: 767-17,978 µg). Recovered volume (34.4±1.6 vs. 17.6±1.4mL; [p<0.001]) and protein (20.7±4.6 vs. 34.3±3.9 µg/100 µL [p<0.04]) differ between 45mL vs. 30mL medium flushed, respectively. Total protein recovered tended to be higher with 45mL (7,197±878µg) than 30mL (4,941±793µg) flushed. Recovered total eggs, viable embryos, blastocyst and expanded/hatched blastocyst rates were higher (p<0.05) with 45mL than 30mL flushed.

Heifers transferred with embryos gave concentration and total protein lower than sham transferred (21.1±3.7 vs. 34.0±4.3 µg/100 µL [p<0.04] and 4,941±793 vs. 7,549±838 µg [p<0.03], respectively). The lowest [protein] were found in flushes with live embryos (15.8±4.7 µg/100 µL) not within sham transferred (35.1±4.2; p<0.01) or degenerated or no embryos recovered (31.8±6.6; p<0.08). Progesterone rise affected embryo recovery, but not [protein], while embryonic stage transferred had no effects.

Flushing with 45mL allows recovering more embryos and protein than 30mL. Recovered protein is sufficient for DiGE gel assays (25µg/processed sample). Live embryos in uterus reduce [protein] in flushes, which likely reflects decreased UF secretion. As this UF shortage seems to be not systemic (no changes in P4 are observed), it could be difficult to observe when only one embryo exists in uterus.
Suboptimal *in vitro* culture (IVC) produces altered gene expression, and reduced quality and cell number in the mouse blastocysts. Embryos retarded by IVC can restore their viability and number of cells during 3 days of delayed implantation produced by induced diapause. This facultative diapause can be experimentally generated by transferring blastocysts to 0.5-dpc oviducts of pseudopregnant females. To evaluate if the long term effect produced by suboptimal IVC in mice can be reduced by inducing diapause, we transferred blastocysts into the oviduct of 0.5-dpc females or into the uterus of 3-dpc female, analyzing number of cell of diapaused blastocysts, embryo viability, weight of born animals during 10 month (80 animals), mortality rate, and male fertility. We found that blastocysts remained in the oviduct until uterotubal junction was opened at 3-dpc, then embryos were implanted in the uterus. During these 2.5 days in the oviduct, blastocysts performed one cell division and then went into diapause until they passed to the uterus. At that time, embryos had double the number of cells than Day-2.5 blastocysts (number of cells at blastocyst stage, 48.8±6 [n=15]; number of cells of blastocysts after 3 days of diapause, 96.4±9 [n=14]). The percentage of born animals was smaller when embryos were transferred into the oviduct (20% in the oviduct vs 29 in the uterus). The percentage of animals that died was also smaller in the group of diapaused embryos after 40 week of age (survival of 94% for diapaused embryos vs 68% for embryos without diapause). However, only those animals obtained from suboptimal IVC embryos that were transfer to the oviduct (diapaused) showed a weight significantly higher than embryos transferred to the uterus. Artificial induction of diapause in human by lowering circulating estrogen to prevent the uterus becoming receptive for implantation may have clinical implications.
Effect of reproductive status on prostaglandin E2 receptor expression in the porcine corpus luteum

Prostaglandin E2 (PGE2) stimulates luteal progesterone secretion through a cAMP-mediated pathway in different species. Subtypes of PGE2 receptors, PTGER2 and PTGER4, are coupled to adenylate cyclase and generate cAMP that activates protein kinase A signaling pathway. Although it has been shown that number of PGE2 receptors was similar in the corpus luteum (CL) of pregnant and cyclic gilts, luteal subtypes of PGE2 receptors have not been determined. Aim of the present study was to evaluate expression patterns of PTGER2 and PTGER4 in porcine CL during the estrous cycle and early pregnancy. CL was collected from gilts on either day 9, 11, 12, or 14 of the estrous cycle and pregnancy (n=4-6 per group). A day x reproductive status interaction was detected for luteal expression of PTGER2 mRNA and PTGER4 protein. PTGER2 mRNA content was comparable on days 9-12 of the estrous cycle and pregnancy. However, on day 14 of pregnancy PTGER2 mRNA abundance was greater versus day 14 of the estrous cycle. Expression of luteal PTGER2 protein was affected by day (p<0.0001), but not reproductive status. In pregnancy, PTGER2 protein expression was maximal on days 12-14 (p<0.05). The abundance of PTGER4 mRNA was affected by day (p=0.048). PTGER4 mRNA content was similar within all studied days of the estrous cycle. During pregnancy luteal PTGER4 mRNA expression gradually increased to reach maximal levels on day 14 of pregnancy. PTGER4 protein content increased from day 9 to 12 of the estrous cycle and then declined 2-fold on day 14 of the estrous cycle. PTGER4 protein abundance was significantly greater (three-fold higher) on day 14 of pregnancy, when compared to all studied days of pregnancy and day 14 of the estrous cycle.

To conclude, PGE2 secreted by endometrium or and trophoblast, after reaching ovaries, could exert a luteoprotective effect through luteal PTGER2 and PTGER4.
Profiles of Pluripotency and Differentiation Factors in In Vitro Fertilized (IVF) Embryos and Their Reprogramming in Cloned Embryos: a special perspective to Embryonic Genome Activation (EGA) in Bovine

In nuclear transplantation (cloning) oocyte ‘reprograms’ differentiated nucleus into an embryonic state, a phenomenon equivocal to maternal to embryonic transition (MET) in normal fertilization. Both of these genomic transitions ultimately result in EGA. However, clones have low birth rate compared to fertilized embryos. Recent advances in direct reprogramming through ectopic expression of pluripotency associated transcription factors have emphasised their role in nuclear reprogramming. Nevertheless, there is no study in bovine, which describes the expression profiles of pluripotency transcription factors during MET in normal embryos and their reprogramming in clones. In order to understand nuclear reprogramming an earlier assessment of embryos/clones is important which is possible immediately after EGA. We hypothesized that the acquisition of a proper gene expression of pluripotency and differentiation factors by the cloned morulae (first stage after EGA in bovine) could be an early indication of appropriate nuclear reprogramming. Therefore, the objectives of this study were a) to chart the expression profiles of important pluripotency and differentiation factors during preimplantation stages of bovine IVF embryos and b) early assessment of the nuclear reprogramming by comparing cloned morulae with IVF controls. In this study we profiled pluripotency factors: OCT4, SOX2, NANOG and STAT3, as well as differentiation factors: HESX1, NODAL, ISL1, ZIC3 and MEIS1 through quantitative RT PCR. We analysed six preimplantation stages including immature and mature oocytes, 4 cell, 8 cell, morula and blastocyst. Moreover, the cloned morulae derived from two donor cell lines with different term development potentials (1.8% vs. 12.7%) were analysed in comparison to donor cells and IVF morulae. This study reveals that the EGA is an important phase which involves both: the silencing of genes of differentiation and the activation of genes of pluripotency—significant for normal embryonic development. Additionally, cloned morulae from both cell lines have undergone significant reprogramming by stage morula.
Piwi proteins, members of the Argonaute family, play a crucial role during germline development and gametogenesis of many animals. Their expression is restricted to germline where they associate with the recently discovered subset of small RNAs known as PIWI-interacting RNAs (piRNAs). Together, they guarantee genomic stability by silencing repetitive sequences, therefore disrupting of Piwi proteins and/or piRNA pathways leads to severe defects in gametogenesis. Piwi proteins in the male germline are well characterized, however relatively little is known about which Piwi proteins are expressed in the vertebrate female germline. Using a bioinformatics approach we identified three Piwi genes in pig (siwi, sili and siwi2) which were predicted to encode a protein of 861, 985 and 863 amino acids, respectively. Additionally we confirmed expression of these three Piwi mRNAs in pig testes using RT-PCR analysis. The aim of this study was to examine the expression of Siwi, Sili and Siwi2 transcripts in the porcine oocyte using Real-time PCR technique.

Ovaries from prepubertal (NCL) and pubertal (CL) gilts were collected in a local slaughterhouse. The obtained Cumulus-oocyte complexes (COC) were selected both morphologically and using the BCB test. Only the COC’s with good morphology were used for the study. Oocytes were denuded and frozen in liquid nitrogen (groups of 25 oocytes per sample). Relative expression of Siwi, Sili and Siwi2 transcripts was measured by real-Time PCR using β-actin as a reference gene. Preliminary results consist of ten samples (five for CL and five for NCL oocytes), each analyzed in duplicate. Siwi transcripts were detected in all samples and Sili transcripts were present in 4 samples of CL and in one sample of NCL oocytes. Siwi2 transcripts were found in one sample of CL and three samples of NCL oocytes. Non significant differences were found between the NCL and CL groups as regards siwi gene expression. The results for sili and siwi2 genes will be confirmed by testing samples with a higher number of oocytes.

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Influence of cortisol on prostaglandin production in bovine endometrium *in vitro*

Glucocorticoids (GCs) are known as anti-inflammatory agents limiting the production of cytokines and prostaglandins (PGs) in various target organs. Previous study showed that interferon-τ (IFNτ) increase activity of 11-HSD1 (enzyme responsible for cortisone to cortisol conversion) and cortisol secretion in endometrial explants and cells (stromal and epithelial). We suggest that cortisol may acts as a luteoprotective factor in bovine endometrium and be mediator of IFNτ actions. The aim of our study was to examine if cortisol has influence on prostaglandin production in bovine endometrial cells. Endometria were taken from cows in early luteal phase of estrous cycle. The epithelial and stromal cells were enzymatically separated. The cells were seeded in 48- and cluster dishes. Stromal and epithelial cells were exposed to cortisol (30nM) or cortisol and IFNτ (30ng/ml). After incubation for 24h media were collected and a prostaglandin E₂ (PGE₂) and F₂α (PGF₂α) levels were evaluated. Cells were collected and frozen at -80°C for Real Time PCR analysis. Expression of genes for enzymes involved in prostaglandins synthesis (prostaglandin synthase 2- PTGS2, prostaglandin E₂ synthase- PGES, prostaglandin F₂α synthase - PGFS) was measured. Results showed that cortisol stimulated PGE₂ production in epithelial cell (P<0.05) but has no significant influence on PGE₂ production in stromal cells. Cortisol decreased PGF₂α production in stromal cells. Moreover this studies showed that expression of genes for PGES was higher comparing to control in epithelial cells (P<0.05) and cortisol inhibited expression for PTGS2 in both stromal and epithelial cells (P<0.05).Glucocorticoids as luteotropic, antyluteolytic and immunomodulatory factors can be mediators of IFNτ actions in bovine endometrial tissues. In bovine uterus cortisol act in antyluteotic manner by selective inhibition of PGF₂α and/or stimulation PGE secretion. Influence of IFNτ PG secretion by up-regulating cortisol activation can results in the maintenance of the corpus luteum during early pregnancy in cattle.
Potential markers of oocyte developmental competence in cumulus cells: comparative analysis of in vitro and in vivo maturation in bovine (Bos taurus)

In vitro maturation (IVM) of oocytes is a crucial step of assisted reproductive technologies in cattle; however, the molecular mechanisms of cumulus cells (CC) contribution to oocyte developmental potential, which is still higher after in vivo maturation, require more investigation. In order to find the potential markers of oocyte developmental competence in cumulus cells, we performed the transcriptomic analysis of CC from cumulus-oocyte complexes (COCs) matured either in vitro (IVM) or in vivo by using bovine 22K oligonucleotide microarray (CRB, Jouy-en-Josas). About 500 genes involved in numerous functions and signaling pathways were differently expressed in these conditions (p < 0.01, ANOVA, correction Bonferroni). We validated then by real-time RT-PCR the differential expression of 16 genes involved in oxidative stress response, cell adhesion, extracellular matrix formation, steroid and lipid metabolism and signal transmission. We then monitored their expression during IVM in different models of oocyte developmental competence, like adult cows versus prepubertal calves, and different maturation mediums which influenced the oocyte developmental competence.

As revealed by analysis of phosphorylation by western blot, PI3-kinase/AKT, TGF-beta and MAP kinases signaling pathways are involved in the regulation of cumulus function and oocyte meiosis. At that, phosphorylation of SMAD2, MAPK ERK1/2, P38 and SMAD4 abundance were lower in CC of COCs matured in vivo as compared to IVM. Gonadotropins and/or growth factors influenced the number of differential genes during IVM, including GSTA1, COX, HAS2, FSHR, TMSB10 and also the phosphorylation of AKT, SMAD2 and JNK1.

Expression of COX2, TMSB10, MMP9 and CAPG varied in relation with animal age. HAS2, SERPINA5, MMP9 and TXNIP over-expressed in CC from oocytes that developed to blastocyst in vitro versus non-developed whereas TMSB10, SDC2, GSTA1 and CAPG expression was unchanged.

In conclusion, several factors in bovine cumulus cells varied in relation with the developmental potential of correspondent oocytes.
Amino acid concentrations in bovine follicular fluid from follicles of different size categories and in TCM-199-based maturation media

Embryo development is strongly influenced by events occurring during oocyte maturation. Although many immature oocytes are capable of completing meiosis in vitro, only a small percentage of the original pool of immature oocytes is competent to continue development to the blastocysts stage and subsequently result in a pregnancy. This is, in part, likely to be due to the non-physiological nutritional milieu to which oocytes are exposed. Improvements in oocyte developmental potential may be achieved by modelling nutrient profiles on those of preovulatory follicular fluid (FF).

The objectives of this study were: to determine the amino acid profile of bovine FF collected from follicles of different size categories (in either the dominant or the growth phase of follicular wave); to compare FF amino acid concentrations with those in the maturation medium (bicarbonate-buffered TCM-199 supplemented with 10% FCS).

Amino acid profiles were determined by reverse-phase HPLC. Follicular fluid were aspirated from slaughterhouse ovaries separately from the large (10–20 mm in diameter) and small (2–9 mm in diameter) follicles in both follicular phases.

Amino acid concentrations in FF from small follicles were markedly higher than those in FF from large follicles. Concentrations of alanine and glycine were highest in FF from small (0.63 and 0.53 mmol/l) like in large (0.43 and 0.39 mmol/l) follicles. Aspartic, methionine and lysine concentrations were lower than other amino acids in FF.

The study demonstrated that 12 from 16 amino acid concentrations were significantly higher in maturation medium than in FF from small and large follicles. Maturation medium had lower arginine and alanine concentrations than FF, whereas valine and lysine were similar.

It was shown that amino acid composition in maturation medium differs markedly from that of follicular fluid and conditions for oocyte maturation are suboptimal. These data could help to create more physiological maturation medium.
Morphological evaluation of three equine oviduct epithelial cell culture systems

In the present experiment, we aimed to establish an *in vivo* like oviductal cell culture system to study embryo-maternal interaction in the horse. The oviducts of 10 cyclic mares (4-14 years old) were collected at a slaughterhouse. Oviduct epithelial cells (OEC) were obtained by scraping the ampullary-isthmic region of the oviduct. Sheets of OEC were washed 3 times by 10 minutes sedimentation in washing medium. Culture was performed in DMEM-F12 with 10% FCS at 38.5°C in 5 % CO₂. Tested cell culture systems were 1) a 50 µl droplet under oil containing 5 vesicles smaller than 200 µm in diameter, 2) a 50 µl droplet under oil with one large vesicle and 3) 20 µl of the cell suspension added to 800 µl of culture medium without oil overlay. From day 0 to day 6, some vesicles from each culture system were processed for histology. Ciliary activity of the cultured vesicles and the homogeneity of colour were assessed. Trypan blue and SYBR14/PI demonstrated that approximately 99 % of the cells were intact during the whole culture period. No vesicles attached to the bottom of the Petri dish. During 6 days, ciliary activity was observed in 99% of the vesicles in the drop culture system. Central darkening was present in 52 % of the small and 72 % of the large vesicles. In the 800 µl culture system, 64 % showed ciliary activity and 68 % central darkening. The age of the mare had no influence (P-value > 0.01). Histology revealed both differentiated and undifferentiated cells from day 1 in all culture systems.

In conclusion, the culture drop system is more similar to the *in vivo* situation than the 800 µl system. Further research is ongoing to identify the influence of hormones on oviduct cell physiology *in vitro*. 
Different ability to form outgrowth between in vitro produced porcine embryos and parthenotes

The establishment of porcine embryonic stem cell (pESC) lines would be a novel tool for animal biotechnology and it would represent a useful model for biomedical research as well as developmental biology studies. In the last years parthenotes received particular attention because they represent an alternative ethical source to obtain ESC lines. However little is known about the biological differences between parthenotes and embryos and further studies are needed in order to better elucidate these aspects. Here we produced in vitro fertilized (IVF) and parthenogenetic embryos and isolated ICMs in order to compare their ability to form outgrowths.

More in detail, blastocysts were subjected to immuno-surgery, using pronase 0.5% (w/v), monkey anti-porcine serum (supplied by Istituto Zooprofilattico Sperimentale Lombardia ed Emilia- Romagna) and Guinea pig complement. ICMs were isolated from lysed trophoblast cells by pipetting and plated onto inactivated STO feeder cells and outgrowth formation was monitored.

We observed that parthenogenetic ICMs generated a significantly higher number of outgrowths than IVF ones (22.16% vs 4.82%). In order to better understand this result we compared the expression levels of the cell adhesion molecules beta integrin-1 and vitronectin in ICMs obtained from the two sources.

A statistically significant difference for the two genes was detected. In particular, the expression of beta integrin-1 was 5.6-fold higher in parthenotes than in IVF ICMs (ΔCt values: 68±0.5 vs 12±0.1). Similarly vitronectin displayed an up regulation 11.7-fold in parthenogenetic embryos (ΔCt values: 82±0.7 vs 7.3±0.2).

Our findings provide the first molecular explanation for the higher efficiency to generate outgrowths exhibited by parthenogenetic blastocysts, as compared to regular IVF embryos.
Effect of cortisol on corpus luteum (CL) function during the estrous cycle and early pregnancy in cows

Cortisol is suggested as anti-inflammatory agent to modulate production and action of cytokines and prostaglandins required for ovulation, luteolysis, successful embryo implantation, pregnancy development and termination. Level of cortisol, is locally regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD)s. Although both 11β-HSDs and GC receptors are expressed in the bovine CL and endometrium throughout the estrous cycle and early pregnancy, the function of GC in CL function regulation of is still controversial. The aim of the study was to demonstrate the effect of exogenous locally produced cortisol on the progesterone (P4) secretion, CL function at the estrous cycle and early pregnancy as well embryo implantation and development in cows in vivo. Cortisol (10 mg) or metyrapone (11β-HSD-type 1 inhibitor; 500 mg) dissolved in vaseline gel were applied into the vagina of heifers daily on Days 15-18 of the estrous cycle (n=18) or pregnancy (n=36). Doses of reagents were establish in preliminary experiments. P4 concentration was measured by EIA. The pregnancy rate was confirmed on Day 28-30 after insemination using USG technique. Application of exogenous cortisol at the end of luteal stage shortened CL function through decreasing P4 secretion (P<0.05). Moreover, inhibition of endogenous, local cortisol synthesis by metyrapone prolonged luteal stage (P<0.05). Conversely, during early pregnancy exogenous cortisol stimulated P4 secretion (P<0.05) and increased the pregnancy rate (75 %) compare to control animals (58%; only gel application). Lower level of P4 and fertility rate (16.7 %) were found in heifers treated with metyrapone (P<0.05). This data suggests that cortisol, dependently on physiological status of cows, modulates CL function influencing P4 secretion. The most important result of this study is that cortisol has positive influence on the CL during pregnancy, supporting embryo implantation and maintaining early pregnancy.
Efficient vitrification of pronuclear-stage rabbit embryos

Embryo cryopreservation is an integral part of applied embryology. The aim of this study was to examine the efficiency of the Solid Surface Vitrification (SSV; Dinnyes et al., 2000 Biology of Reproduction, 63, 513-518) technique on post-warming in vitro developmental capacity of in vivo versus in vitro produced pronuclear-stage rabbit embryos. The protocols for animal care and handling were approved by Ethics Committee. Oocytes, sperm and zygotes were collected from matured Hycole hybrid rabbits. The in vivo or in vitro (by IVF) produced zygotes were vitrified with SSV technique (35% EG, 5% polyvinyl-pyrrolidone, 0.4 M trehalose) and were immediately warmed and rehydrated. Following five days of in vitro culture, the developmental rate was recorded and the blastocysts’ cell nuclei were counted by Hoechst staining. At least three replicates were performed. The blastocyst rates of the non-vitrified zygotes were significantly (P<0.05) higher (in vivo 78% vs. in vitro 76%) than that of vitrified ones (in vivo 32% vs. in vitro 36%). Although in vivo or in vitro produced blastocyst rates did not differ, cell numbers of blastocyst developed from either fresh (121.6±4.9) or vitrified (107.3±16.8) in vivo zygotes were higher than that of the in vitro ones (fresh 105±11.5 and vitrified 91.4±9.6). In conclusion, the SSV technique was efficient to cryopreserve both in vivo or in vitro fertilized rabbit zygotes. Supported by “Plurabit” (OMFB-00130/2010 ANR-NKTH), EU FP6 “Clonet” (MRTN-CT-2006-035468), Hungarian-Chinese Bilateral project (TÉT CN-56/2007) and EU FP7 “RabPStem” (PERG07-GA-2010-268422).
Transcriptome changes at the initiation of elongation in the bovine conceptus: role of solute carriers

The process of conceptus elongation is driven by the transport of maternally derived molecules into the uterine lumen; evidenced by the fact that elongation does not occur during in vitro culture and is suppressed in vivo in the absence of maternal uterine glands. The solute carrier (SLC) superfamily is a large group of integral membrane transport proteins whose primary function is to facilitate small molecule exchange across cell membranes. It is hypothesized that embryonic origin will alter the expression of the SLC family members which contributes to impaired conceptus elongation. Using the bovine Affymetrix microarray, the expression of SLC transcripts were compared between in vivo and in vitro derived blastocysts on Day 7 and in vivo and in vitro derived embryos on Day 13 following embryo transfer. Comparison of Day 7 and Day 13 in vivo derived embryos revealed 444 differentially expressed genes (DEGs) compared to the 1,341 identified as different between Day 7 and 13 in their in vitro counterparts. Of these DEGs, two out of the 444 in vivo DEGs were SLCs (SLC28A3, SLC29A3) while in the in vitro group 28 transcripts of the SLC family related to amino acids, oligopeptides, metal ions, sugars, organic anion transport and transport across mitochondrial membranes were differentially expressed. A further 465 DEGs changed between Day 7 blastocysts and the initiation of conceptus elongation on Day 13, irrespective of embryonic origin and eight of these DEGs were SLCs family members (SLC25A24, SLC25A12, SLC44A4, SLC13A4, SLC16A3, SLC7A5, SLC6A8, SLC5A11). Expression of specific SLCs are likely important for conceptus survival, growth, and development. The large number of differentially expressed SLCs in in vitro embryos between Day 7 and 13 likely alters small molecule uptake and may impede conceptus elongation. Their role in maternal conceptus cross-talk during early pregnancy warrants further study.
DNA Methylation Dynamics in Rabbit Zygotes
Developed in Vivo or In Vitro

DNA methylation reprogramming (DMR) in early embryo has been considered essential for further differentiation and development (1). In mouse, embryonic genome activation (EGA) spans over a single cell cycle, and occurs after extensive epigenetic modifications of parental genomes, especially after demethylation of the paternal pronucleus (PP) DNA. In most other mammals, EGA is more progressive and early DMR is still under investigation (2). Because DNA methylation alterations have been attributed to in vitro culture and may alter normal development (3,4), we chose to analyse DMR in the rabbit which allows easy comparative studies between in vivo-developed (IVVD) and in vitro-cultured (IVTC) embryos. In this species the variation of DNA methylation was never quantified in both maternal (MP) and paternal pronuclei, and PP demethylation is still controversial (5,6). Moreover the precise comparison between in vitro culture and in vivo development has not been performed. This study reported the dynamics of DMR in the MP and PP of IVVD zygotes. A total of 300 zygotes were recovered between 14 and 24 h post-coitum, classified according to pronuclear morphology and analysed by 5-methyl cytosine immunostaining and fluorescence quantification. The DMR was found to be different between MP and PP in IVVD zygotes. The MP displayed a constant methylation over the one cell stage, resulting from a maintenance methylation over DNA replication. Conversely, the PP exhibited a partial demethylation over the one cell stage. This demethylation resulted from both an active DNA demethylation before replication, and no, or only a partial maintenance methylation during replication. IVTC zygotes displayed somewhat different DMR dynamics compared to IVVD zygotes. Taken together, the extend of our in vivo study paves the way to a precise analysis of the consequences of different in vitro culture conditions.

References:
Leptin, the product of the *ob* gene, is involved in the control of reproductive functions. It is a regulator of puberty onset, can influence fertility, implantation, maintenance of gestation, embryo growth and development. At the level of the ovary, the leptin might take part in the ovulation process, corpus luteum formation, angiogenesis and steroidogenesis. Recently, leptin gene and protein expressions have been found in the porcine corpus luteum during early pregnancy (days 14-16 and 30-32). Moreover, it was shown that LH and steroids participate in the regulation of leptin mRNA expression in porcine luteal cells on days 14-16 of pregnancy.

The aim of these studies were: 1] to examine the effects of LH, E$_2$ and P$_4$ on leptin gene expression in porcine luteal cells on days 30-32; 2] to compare the expression levels of leptin transcript in the porcine oviduct during two stages of pregnancy corresponding to the beginning (days 14-16) and the end (days 30-32) of the implantation process. Luteal cells after preliminary culture (48 h) were treated for 24 h with LH, E$_2$ and P$_4$. Total RNA was isolated from cells or oviduct and transcribed into cDNA. The expression of leptin mRNA was analysed by Real-Time PCR methods.

It was shown that: 1] LH (1 ng/ml), E$_2$ (2 ng/ml) and P$_4$ (100 ng/ml) significantly stimulate leptin gene expression in porcine luteal cells on days 30-32 of gestation; 2] leptin mRNA expression is localised in the porcine oviduct on days 14-16 and 30-32 of gestation. There were no significant differences in the level of leptin transcript in the oviduct when comparing these periods of early pregnancy. These data indicate that LH and steroids are involved in the regulation of leptin mRNA expression in porcine luteal cells and suggest that leptin can affect the function of luteal cells and the oviduct during gestation.

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Sperm membrane proteins critical to stallion fertility and sperm function in the female reproductive tract

A stallion’s intrinsic fertility is critical to breeding success and should, ideally, be predicted in advance. Currently, ‘breeding soundness evaluation’ (BSE) consists of physical examination and a basic semen quality analysis. While a BSE will identify stallions clearly lacking the capacity for adequate fertility, it will fail to identify other seriously sub-fertile animals. In vivo, fertilizing sperm must interact correctly with oviduct epithelial cells, capacitate and bind to and penetrate the zona pellucida, events that depend critically on orderly functioning of the sperm plasma membrane and its constituent proteins.

The aim of this study was to determine whether sub-fertility in some stallions can be explained by inability of their sperm to capacitate; sperm head plasma membrane protein profiles would then be compared between fertile stallions and sub-fertile stallions with evidence of capacitation failure. Semen was collected from approved breeding stallions with adequate semen quality, and categorized as fertile or sub-fertile based on seasonal pregnancy rates. Sperm were washed through a Percoll gradient and incubated under capacitating conditions (with HCO³⁻ and BSA) for 4 hours. The capacitation status of the sperm was then analyzed using an anti-phosphotyrosine antibody. In five of fourteen sub-fertile stallions, sperm failed to capacitate under these conditions, with < 5 % of sperm recorded as live and capacitated; these values were significantly lower (P>0.001) than for fertile stallions. Four fertile and four sub-fertile stallions with inadequate capacitation were then selected for sperm plasma membrane proteome analysis. The apical plasma membranes were isolated by nitrogen cavitation and sequential centrifugation; plasma membrane proteins are currently being compared quantitatively using 8plex iTRAQ. It appears that inability of sperm to capacitate is a cause of poor fertility in a sub-set of stallions. Proteomic analysis should help determine how variations/modifications in sperm plasma membrane proteins contribute to this problem.
Individual serum free culture of bovine embryos is producing high blastocyst rates

Paracrine communication between group cultured embryos is important during the first cleavage stages in mice\(^1\) and only later on (98 and 144hpi) in pig\(^2\). The aim of this study was to identify if the most critical period of paracrine communication during bovine embryo culture was situated before or after embryonic genome activation (EGA).

Bovine presumed zygotes (n = 1440; 3 replicates) were cultured in modified synthetic oviduct fluid (SOF) supplemented with ITS and 0.4% BSA\(^3\) either in groups of 25 (50µl droplets, 2 groups) or individually (20µl droplets, 2 groups). Serum free culture was used for further analysis of the conditioned medium. Cleavage was evaluated in all groups at 45 hours post insemination (hpi). At 80hpi, half of group cultured embryos were transferred to new individual droplets (GR-IND), whereas half of individually cultured embryos were transferred to new group culture droplets (IND-GR). The remaining embryos were kept in their original droplets (GR-GR and IND-IND). Blastocyst development was evaluated at 8 dpi.

Cleavage rate for group cultured embryos was 68.1±1.91% at 45hpi and 75.0±1.76% at 80hpi. Furthermore 75.8±1.75% and 79.0±1.68% of individually cultured embryos cleaved at 45 and 80hpi, respectively. Blastocyst rate was significantly higher in IND-GR (31.7±3.28%) and IND-IND (29.4±2.64%) in comparison with GR-GR (22.3±2.41%) and GR-IND (23.4±2.44%) embryos (p<0.05).

Whereas individual embryo culture is usually associated with low blastocyst rates, we demonstrated that serum free culture\(^3\) is able to support normal blastocyst development of individually cultured bovine embryos, and that individual culture before EGA is even beneficial for embryo development. Ongoing research is focussing on embryonic secretions in order to unravel the complex cooperative communication between embryos in vitro.

Centriole de-novo formation, number and distribution during in vitro development of parthenogenetic and IVF pig embryos

Each centrosome is formed by a pair of tubular organelles, the centrioles, surrounded by pericentriolar material. Centrosomes duplicate before mitosis, therefore, at the time of fertilization, each gamete provides either the centrioles or the pericentriolar material, so that a single centrosome can duplicate before the first mitosis. Typically the oocytes provides the pericentriolar material and the sperm provides the centrioles.

In this study, we compared the centrosome formation between pig embryos obtained by IVF and parthenotes, where sperm and its centrioles are not present.

Centriole number was assessed by immunocytochemistry using an anti-centrin antibody. In IVF embryos, we observed the presence of a normal centrosome at completion of the first mitosis. In parthenotes, we were unable to detect any centriole before the 4-cell stage. From this stage onward centrioles were detected. However, the vast majority of parthenote blastomeres (around 83%) showed abnormal numbers of centrioles (predominantly 3/blastomere). In contrast, 98% of IVF embryos displayed the expected arrangement of 1 or 2 centrioles/blastomere.

The presence of 3 or more centrioles determines the formation of multipolar spindles that are consistent with the high incidence of aneuploidy described in parthenotes of all species. Aneuploidy in biparental cells activates the p53/p21 pathway that leads to cells cycle arrest and apoptosis. However, parthenotes can develop in utero and proliferate until around implantation. In order to better elucidate this aspect, we investigated p53 and p21 expression. We observed a significant down-regulation in parthenotes (59.6-fold and 10.2-fold, respectively).

Our data indicate that the absence of paternal centrioles causes an altered centrosome formation possibly causing centrosome amplification and aneuploidy. The normal development observed despite such severe alterations in vitro, and in vivo, is likely to be due to an adaptation mechanism, based on the down regulation of the p53/p21 pathway.
Osteopontin (OPN) plays a critical role in the adhesion-phase of implantation, and signal transduction at the uterine-placental interface, in various species. In the mare, expression of OPN and its receptors (CD44 and integrin αVβ3) has not been reported. However, since equine pregnancy is characterized by a prolonged pre-implantation phase (40-45 days), it is a potentially valuable species in which to study the adhesion process. The aim of this study was to determine whether OPN, CD44 and integrin αVβ3 are expressed in the mare’s uterus and, if so, to examine the temporal pattern of expression in the endometrium of both cycling and pregnant mares, and in the early conceptus. Endometrial biopsies were recovered from 4 mares at each of days 7, 14 and 21 of gestation, days 7 and 14 of dioestrus, and during late oestrus. Corresponding conceptuses were retrieved by uterine lavage (day 7) or using a video-endoscopically guided net (days 14 and 21). Expression of mRNA for OPN, CD44 and integrin αVβ3 was analysed by real-time PCR, with relative gene expression calculated with respect to 3 stable reference genes using GeNorm.

Endometrial OPN mRNA expression decreased during oestrus (p<0.05), whereas an apparent gradual decrease in OPN expression as pregnancy progressed was not significant. By contrast, endometrial expression of CD44 increased during oestrus (p<0.05), and integrin αVβ3 mRNA expression was up-regulated (p<0.05) on day 14 of both the cycle and pregnancy. In the conceptus, expression of OPN, CD44 and integrin αVβ3 increased markedly on day 21 (p<0.05). The combination of apparent progesterone-dependency of endometrial OPN expression and up-regulation of expression for OPN and its receptors in trophoblast between days 14 and 21 leads us to propose that OPN contributes to trophoblast-endometrium adhesion from around the disappearance of the blastocyst capsule (days 18-22 of gestation).
Expression and localization of prokineticin 1 and prokineticin receptor 1 in the porcine endometrium and conceptus.

Embryonic-maternal dialogue requires local mediators within endometrium. Studies in the human have shown that prokineticin 1 (PROK1) plays a role in conceptus receptivity and implantation. PROK1 and its receptor PROKR1 expression is elevated in the first-trimester decidua, compared with nonpregnant endometrium. It is suggested that in the human endometrium, PROK1 can act in an autocrine and paracrine manner through PROKR1 to regulate host implantation-related genes. Therefore, the aim of the studies was to elucidate the expression pattern of PROK1 and PROKR1 proteins in the porcine endometrium and conceptus. Endometrial samples were collected from gilts on either day 9, 11, 12, or 15 of the estrous cycle (n=29) and pregnancy (n=22). Moreover, conceptuses and trophoblasts (n=20) were obtained from gilts on days 12-15 (n=6), 18-20 (n=7) and 22-25 (n=7) of pregnancy. Protein expression of PROK1 and PROK1 in endometrial and conceptus samples was studied by Western blot analysis. Additionally, localization of these proteins in uterus was studied by immunohistochemistry. On day 15 of pregnancy (beginning of implantation) endometrial expression of PROK1 protein was significantly elevated when compared to all studied days of pregnancy and the estrous cycle. Expression of PROK1 on day 15 of pregnancy was 6-fold greater (p<0.01) than on day 9 of pregnancy (preimplantation period). Endometrial expression of PROKR1 did not change significantly during the estrous cycle and pregnancy. PROK1 and PROKR1 proteins were localized in luminal and glandular epithelium, stroma and blood vessels of endometrium. PROK1 and PROKR1 were expressed in conceptuses in all studied stages of pregnancy. These results suggest the involvement of PROK1-PROKR1 signaling in the embryo-maternal dialogue during the implantation window and early pregnancy in the pig.

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We have recently shown that lysophosphatidic acid (LPA) can be produced by the bovine endometrium and corpus luteum. Moreover LPA influenced secretory functions of these organs both in vitro and in vivo during estrous cycle and early pregnancy. However, there is no data if LPA can be produced by the bovine embryo. The objective of the present study was to examine protein localization (immunofluorescent staining) and mRNA transcript quantification (Real-time PCR) for LPA synthesizing enzymes (Phospholipase A2 - PLA2, Autotaxin - AX) and LPA receptors (LPAR1, LPAR2, LPAR3 and LPAR4) in the bovine embryo on day 5 (the time of maternal genome presence) and 7 (the time of embryonic genome activation) of in vitro culture. We confirmed the presence of protein and mRNA expression for PLA2 and AX at both examined phases of in vitro embryo culture. We also found the presence of protein and dynamic mRNA expression for LPAR1, LPAR2, LPAR3 and LPAR4 at both examined phases of in vitro embryo culture. Moreover, we found dominant mRNA expression for LPAR3 at both examined phases of embryo development. In conclusion we demonstrated that the bovine embryo, additionally to the uterus and CL is a source and a target for LPA in the cow.
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**Gonadotrophins and prolactin regulate endometrial 17β-estradiol production in pigs during early pregnancy (days 10 to 11, 12 to 13 and 14 to 16)**

Biosynthesis of steroid hormones occurs in porcine uterine tissues during both early pregnancy and the estrous cycle. In pregnant pigs the most potent estrogen is 17β-estradiol (E$_2$) which is considered to be the crucial factor for recognition and maintenance of early pregnancy. We hypothesized that the gonadotrophins (luteinizing hormone- LH, follicle-stimulating hormone- FSH) and prolactin (PRL) may be regulatory factors for uterine E$_2$ production. Therefore we investigated the effect of LH, FSH and PRL on in vitro E$_2$ release from pregnant porcine endometrium. Slices of endometrium were isolated from uteri of post pubertal pigs on days 10 to 11, 12 to 13 and 14 to 16 of pregnancy. The isolated slices were pre-incubated in 2 ml of M199 medium for 18 hours in atmosphere of 95% O$_2$ + 5% CO$_2$ in 37 °C and then incubated for 6 hours with control medium, LH, FSH and PRL (hormones in doses of 10 ng/ml and 100 ng/ml). Concentration of E$_2$ in medium was determined with radioimmunoassay. During days 10 to 11 of pregnancy none of experimental factors changed E$_2$ release from endometrium. On days 12 to 13 of pregnancy FSH in dose of 100 ng/ml and PRL in the dose of 10 ng/ml decreased E$_2$ secretion from examined tissue (p < 0.05). During days 14 to 16 of pregnancy only FSH in dose of 100 ng/ml reduced E$_2$ release from endometrium (p < 0.05). These data show that FSH and PRL may be involved in regulation of endometrial E$_2$ release and fluctuations of E$_2$ concentration in uterine fluid between days 12 and 16 - the critical period for maternal recognition of pregnancy and upholding of porcine gestation.

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**Differential apoptotic staining of bovine blastocysts based on double immunofluorescent CDX-2 and active caspase 3 staining**

The transcription factor CDX-2 is localized in the nucleus of trophectoderm (TE) cells but absent in the inner cell mass (ICM), and this feature can as such be used as a basis for differential staining. Since both ICM/TE ratio and apoptotic cell ratio are important blastocyst quality parameters, the aim of the present study was to test a double immunofluorescent staining using antibodies against CDX-2 and active caspase 3.

Day 8 *in vitro* produced bovine blastocysts were fixed in 4% paraformaldehyde during 20 minutes at room temperature and afterwards permeabilized with 0.5% Triton-X + 0.05% Tween in PBS overnight at 4°C. The DNA of the blastomeres was denaturalized by exposure to HCl. Blocking was performed overnight in 10% goat serum + 0.05% Tween in PBS at 4°C. Next, embryos were incubated in primary CDX2 antibodies (Biogenex, San Ramon, USA) for 3 days at 4°C. After washing for 30 minutes at room temperature, they were incubated in rabbit anti-caspase 3 (Cell Signaling Technology, Leiden, The Netherlands) overnight at 4°C. After another wash step, the blastocysts were incubated in secondary antibodies Goat anti-mouse Texas Red (1:100) for 3 days at 4°C and Goat anti-rabbit FITC (1:100) for 1 hour at room temperature. Finally, the embryos were transferred to PBS-BSA containing 1:100 nuclear stain (Hoechst 33342) to visualize all nuclei (ICM + TE). Evaluation by fluorescent microscope allowed a perfect distinction between ICM and TE cells as well as clear detection of apoptotic cells by active caspase positivity in the cytoplasm.

In conclusion, this double immunofluorescent staining represents an easy and repeatable method for differential apoptotic staining of bovine blastocysts.
The IL-1 system contains interleukin 1β (IL-1β), IL-1 receptor type I, IL-1 receptor antagonist and IL-1 receptor accessory protein (IL-1RAP). Our previous studies have shown that IL-1β may regulate synthesis and secretion of prostaglandins and steroids in porcine corpora lutea (CLs). Thus, to clarify the mechanism of IL-1 system action in porcine CLs we determined the mRNAs expression of IL-1β and IL-1 RAP in CLs of gravid pigs. Ovaries were isolated from pregnant pigs – Days 9 (n=4), 10-11 (n=5), 12-13 (n=5) and 15-16 (n=3). The CLs were separated, frozen in liquid nitrogen and stored in -80°C until RNA isolation. Total RNA was extracted from CLs using Total RNA kit and Real Time PCR was performed using Power Sybr Green master mix following the manufacturers’ instructions. To evaluate mRNA level were used specific primers: sense 5’-TGCCAACGTGCAGTCTATGG-3’ and antisense 5’-TGGGCCAGCCAGCACTAG-3’ for IL-1β and sense 5’-AAATGCCAAAGGGGAGGTT-3’ and antisense 5’-TGCTGTGTGCATCCATTACC-3’ for IL-1RAP. Results obtained from Real Time PCR analysis were normalized against the housekeeping gene - GAPDH. The luteal mRNA expression of IL-1β was the lowest and highest on Days 10-11 and 15-16 of pregnancy, respectively(p<0.05). The transcript level of IL-1RAP was low on Days 9-13 (p<0.05) and increased on Days 15-16. In conclusions: 1) this study showed the presence of IL-1β and IL-1RAP transcripts in porcine luteal tissues, 2) the highest abundance of IL-1β and IL-1RAP mRNAs was observed on Days 15-16 of pregnancy.

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miRNAs play a role in the regulation of the Drosophila female mating response

In *Drosophila*, like most insects, mating induces a rapid change in the female’s physiology and behavior. In the hours after mating, major changes in gene expression and reproductive tract (RT) morphology occur. Immediately after mating, 79 genes in the lower RT exhibit a change in expression level, 70% of which are down-regulated. By 6 hours post-mating, major changes in expression profile of the female lower RT is evident. The rapid changes in mRNA and protein levels after mating, and in particular the lack of correlation between mRNA and protein levels for some genes, led us to hypothesize that the female response to mating involves post-transcriptional regulation by miRNAs. To test this hypothesis, we performed a bioinformatics analysis using expression profiling data of the lower RT of unmated and mated females. The analysis suggests that miRNAs negatively controls mRNA levels after mating in the lower RT, namely, there are miRNAs that are induced by mating. As an initial test for a possible role of miRNAs in reproduction, we reduced the function of all miRNAs by driving RNAi against *Dicer-1* and *Drosha* in the whole female body pre-mating. A clear reduction in female fecundity and fertility was observed in the first day after mating. We further dissected the effect of reduced function of all miRNAs in the lower RT, specifically in the Spermathecae Secretory Cells (SSC), both pre-mating and post-mating. At both examined time points, reduced function of all miRNAs in the SSC reduced female fecundity 6 hours post-mating but had no effect of female fertility. We thus conclude that reduced function of miRNAs in the SSC affects regulation of the initial activation of ovulation/egg-laying. Our results suggest a role for miRNAs in reproduction and more specifically in RT maturation and in the very early mating response.
Introduction: Many female species store spermatozoa in the reproductive tract prior to fertilisation. Sperm storage occurs via the temporary attachment of spermatozoa heads to the apical oviductal epithelial cells (OECs) membrane. Sperm viability is maintained by such sperm-OEC associations. Using a recombinant bovine HSPA8 generated in an E.coli over expression system in our laboratory, we showed that HSPA8 is able to enhance sperm survival (assessed by membrane integrity based viability assays) as early as 15 min post-exposure to spermatozoa. The current investigation examined the effect of extracellular HSPA8 on sperm membrane fluidity as the potential mechanism behind the viability enhancing effect of HSPA8.

Methods: Fresh spermatozoa from three boars were diluted in Beltsville thawing solution, washed using a Percoll gradient and diluted to 10^6 spermatozoa/ml in Tyrod’s albumin lactate and pyruvate solution. Diluted semen samples were loaded with 5-(N-octa-decanoyl) aminofluorescein (ODAF) 12.5µM, as a fluorescent lipid probe. Fluorescence recovery after photobleaching (FRAP) analysis was performed on acrosomal and postacrosomal domains of live stained spermatozoa (n=6) after 15 min co-incubation with 0 and 0.5µg/ml HSPA8 at room temperature. HSP70 protein was also included as control protein sample. Diffusion coefficient (D) and recovery% (R%) values were recorded.

Results: HSPA8 significantly increased the R% and D values (means ± SEM) of acrosome (60 ± 0.5 vs 70 ± 1[p=0.007] and 27.73 ± 1.15 vs 49.6 ± 3.5 [p<0.001], respectively) and postacrosome (55 ± 1 vs 65 ± 1.5 [p=0.009] and 24.44 ± 2.5 vs 43.12 ± 4 [p<0.005], respectively) of live spermatozoa after 15 min exposure time, compared to control samples. In contrast, the addition of HSP70 to sperm samples did not influence sperm membrane fluidity on any of the two regions.

Conclusion: These findings indicate that recombinant bovine HSPA8 increases sperm membrane fluidity in a specific manner. Alterations in sperm membrane microviscosity might be the mechanism which mediates HSPA8-induced enhanced sperm survival.
Nano-LC-MS/MS based proteome profiling of bovine oocytes

Assisted reproduction techniques (ARTs) become increasingly important both in veterinary and in human medicine. Since ARTs are still associated with low conception and birth rates, it is necessary to improve the understanding of biochemical processes underlying oocyte maturation and embryonic development. During oocyte growth in the developing ovarian follicle, the oocytes accumulate significant amounts of proteins required for fertilization and zygote development. Our work focuses on the qualitative characterization of this protein reservoir by a LC-MS/MS proteome approach of bovine oocytes. Although proteomics of mammalian oocytes is challenging due to very low sample amounts 1221 proteins could be identified with high confidence (FDR < 1) using this strategy. However, beside the pure identification of proteins within the oocyte proteome, the determination of their concentrations is a pre-requisite in order to get a deeper view of their role during oocyte maturation and early embryogenesis. Therefore, a further aim of this study was to establish a method allowing absolute quantification of proteins within oocytes. One of the most powerful techniques to perform mass spectrometry based absolute quantifications is the so called Selected Reaction Monitoring. This technique routinely used to quantify small molecules, was recently expanded to the quantification of proteins. As a first step and as a proof of concept three proteins which are expected to play an important role during early embryogenesis were selected for quantification in bovine oocytes: i) the Y-box protein 2 (YBX2) ii) the IGF 2 mRNA-binding Protein 3 (IF2B3) and iii) the Programmed Cell Death Protein 5 (PCDP5).
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Whole Genome resequencing of Estonian Dairy cow
(Estonian Holstein)

The aim of our study is to find relevant biomarkers related to the outcome of somatic cell nuclear transfer cloning in dairy cattle by comparing different donor cell lines used. The recent availability of a bovine reference genome has made a huge step forward in research regarding bovine genomics.

The bovine reference genome currently available from public databases is one of a Hereford female (Btau_4.0).

We present here the whole genome resequencing of Estonian Holstein dairy cow as the model animal in our study, using next generation sequencing. Whole genome sequence of a female Estonian Holstein was generated using massively parallel sequencing technology. 700 million short sequencing reads were obtained from 50-bp mate-paired library with a mean insert size of 1500 bp.

All data was aligned against Btau_4.0 Hereford reference sequence using Bioscope v1.2. We were able to map over 75% of the reads to the reference, that is 42.3 gigabases of sequence resulting in an average of 16-fold coverage.

Further analysis of our data is ongoing and the results will be presented during the 3rd Annual GEMINI Meeting.
The aim of present study was a development of easy somatic cell nuclear transfer protocol for bovine cloning using in vitro matured oocytes and fetal fibroblasts. Oocytes were matured in TCM media supplemented with fetal calf serum and Intervert PG600 hormone preparate. Oocytes were enucleated in 0.5% BSA/PBS solution “blindly” using micromanipulators. Fibroblast isolated from two months old fetus were used as nuclear donors. Fibroblast cells were microinjected into oocyte periviteline space in 0.5% BSA/PBS solution. Cytoplast donor cell complexes were fused in Eppendorf isoosmolar fusion buffer using Eppendorf fusion machine parameters 65V 20µsec 2p. Fused complexes were chemically activated with incubation of Ca-ionophore (5µ/ml) SOF (Minitüb) solution for 5 min. Following activation the oocytes were placed 2 mM DMAP SOF medium for 5 h incubation. Embryos were cultured of SOF medium for seven days at the blastocyst stage. On the day five one group of morulas were hatched with micromanipulator in 0.5% BSA/PBS solution and other group were not hatched. For embryo transfer 33 virgin Holstein breed heifers treated twice at 14- days interval with 25 mg injection of PGF$_{2\alpha}$ were used. Total of 39 clone-embryos were transferred- five recipients with total 11 embryos with zona pellucida and 14 recipients with total of 28 hatched embryos. None of five recipients became pregnant after transfer of clone-embryos with zona pellucida. From transfer of hatched embryos the pregnancy rate was 42.9% (6/14). All of six pregnancies were lost during two months.
Resilin a rubber-like protein is localized in the Drosophila female reproductive tract

Resilin is a rubber-like protein found in specialized regions of the cuticle of most insects, providing low stiffness, high resilience and efficient energy storage. Its function and high resilience resembles the elastic protein, elastin, which is present in vertebrates. Resilin is best known for its roles in insect flight and the remarkable jumping ability of fleas and spittle bugs.

The female reproductive tract of the fruit fly, Drosophila melanogaster, is surrounded by contractile circular striated muscle fibers. Their contractions are linked to ovulation, egg transport along the reproductive tract and finally oviposition of the fertilized eggs. However, we know little about how epithelial cells expand during the egg passage through the reproductive tract. We hypothesized that the expansion of the epithelial tissue along the female reproductive tract during tonic stretch is facilitated through cell surface unfolding and the presence of resilin and/or other possible mechanisms.

To determine whether resilin plays a role in egg passage through the reproductive tract, we first examined the spatial distribution of resilin in the female reproductive tract pre- and post-mating. We found that resilin is present in the lateral oviducts and lower common oviduct. After mating, the spatial distribution of resilin was unaltered, but the level of resilin increased significantly in the lateral oviducts. To better understand the function of resilin we next used RNA interference (RNAi) to knock down the resilin synthesis in the oviduct of females and examined their reproductive success. We will present and discuss the significance of resilin localization in the female reproductive tract; and how the study of a rubber-like protein in the female fly may contribute to understanding of reproductive tract function in other organisms, such as farm animals.
Ovarian folliculogenesis is a long and complex process in which both the endocrine- and immune system have significant roles. Besides cellular autoimmunologic processes also humoral processes have important parts to play in the development of female infertility. Aberrances in the humoral immune system may lead to impairments in folliculogenesis and endometrial receptivity. Cytokines, as one of the modulators of the immune system, take part in the regulation of the ovarian cycle, such as supporting follicular growth, infiltration and activation of leucocytes necessary for ovulation and tissue remodelling during luteinization and luteolysis. In previous studies we have shown the expression of several cytokine genes in granulosa cells during controlled ovarian hyperstimulation (COH) in in vitro fertilization (IVF) treatment. However, further studies on the associations of follicular fluid cytokines with oocyte and embryo quality and IVF treatment results are still needed. We therefore aimed to examine the effect of follicular fluid cytokine and other biomarker levels, in infertile women, on oocyte and embryo quality and also pregnancy results after IVF. For this 156 follicular fluid samples from women undergoing IVF in Nova Vita Clinic were analysed using Bender Medsystems FlowCytomix technology in combination with Beckman Coulter FC500 flow cytometer and CXP software. As a result of the study 16 biomarkers secreted into the follicular fluid were detected: RANTES, IL-8, MIP-1α, MIP-1β, IL-23, IL-1β, TNF-α, IL-18, IL-12 (p70), MCP-1, IFN-α, IFN-γ, IL-6, G-CSF, APO-1/Fas ja CD44 var(6). Also, the profiles of these biomarkers were compared with infertility etiology, COH and IVF outcome.
Apoptosis in bovine cumulus cells – associations with follicle diameter and COC quality.

It is known that only 30-40% of bovine oocytes reach the blastocyst stage when matured and fertilized in vitro (Lonergan, 2003). It is probably due to the inferior quality of oocytes collected from abattoir ovaries (Rizos, 2002a, b, 2003). Therefore selection of good quality oocytes is an important step in IVP procedure. Regardless of some contradictory reports, apoptosis in cumulus cells (CC) is considered a good, non-invasive marker of COC quality. It is interesting especially in the light of human IVP data presenting associations between CC apoptosis and the number of retrieved oocytes, embryo quality at 44hpi and blastocyst development (Lee, 2001, Corn, 2005).

The aim of the present study was to investigate associations between apoptosis in cumulus cells and two commonly used parameters related to COC quality (follicle diameter, COC morphology). COCs were aspirated from individual follicles of known diameter (<6mm, 6-8mm, >8mm) and their morphology was evaluated (1 to 4 grade depending on the ooplasm homogeneity and CC quality; Stojkovic, 2001). The experiment comprised 12 groups of COCs (eg. >8mm grade 1; <6mm grade 4 etc). The external layers of CC were subjected to analysis of apoptosis by TUNEL.

At the present stage of the experiment, CC from 55 complexes were analysed and apoptosis was detected in 47% of them (26/55). The average apoptotic index was 5.03 and varied from 0 to 40. Statistical analysis showed no correlation between presence of apoptotic CC and follicle diameter or COC’s grade. It was also the case when mean apoptotic index for each experimental group was taken under consideration. Our work is currently focused on collecting data on an uniform number of CC samples in each experimental group what will yield more reliable results.

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The foetal and postnatal effects of periconceptional hyperglycaemia using a rabbit model

Our aim was to analyze the effects in offspring of maternal hyperglycaemia during the periconceptional period.

Diabetes was induced (group D) with alloxan in adult does one week prior to mating. Glycaemia was maintained between 3 and 5 g/l with 2 daily subcutaneous insulin injections. Embryos were collected on Day 4 post-coitum. In a first experiment, embryos from D (n=11) and control (n=13) groups were transferred respectively to the right and the left horn of 3 recipients. Recipients were euthanised on Day 28. In a second experiment, 68 embryos from 17 D females and 98 from 16 controls were transferred to 26 non-diabetic recipients. Foetal development was monitored by ultrasound. At birth, litters were equilibrated in number. 15 D and 7 C pups were euthanized at weaning. The remaining pups were allocated to one of two feeding groups (control or cafeteria diet). Bodyweight, adiposity, and glucose metabolism were monitored until sacrifice at 5 months of age.

Foetal development was not different between D and C groups. Foetal, placental and organs weights did not differ at Day 28, except for brain weight which was significantly lower in D foetuses (p<0.05). There was no difference in litter size at birth, but birthweight was significantly increased in D offspring (p<0.05). Pups caught up and there was no difference in weight after 14 days. At one month of age, in male D offspring, fasting glycaemia was significantly lower (p<0.01), plasma IGF1 was significantly increased (p<0.05) and kidney/body weight ratio was significantly reduced. From 12 weeks of age, body weight becomes significantly different between D and C groups and according to diet (p<0.005, ANOVA), and fasting glycaemia is significantly higher in the cafeteria group (p<0.04).

This suggests that maternal hyperglycemimia during the periconceptional period affects glucose metabolism and organ development in offspring, with sexual dimorphism.
Elevated non-esterified fatty acid concentrations affect bovine oviductal epithelial cell viability and sperm binding

Maternal metabolism may influence oviduct micro-environment and physiology. Therefore, bovine oviductal epithelial cell (BOEC) monolayers were exposed to high non-esterified fatty acid (NEFA) concentrations and were evaluated for cell number, cell viability and sperm binding capacity. BOECs were mechanically isolated (4 replicates) from slaughterhouse oviducts and cultured serum-free for 6 days until confluent monolayer formation. On day 7 BOEC monolayers were exposed for 24h to 1) NEFA free conditions = CONTROL, 2) elevated stearic acid concentrations = HIGHSA (240 µM SA), or 3) elevated NEFA concentrations = HIGHCOMBI (780 µM of total NEFA, i.e. oleic, stearic & palmitic acid). On day 8 sperm binding (glutaraldehyde fixation after co-incubation and visualization under phase-contrast inverted microscope), cell number and cell viability (trypan blue) were assessed. The results showed that total cell numbers (± SD) (25499±15582 and 21665±12665 cell/ml) and cell viability (92.6%±5.31 and 89.09%±24.08) in HIGHCOMBI and HIGHSA conditions respectively were significantly lower compared to the CONTROL group (36749±11795 cell/ml and 98.6%±1.89, respectively) (P < 0.05). Addition of SA (17.65±10.44) or the NEFA mixture (10.84±8.61) reduced the number of spermatozoa bound per 0.026 mm² monolayer compared to the CONTROL group (21.1±8.08). These in vitro results suggest that high NEFA concentrations in blood, which have been linked to several maternal metabolic disorders and subfertility, may alter oviduct cell viability and sperm binding, thereby possibly affecting gamete transport, fertilization and early embryonic development.
Maternal nutrition during gestation can alter metabolic processes involved in fetal growth regulation increasing adult onset disease in the offspring. For example, feeding mothers a high fat/high cholesterol diet (HFD) not only results in maternal obesity but also predisposes their offspring to obesity and cardiovascular and metabolic dysfunction (1). We previously reported that short-term HFD exclusively during the preimplantation period already introduces detectable changes before implantation. Blastocyst development was reduced and local glucose handling within the embryo and maternal reproductive tract was compromised (2). We now examined maternal systemic status and embryo growth more closely.

Short-term (plug-3.5 days) HFD did not induce maternal obesity, likely due to similar caloric intake when compared to chow control mothers. Systemic insulin/glucose homeostasis was perturbed in HFD-fed mothers with increased serum-insulin levels and absence of the expected inverse relationship to serum glucose levels. Interestingly, no relationships were found between maternal weight, food intake or glucose handling and blastocyst numbers produced. Blastocysts obtained after 3.5 days of maternal HFD had more cells in both lineages, ICM and TE, compared to controls. Moreover, both glucose transporters examined (GLUT-1 and GLUT-3) were upregulated at cell membranes whilst amino acid transporters remained constant.

Overall, we present evidence that acute fat/cholesterol overnutrition perturbs systemic maternal glucose/insulin homeostasis but does not (yet) coincide with obesity. However, less blastocysts develop and those that do have more membrane-localised glucose transporters and more cells in both lineages. We now propose that acute maternal HFD changes maternal metabolism sufficiently to induce selection for embryos that can adapt to these alterations by growing more. This may provide a route how transient maternal metabolic imbalances, if occurring during the selective preimplantation phase, may translate into longer-term consequences for the developing conceptus.

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Fat content in the diet affects early-embryo weight in sows with gene polymorphisms for leptin and melanocortin receptors

Obesity and nutritional habits are associated with incidence of infertility, early miscarriages and alterations of foetal growth. Current study aimed to determine possible effects of life-time fat feeding on rates of ovulation, embryo implantation and later incidence of embryo mortality and intrauterine growth alterations at early pregnancy stages. Iberian sows, being a breed with a high potential for obesity due to gene polymorphisms in leptin and melanocortin receptors, were used as a model. Current study included 14 sows that were fed either with a standard diet having 2.8% of fat (group C, n=7) or with a fat-enriched diet (6.3%, group HF) from 18 weeks-old. At 47 week-old the animals become pregnant and at Day 25 of pregnancy, entire genital tracts were collected and weighed. Thereafter, ovulation rate was assessed and embryos were recorded, weighed and measured (longitudinal and transversal diameters of the embryos and their vesicles). There were not found significant differences between groups in number of ovulations (13.6±0.4 for C vs 12.4 ±1.0 for HF), number of conceptuses (8.0±1.5 for C vs 8.3 ±0.9 for HF) and, therefore, in the rate of implantation and survival (58.1 for C vs 66.0% for HF). However, the weight of the uterus, having a similar mean number of conceptuses, was higher in HF sows (2714.8 ± 199.6 vs 2152.5 ± 283.9 g for C; P<0.05). Individual assessment of the conceptuses showed that their sizes vesicles were similar between groups; however their weight was higher in HF group (3.7 ± 0.1 vs 3.2 ±0.1 g for C; P<0.05). Such difference was mainly related to a higher weight of the proper embryo (1.2 ± 0.0 vs 0.9 ±0.1 g for C; P<0.0005), having similar sizes in both groups, since the weight of the extra-embryonic annexes and the vesicle fluid did not reach statistical differences.
The repeat breeder cows are defined as a clinically healthy animals, which have failed to conceive after at least three inseminations. Information concerning prevalence and causes of repeat breeding is still limited. Many factors contribute to repeat breeding, however, recently the increased importance of the altered uterine environment has been shown.

The aim of the study was to evaluate the prevalence of subclinical endometritis in repeat breeding dairy cows.

The study was carried out in 5 dairy herds on 902 cows. 207 (22.0%) cows were inseminated at least 3 times and still were not pregnant. They were examined by vaginoscopy, rectal palpation and ultrasonography. Heat intervals of repeat breeding cows were calculated. 112 of 207 (54.1%) of repeat breeding cows were clinically inapparent. Using the cytobrush technique, endometrial epithelium samples were obtained from these cows. Swab samples from the uterus were also collected for microbiological examination. The smears were stained by Papanicolau stain and a total of 100 cells were counted to determine the percentage of polymorphonuclear leucocytes (PMNs). A threshold of 10 % PMNs was used for the diagnosis of subclinical endometritis. With the cytobrush technique 45 (40.2%) of clinically healthy cows were diagnosed having a subclinical endometritis. Only 62.7% of swab samples from the uterus of these cows were positive for bacteria. The most prevalent bacteria were Streptococcus sp. (42.8%), E. coli (16.1%), and A. pyogenes (5.4%). Most cows with subclinical endometritis showed normal or prolonged regular intervals between heats.

This study showed that the subclinical endometritis is a substantial cause of repeat breeding in the dairy cows. The high percentage (37.3%) of bacteriologically negative samples from uterus of cows with subclinical endometritis suggests the nutritional background. However, the future investigations on the composition of uterine secretion are needed to clarify the nature of this disease.
The consequence of high non-esterified fatty acid concentrations during oocyte in vitro maturation on bovine embryo quality and viability

Metabolic disorders such as obesity, type 2 diabetes and negative energy balance have been linked to subfertility. Elevated non-esterified fatty acid (NEFA) concentrations may explain this as they can reduce oocyte developmental competence. It is unclear whether elevated NEFAs during maturation can affect embryo quality. We have measured this by the following quality parameters; cell number (CN), apoptotic cell ratio (ACR) and amino acid turnover. During serum-free maturation, 734 bovine COCs were exposed to 1) CONTROL (150 µM of total NEFA, i.e. oleic, stearic & palmitic acid), 2) elevated stearic acid = HIGHSA (75 µM SA) and 3) elevated NEFA = HIGHCOMBI (425 µM total NEFA). Following IVF, zygotes were cultured in SOF + 5% FCS for 7 days. Blastocysts were evaluated for CN (propidium iodide) and ACR (TUNEL). 134 embryos were cultured singly in 5 µL droplets for 24h. The amino acid composition in spent and blank medium was measured by HPLC.

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<th>CONTROL</th>
<th>HIGHSA</th>
<th>HIGHCOMBI</th>
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<tr>
<td>Blastocyst (%)</td>
<td>25.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>oocytes</td>
<td></td>
<td></td>
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<tr>
<td>CN</td>
<td>125.8±29.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>105.4±24.7&lt;sup&gt;$&lt;/sup&gt;</td>
<td>104.7±26.1&lt;sup&gt;$&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACR (%)</td>
<td>0.11±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
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Superscripts a,b: P<0.05; superscripts *,$: P=0.08

HIGHCOMBI embryos showed elevated amino acid consumption (factor 1.8) (P<0.01), production (factor 1.7) (P=0.02) and total turnover (factor 1.7) (P<0.01) compared to CONTROLs. HIGHSA embryos displayed higher amino acid consumption (factor 1.9) (P<0.01) and turnover (factor 1.6) (P<0.03) compared to CONTROL embryos. Our results suggest that maternal metabolic disorders, associated with high NEFA concentrations, hamper fertility through a reduction of oocyte developmental competence, reduced embryo quality and an upregulated embryo metabolism, previously been associated with elevated DNA damage and low embryo viability.
The insulin-like growth factor axis profoundly regulates embryonic growth and differentiation. In addition to the endocrine effects by circulating IGFs, the locally produced IGF exert paracrine and autocrine effects on embryonic cell proliferation.

We have investigated the influence of maternal type 1 diabetes (T1D) on IGF1 and 2 expression during rabbit preimplantation development. IGF levels were analysed by real time PCR and ELISA in the maternal serum, liver and uterine tissues and in 6 day old blastocysts.

While the hepatic IGF1 expression and maternal serum levels were decreased in the T1D females, the IGF1 and IGF2 expression increased significantly in uterine tissues and blastocysts. Concordantly with the increase in RNA expression, the IGF1 protein amount in diabetic blastocysts was approx. 2fold higher than in controls (2.81 rU vs. diabetic 4.59 rU, p=0.08)

To closer investigate preimplantation diabetic programming, 4 day old rabbit blastocysts developed in diabetic females were transferred into healthy synchronized females. In 28 day old foetuses and 4 weeks old pups, IGF1 and insulin concentrations were quantified in blood serum. In 4 weeks old male pups the IGF1 serum levels were significantly increased whereas the serum insulin levels were unaltered.

Our data show that the IGFs axis in the reproductive tract and even in the blastocyst was shifted to an upregulation of the paracrine and autocrine IGF production in response to maternal diabetes. A period of only 4 days of embryonic development in a diabetic milieu is sufficient to increase IGF1 levels in male offspring. These results indicate that the IGF system is highly sensitive to external metabolic and hormonal changes during the preimplantation period and that these effects are long lasting and sex dependent.

Supported by DFG NA 418/4-2 and the Wilhelm Roux Programme of the MLU Faculty of Medicine
Transgenerational effects of a maternal hyperlipidic hypercholesterolemic diet on the morphology of placentas, in a rabbit model

The maternal metabolic environment is crucial to the short and long term development of the offspring. The administration of a hyperlipidic hypercholesterolemic diet before and during pregnancy in a rabbit model decreases the placental weight and causes IUGR. Here, we have focused on the consequences of this diet on the morphology of the placentas of offspring (F2).

Female rabbits (F0) were fed with a control diet (C) or a high fat diet (H) (6% of soybean oil and 0.2% cholesterol) from 10 weeks of age, throughout pregnancy and lactation (breeding at 17-18 weeks). After weaning, female offspring (F1) received a control diet (groups CC and HC) or a high fat diet (groups CH and HH). They were bred at 18 weeks of age with control males. At 28 days of gestation, placentas corresponding to the fetal offspring (F2) were processed for histology and transmission electron microscopy.

Histological analysis of CH and HC placentas revealed an abnormal accumulation of numerous light vesicles localized in the trophoblast layer and involved in materno-fetal exchange, whereas no vesicle was present in the syncytiotrophoblast of the CC placentas. The HH syncytiotrophoblast was characterized by the presence of both light and dark vesicles. Ultrastructural analysis of CH, HC and HH placentas demonstrated that light vesicules correspond to a large number of lipid droplets. Moreover, dilated smooth endoplasmic reticulum was observed in HC placentas, while the dark vesicles observed in the trophoblast layer of HH placentas were identified as multilamellar lipid lysosomes.

These data establish that a maternal high fat diet can modify the structure of the placenta of the second generation offspring (F2). Consequently, the effects of maternal nutritional imbalance are not limited to the first generation offspring but can be passed on transgenerationally to the second generation.
Increase of essential amino acids in the bovine uterine lumen during preimplantation

In animal husbandry, metabolic imbalances may not only negatively affect health and productivity of dairy cattle during early lactation, but might also alter the nutrient composition of the uterine fluid. Amino acids (AA) are crucial for the developing conceptus prior to implantation. We here present a comprehensive and quantitative analysis of the physiological AA composition in the uterine lumen of early pregnant cattle to provide insights into the requirements of the bovine embryo. At days 12, 15 and 18 post-estrus, uteri of synchronized Simmental heifers were flushed for the analysis of 41AA and derivatives by LC-MS/MS. The ipsilateral endometrium was sampled for quantitative PCR. Most notably, all essential AA accumulated with proceeding pregnancy while the trophoblast undergoes continuous elongation. In addition, we detected elevated concentrations for most non-essential proteinogenic AA. The transcript abundance of the the His/peptide transporter solute carrier 15A3 (SLC15A3) was significantly increased at day 18 of pregnancy in vivo, and stimulated by interferon-tau predominantly in stroma cells in vitro. Apart from SLC15A3, the increasing AA content in the uterine fluid was not accompanied by an increased transcript abundance of specific amino acid transport systems in the endometrium of pregnant animals. This may point towards a distinct relevance for an adjusted blood flow, allowing an adequate supply of nutrients. Our results show an increased concentration of AA most likely to optimally provide the elongating pre-attachment conceptus with nutrients during this eminent phase. By any means, the presented data provide the basis to further question if local deprivation in case of imbalances in nutrient supply occurs with possible negative consequences on early embryo development.
Maternal metabolism and the influence on fertility: a study of the follicular microenvironment

Growing evidence suggests that maternal metabolic disorders have an impact on the oocyte’s developmental capacity, thereby possibly affecting the early embryonic stages of development and the health of the offspring. The hypothesis of this study was firstly that an altered maternal metabolism may be reflected in the composition of the follicular fluid (FF) and secondly that an alteration of this microenvironment may affect oocyte quality. Follicular fluid and blood samples from 113 women undergoing assisted reproduction were collected and analyzed for urea, total protein, albumin, cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, non-esterified fatty acids (NEFAs), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), glucose, lactate, C-reactive protein (CRP), insulin and insulin like growth factor 1 (IGF-1). Significant correlations between blood and FF values could be found ($P < 0.05$) for urea, total protein, albumin, cholesterol, HDL-cholesterol, triglycerides, NEFAs, ApoA1, glucose, lactate, CRP, insulin and IGF-1. Mean FF concentrations for all parameters, with the exception of urea and lactate, were lower than the blood concentrations and were not affected by body mass index (BMI). Less oocytes were aspirated from obese women compared to non-obese patients. Correlations between individual biochemical parameters and oocyte parameters were weak. These results indicate that the biochemical composition of the blood is at least partly reflected in the composition of the FF, thereby possibly affecting oocyte quality. Additional and extensive studies should further illuminate correlations between blood and FF biochemical compositions and the complex interaction between the FF and the oocyte.
Dynamic Changes of Messenger RNA Profiles of Equine Endometrium during the Oestrous Cycle

The equine endometrium exhibits characteristic morphological and functional changes during the oestrous cycle, which are mainly regulated by progesterone (P4), oestrogens and oxytocin. So far there is no published systematic study of gene expression changes in equine endometrium during the oestrous cycle. Thus, we studied the response of the endometrium to this changing hormonal environment at the transcriptome level using the Agilent Equine microarray. Endometrial biopsy samples were recovered from five mares (Bavarian Warmblood) on five points of time during the cycle, namely day 0 (oestrus shortly after ovulation), day 3 (metoestrus), day 8 (dioestrus), day 12 (late dioestrus), and day 16 (early oestrus). Biopsy samples were divided and subjected to isolation of RNA for transcriptome analysis and to the analysis of tissue composition (quantitative stereology). In addition, blood samples were collected to determine serum progesterone levels for every biopsy sample. Statistical analysis of microarray data revealed more than 10,000 differential probes (SAM, multiclass, FDR 1%) corresponding to approx. 5,750 different genes. To obtain groups of genes with similar gene expression profiles a clustering of the transcripts based on their expression changes during the cycle was performed. This analysis revealed 8 major expression profiles: 1) mRNAs with highest levels in early oestrus (day 16); 2) highest levels at day 16 and day 0; 3) highest levels from day 0 to day 3; 4) highest levels at day 3; 5) highest levels from day 3 to day 8; 6) highest levels at day 8; 7) highest levels from day 8 to day 12; and 8) highest levels at day 12. Detailed bioinformatics analysis of the single gene clusters is in progress. First conclusions are that i) a huge number of genes shows different expression levels in equine endometrium during the oestrous cycle and ii) a variety of different profiles was found indicating highly dynamic changes in the endometrium also within the classical phases of the oestrous cycle.

This study was supported by the German Ministry for Education and Research (BMBF, FUGATO-plus, COMPENDIUM), the German Research Foundation (DFG, Research Training Unit 1029 Functional Genome Research in Veterinary Medicine), and the ReproZentrum of the LMU Munich.
Transcriptomal profiling in porcine oviduct is regulated by the oestrus cycle

The oviduct is a part of the female reproductive tract involved in relevant processes such as gamete maturation, sperm capacitation, fertilization and early embryo development. It is well known that oviductal physiology undergoes cyclical changes under the influence of steroid hormones. However, the molecular mechanisms underlying oviductal regulation are not fully understood. The aim of this study was to identify the gene expression profile in the porcine oviduct at different cycle stages. Specifically, we aimed to know the changes in the ampullar-isthmic junction, since our focus of interest was the environment where the fertilization takes place.

Oviducts were classified into prepuber (containing only follicles 1-2 mm in diameter), preovulatory (containing 6-12 follicles 8-12 mm in diameter), postovulatory (containing 6-12 hemorrhagic corpora) and luteal phase (containing 6-12 corpora lutea). Total RNA was extracted using the “TRIZOL method” and hybridization was performed onto Porcine Gene Expression Microarray (Agilent) containing more than 44,000 probes. Microarray data were analyzed using bioinformatics methods such as principal component analysis (PCA) and selection of differentially expressed genes.

The results of PCA analysis showed a clearly different gene expression profile among the different phases. The differentially expressed genes analysis indicated that a total of 148, 187 and 353 genes were up-regulated in prepuber, preovulatory and luteal phases respectively; however, 137, 108 and 280 genes were down-regulated in prepuber, preovulatory and luteal phases respectively compared to the postovulatory phase.

In conclusion, the results of this study demonstrate that gene expression profiling of the ampullar-isthmic junction of porcine oviduct is clearly regulated to the oestrus cycle. These data provide new insights into the biological mechanisms involved during the fertilization. This study was supported by Fundación Séneca (CARM11996/PI/09).
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