4th International Rabbit Biotechnology Meeting

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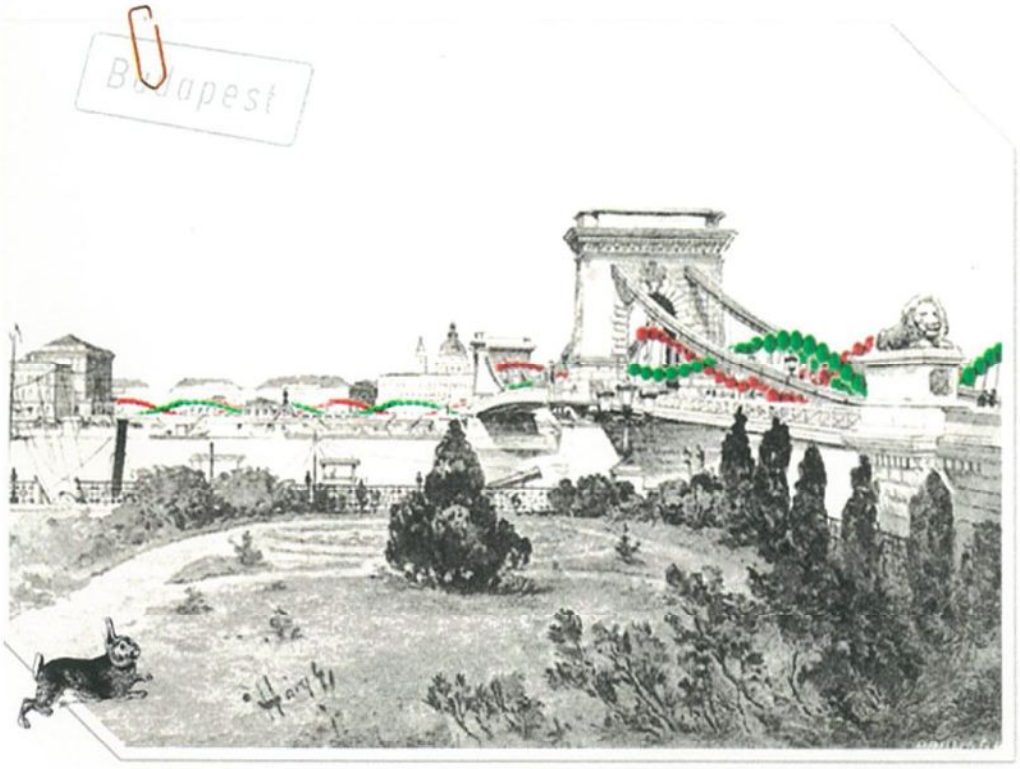

# PROGRAM

| 30th June 2011 | | |
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| **Opening remarks** | | |
| 9:30 | **Dudits Dénes** | Vice President of Hungarian Academy of Sciences |
| **Session 1**: Rabbit genom, selection, conservation<br>Chairman: Peter Chrenek, Jose Vicente | | |
| 10:00 | **Veronique Duranthon** | Rabbit embryo as a model for genome reprogramming over preimplantation development |
| 10:30 | **Peter Chrenek** | Quality of rabbit vitrified/thawed transgenic embryos |
| 11:00 | **Coffee break, posters** | |
| 11:30 | **Csaba Pribenszky** | High pressure treatment in rabbit semen preservation |
| 12:00 | **Emmanuelle Koch** | Fetal programming analysis in the rabbit model |
| | **Lunch** | Pálinka Bistrot |
| **Session 2**: Rabbit models to study human diseases<br>Chairman: Michael Brunner, Kazuhito Jamaguchi | | |
| 14:00 | **Anne Navarette-Santos** | Rabbit as a model of embryonal development in type I diabetes women |
| 14:30 | **Koike Tomonari** | Influence of human apoAII gene on lipoprotein metabolism and atherosclerosis in transgenic rabbits |
| 15:00 | **Katja Odening** | The transgenic rabbit as a model to study the mechanisms and treatment of inborn arrhythmias |
| 15:30 | **Kazutoshi Nishijima** | Assessment of energy expenditure in rabbit with doubly-labeled water method |
| 18:00 | **Danube Corso-Budapest sightseeing cruise** | Departure from Budapest, Vigadó tér, Landing Stage, Pier 5 or 5/A |

| 1th July 2011 | | |
|---|---|---|
| **Session 3**: Novel findings in rabbit ES and iPS cell establisment<br>Chairman: Pierre Savatier, András Dinnyés | | |
| 9:00 | **Arata Honda** | Generation of Induced Pluripotent Stem Cells in Rabbits: Potential experimental models for human regenerative medicine |
| 9:30 | **Pierre Savatier** | Naive and primed pluripotent stem cells in the rabbit |
| 10:00 | **András Dinnyés** | Progress and bottlenecks towards generating germline chimera forming induced pluripotent stem cells in rabbit |
| 10:30 | **Coffee break, posters** | |
| 11:30 | **Elen Gócza** | Pluripotency markers in early rabbit development and embryonic stem cells |
| 12:00 | **Pouneh Maragechi** | Stem cell specific miRNA expression in rabbit embryos and embrionic stem cells |
| | **Lunch** | Pálinka Bistrot |
| **Session 4**: Second generation methods in rabbit transgenesis<br>Chairman: Valeri Zakhartchenko, Zsuzsanna Bősze | | |
| 14:00 | **Rainer Ebel** | The zink finger nuclease technology and its perspectives in rabbit transgenesis |
| 14:30 | **Valeri Zakhartchenko** | Pluripotent and multipotent stem cells for cell-mediated transgenesis in rabbits: Chimeric and nuclear transfer animals |
| 15:00 | **Zsuzsanna Bősze** | The IgG binding Fc receptor transgenic rabbits created through BAC transgenesis |
| 15:30 | **László Hiripi** | Sleeping Beauty mediated transgenesis in rabbit |
| 16:00 | **Zsuzsanna Polgár** | Nuclear transfer technology in rabbits |

## RABBIT EMBRYO AS A MODEL FOR GENOME REPROGRAMMING DURING PREIMPLANTATION DEVELOPMENT

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Early mammalian development is very complex on a molecular point of view. The challenge for the embryo is to manage the maternal-embryo transition: that is to reprogram the highly differentiated and transcriptionally silent gametic genomes into a unique totipotent (able to give rise to a whole individual) and transcriptionally active embryonic one. Such a function has consequences well beyond early development, and concerns in fact full term development and adulthood. It supposes the involvement of maternal information into huge epigenetic modifications of the gamete-inherited and newly formed embryonic genomes. Rabbit appears as a pertinent model for these early epigenetic modifications because the kinetics of its embryonic genome activation is more representative of most mammals than the mouse one. It is also an interesting model for the effects of environment on these epigenetic changes because in vivo developed rabbit embryo are easily available, and in vitro preimplantation development is efficient. We have developed an analysis of the epigenetic changes that occur after fertilization in this species. Data will be presented first concerning the dynamics of DNA methylation levels rabbit genomes after fertilization and during preimplantation development. In the maternal pronucleus, the methylation level remained constant during the first cell cycle. Conversely the paternal pronucleus is submitted to partial but active demethylation. During cleavages, embryonic genome is progressively demethylated until the morula stage in vivo. These events are affected by in vitro environment. Recently, 5-hydroxymethylcytosine (5hmC), derived from enzymatic oxidation of 5-methylcytosine, has been proposed as a potential intermediate in active DNA demethylation in mouse embryos. We performed experiments to check whether 5hmC is present into one-cell, two-cell, and later cleavage-stage embryos and whether this could be related the DNA demethylation process we observed at these stages. Then in order to evaluate how chromatin architecture affects and even regulates gene expression during embryonic genome activation, we studied the distribution of pericentric/centric heterochromatin, a peculiar region within nuclei, known to form higher-order chromatin structures. After fertilization, we observed that these sequences are first dispersed within the nuclei of early stage embryos and that non-randomly distributed foci progressively appeared in embryonic nuclei by the 8 cell stage only, suggesting a relationship with the onset of transcription. Lastly we analyzed X chromosome inactivation in rabbit embryos which appears to be regulated in a very different way compared to the mouse model. Especially, the expression of the Xist non coding transcript responsible for this inactivation is biallelic in rabbit as in human and opposite to the mouse where it is submitted to imprinting. Moreover, the relationship between X inactivation and establishment of pluripotent cells in rabbit is very different from the most studied mouse model.

## EFFECT OF MICROINJECTION AND VITRIFICATION ON THE RABBIT TRANSGENIC EMBRYO QUALITY

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The aim of our study was to investigate the influence of vitrification on the developmental rate, embryo cell number, number of blastomeres in ICM area (differential staining), apoptotic index (TUNEL), embryo diameter and ultrastructural morphometry of embryonal organelles (transmission electron microscopy, TEM) of transgenic rabbit embryos carrying either endogenous (human factor VII, hFVIII) or exogenous (EGFP) genes. The EGFP-positive embryos were produced by the microinjection of foreign gene into the pronucleus. The transgenic hFVIII gene-positive embryos were produced by mating of homozygous transgenic rabbits and flushing at the 1-cell stage. Vitrification of embryos was done in the medium contained 40% ethylene glycol, 18% ficoll 70 and 0.3 M sucrose.
Hatching blastocyst rate of vitrified/devitrified transgenic embryos (68.00% and 69.00 %) was differed significantly ($p<0.001$) from those in control (100.00 % resp.). Significant difference ($p<0.05$) in the embryo cell count between control (117.00 ± 36.00) and vitrified (141.00 ± 34.80) hFVIII-positive embryos was found. The higher proportion of ICM cells (32.00 %) and the embryo diameter (130.85 ± 10.90) were found in the control group compared to transgenic ones. Apoptotic index was significantly higher ($p<0.01$) in the control group (2.50%) and vitrified the EGFP-positive embryos (2.90%) compared to vitrified, hFVIII-positive embryos (0.70%). The vitrified/devitrified EGFP-positive and EGFP-negative embryos exhibited slight accumulation of cellular debris and lipid droplets compared to control embryos. More severe alterations were detected in membrane structures of the treated embryos, mostly in the cytoplasmic envelope, trophoblastic microvilli, junctional contacts and mitochondria. Our results demonstrate that microinjection and vitrification given in combination, rather than alone, significantly decrease ($p <0.001$) quality of rabbit transgenic embryos.

This work was supported from the grant of Slovak Research and Development Agency: APVV LPP-0119-09.

## SUBLETHAL STRESS INDUCED TOLERANCE IN GAMETES AND EMBRYOS AND ITS UTILIZATION IN ASSISTED REPRODUCTIVE TECHNOLOGIES

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Sublethal stress induced response with a temporary increase in a general, relatively non-specific resistance to various further stresses has been observed in almost all levels of life, from bacteria to multicellular organisms including humans. The first reaction for stress is the 'fight-or-flight' or the 'acute stress response', described by Cannon (1915). This response was later recognized as the first stage of a general adaptation syndrome that regulates stress responses among vertebrates and other organisms (Selye 1936). On the cellular level, the reaction incorporates sensing, assessing and then counteracting stress-induced damage, consequently temporarily increasing tolerance to such damage. Proteins that are involved in the key functions of the stress response are conserved in all cells and participate in cellular functions including protein, DNA, chromatin and cytoskeleton stabilization and repair, cell cycle control, redox regulation, energy metabolism, fatty acid-, lipid metabolism and elimination of damaged proteins. However, if the stress level is over the limit of tolerance, programmed cell death (apoptosis) or necrosis occurs (Hansen 2007).
Experiments with the application of controlled sublethal stress to precondition cells have shown significant increase in cell function in several biotechnical procedures. As for the stressor high hydrostatic pressure (HP) was used because of its unique physical features like: 1) it acts immediately and equally at every point of the sample without gradient effect; and 2) it can be applied with the highest precision and safety. Studies demonstrated that by utilizing a well-defined and properly applied HP stress treatment to reproductive cells before insemination, cryopreservation, enucleation, or in vitro maturation/culture, post-thawing survival, continued in vitro development, blastocyst ratio, and cell number, pregnancy, and/or farrowing rates improved considerably compared to untreated controls. The optimal stress treatment may be different according to species, cell types, and developmental stages. Although the biological mechanism is still unclear, several processes including alteration of the protein profile and biochemical balance of cells, production of shock proteins, and temporary arrest of the cell cycle might explain the observations. The birth of healthy piglets, calves, mice from treated fresh or frozen-thawed semen or from the transfer of embryos reconstructed from treated recipient eggs for SCNT demonstrates the in vivo safety of the procedure. In mice, two-generation studies have demonstrated that offspring from HP treated embryos have normal reproductive function. Results and protocols were reviewed in Pribenszky et al. (2010): Cells under pressure: how sublethal hydrostatic pressure stress treatment increases gametes' and embryos' performance? (Reproduction, Fertility and Development 23, 48–55.). Since rabbit sperm has not been examined earlier, we optimized this treatment for wild type and transgenic rabbit semen samples.
The new principle, i.e., to improve the stress tolerance by a defined sublethal stress may outline a completely new strategy in assisted reproductive technologies with remarkable theoretical and practical consequences.

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## DIET-INDUCED ADIPOSITY LEADS TO EARLY MAMMARY GLAND DEVELOPMENT DURING PREGNANCY IN THE RABBIT

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Epidemiological and animal studies have suggested that alterations to hormonal and metabolic environments during puberty can induce adverse effects on both lactation and mammary tumorigenesis (de Assis *et al.*, 2006). Obese women have an increased risk of failing to initiate successful breastfeeding and can experience a premature cessation of lactation (Riva *et al.*, 1999; Rasmussen *et al.*, 2004). The effects of obesity on lactogenesis were highlighted during early studies in the rat, where this pathological condition was shown to reduce the chances of a successful outcome regarding pregnancy and lactation (Rolls *et al.*, 1984). More recent studies have suggested that impaired mammary gland development and lactogenesis are possible causes of lactation failures and the high mortality rates observed among the pups of obese dams. In cattle, evidence concerning the relationship between growth rate, mammary growth and milk yield has led to the conclusion that an increased growth rate due to high feeding levels before the onset of puberty may impair subsequent pubertal mammary development and milk potential (Sejrsen, *et al.*, 1997). Moreover, high energy feeding decreases mammary epithelial cell proliferation in areas of active ductal expansion at puberty (Davis Rinker *et al.*, 2008).

We have further investigated the impact of obesity on mammary gland development. A rabbit model of diet-induced obesity was developed, by feeding female rabbits with a high fat/high sugar (OB) diet (+276% fat and +269% sugar when compared with the control (C) diet), from 8 weeks of age until mid-pregnancy (day 14 of pregnancy, around the age of 24 weeks). Body weight gain between 21 weeks of age and day 14 of pregnancy was significantly higher (+10%) in OB animals, and was mainly associated with the development of more adipose tissue.

Mammary gland morphology was altered in the OB group. Alveolar structures had invaded the entire fat pad, whereas they were more clustered in the control group. Moreover, on day 14 of pregnancy, the mammary ducts were made up of a cell monolayer, were dilated and filled with dense products. Numerous microvillosities were located in the apical region of mammary epithelial cells. Electron microscopy analysis revealed that casein micelles were present in the lumen of alveolae. Immunohistochemical studies of HF mammary tissue revealed a more abundant accumulation of the major rabbit milk proteins, alphaS1-casein, kappa-casein and Whey Acidic Protein (WAP), in both the alveolar lumina and secretory ducts. Milk protein synthesis in mammary tissue extracts was quantified by Western blot analyses. A clear accumulation of milk proteins was observed in OB animals but was almost undetectable in the controls. As revealed by BodiPy staining, lipids had also accumulated in the lumens and ducts. Taken together, these analyses revealed an early secretory phenotype in the OB group.

These results show that diet-induced obesity, beginning before puberty, alters mammary gland development at mid-pregnancy in the rabbit. On day 14, mammary tissue in obese animals displayed a morphological aspect and functional profile similar to that normally observed at the end of pregnancy. Preliminary data indicated that these abnormalities affecting mammary development are not associated with agalactia at term, but that milk quality is affected and may influence the predisposition of offspring to obesity.

## RABBIT AS A MODEL TO STUDY EMBRYONIC DEVELOPMENT IN TYPE 1 DIABETIC WOMEN

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Worldwide the incidence of diabetes mellitus is increasing drastically. This metabolic disease is characterised by high levels of blood glucose resulting from defects in insulin production, insulin action, or both. Type 1 or insulin-dependent diabetes mellitus (IDDM) has been found to negatively affect pregnancy by causing miscarriage and poorer prenatal outcomes in humans. Prior to implantation embryo development is regulated by maternal and embryonic factors, interacting at the cellular level without the placental barrier. The production and release of maternal factors like insulin, insulin like growths factors (IGFs) and glucose play a major role in maintaining cell viability and survival of the embryo. Insulin and IGFs regulate metabolic and mitogenic effects during early embryo development.

We used the rabbit as a model for diabetes mellitus in pregnancy. This model allows studying the influence of type 1 diabetes on maternal reproductive organs and their endocrine regulation on one side and on blastocyst development and molecular adaptation on the other. The gastrulating rabbit blastocyst is the largest mammalian preimplantation embryo and shows higher similarities to the human than other laboratory animals. The embryo-maternal interactions and early embryo development are well studied in the rabbit and provide therefore useful morphological and molecular tools.

In female rabbits diabetes was experimentally induced by alloxan. Alloxan treatment results in a complete loss of endogenous insulin (hypoinsulinaemia) followed by elevated blood glucose levels about 14mmol/L (hyperglycaemia) about 2 days after treatment. As an effect of the maternal diabetes, the number of blastocysts per female was reduced (by 40%) and blastocyst development itself was delayed as shown by retarded gastrulation. The uterine secretions of diabetic females contain less insulin, a 3fold higher glucose concentration and increased amounts of aliphatic branched chain amino acids (BCAAs), demonstrating that embryos usually grow up in hypoinsulinaemic and hyperglycaemic conditions. Due to these changes in uterine environment the rabbit embryo is constrained to metabolic adjustments and deceleration of growth. Compensatory, the expression of uterine and embryonic IGFs and adiponectin is increased, indicating that the embryo compensates for insulin deficiency. We suppose that the molecular mechanisms of embryonic metabolic and developmental adaption encompass the insulin-IGF-adiponectin signalling network with the key transcription factor CREB. Blastocysts from diabetic females showed a significantly reduced CREB phosphorylation and an increased adiponectin expression, compensating for the lack of maternal insulin in order to maintain the embryonic glucose uptake. Our hypothesis is supported by findings in in vitro cultured blastocysts from healthy rabbits as well as from diabetic rabbits that showed a stimulatory function of adiponectin on embryonic glucose uptake.

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## APOLIPOPROTEIN-AII PLAYS AN IMPORTANT ROLE IN LIPID METABOLISM AND ATHEROSCLEROSIS

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Apolipoprotein AII (apoAII) is the second major apolipoprotein in high-density lipoprotein (HDL). However, the physiological functions of apoAII in lipoprotein metabolism have not been fully elucidated. In the current study, we generated human apoAII transgenic (Tg) rabbits, a species that normally does not have an endogenous apoAII gene. Plasma levels of human apoAII in Tg rabbits were ~30 mg/dl, similar to the plasma levels in healthy humans. The expression of human apoAII in Tg rabbits resulted in increased levels of plasma triglycerides, total cholesterol, and phospholipids accompanied by a marked reduction in HDL-cholesterol levels compared with non-Tg littermates. Analysis of lipoprotein fractions showed that hyperlipidemia exhibited by Tg rabbits was caused by elevated levels of very-low-density lipoproteins (VLDL) and intermediate-density lipoproteins. Furthermore, postheparin lipoprotein lipase activity significantly decreased in Tg rabbits compared with non-Tg rabbits. These results indicate that apoAII plays an important role in both VLDL and HDL metabolism, possibly through the inhibition of lipoprotein lipase activity. ApoAII Tg rabbits may become a new model for the study of human familial combined hyperlipidemia. In the second experiment, we fed Tg and non-Tg rabbits with a cholesterol diet for 16 weeks and found that although Tg rabbits exhibited higher levels of plasma lipids than non-Tg rabbits but the extent of aortic atherosclerosis was not changed. We are now investigating the possible molecular mechanisms underlying apoAII-mediated atheroprotective effects.

## TRANSGENIC LONG-QT SYNDROME TYPE 1 AND 2 RABBITS MIMIC THE HUMAN PHENOTYPE AND CAN HELP TO ELUCIDATE MECHANISMS OF ARRHYTHMOGENESIS IN LONG-QT SYNDROME

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The long-QT syndrome (LQTS) is an inherited arrhythmogenic disease characterized by an impaired cardiac repolarization due to loss-of-function mutations in cardiac $K^+$ channels. Patients have prolonged QT intervals and are prone to develop polymorphic ventricular tachycardia (pVT) and sudden cardiac death (SCD). Mouse models of LQTS fail to mimic the human phenotype since different $K^+$ currents determine cardiac repolarization. Rabbits, however, have similar repolarizing $K^+$ currents as humans. We therefore reasoned that studying the impact of mutations of human ion channels in rabbits would provide important additional mechanistic insights into arrhythmogenesis in LQTS. We have thus generated transgenic rabbits overexpressing dominant-negative pore mutants of the human KvLQT1 (KvLQT1-Y315S, LQT1, loss of IKs) or HERG channels (HERG-G628S, LQT2, loss of IKr) selectively in the heart. These rabbits mimic the human phenotype with QT prolongation, spontaneous pVTs and SCD – as well as similar sex differences as observed in human patients with longer QTc intervals in females and a high arrhythmogenic risk during the postpartum period.

We have used these transgenic LQT1 and LQT2 rabbits to investigate mechanisms of arrhythmogenesis and to elucidate how the arrhythmogenic phenotype can be manipulated by ion channel-blocking or -activating agents and different sex hormones using in vivo telemetric ECG recording, in vivo electrophysiological studies, ex vivo optical mapping, ex vivo monophasic action potential measurements, cellular patch clamping, and biochemical methods.

We could identify a spatially heterogeneous prolongation of action potential duration (APD) – e.g. a pronounced dispersion of repolarization – as a main mechanism of arrhythmogenesis and observed that several drugs or hormones exert their pro- or anti-arrhythmic effects by modifying this dispersion of repolarization.

Conclusion: Transgenic LQT1 and LQT2 rabbits mimic the human LQTS phenotype and could serve as an in vivo model in which to assess pro- and anti-arrhythmic effects of drugs and the underlying mechanisms of arrhythmogenesis in LQTS.

## ASSESSMENT OF ENERGY EXPENDITURE IN RABBIT WITH DOUBLY-LABELED WATER METHOD

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Rabbit can become a relevant animal model to research human metabolic syndrome and obesity, since rabbit has several characteristics similar to human in metabolism. Analysis of the energy metabolism is essential to clarify mechanisms of obesity. Doubly-labeled water (DLW; $^{2}H_2O$ and $H_2{}^{18}O$) method has been widely accepted as a gold standard method to assess energy expenditure and body composition in wild animals and free-living or hospitalized humans without any restraint. However, to our knowledge, there is no previous study which applied the DLW method into rabbit studies. Thus, we investigated the application of DLW method to develop an optimum assessment of energy expenditure in rabbits. DLW was injected (0.92g/kg body weight, i.v.) into Japanese White rabbits (male, 26 weeks old). Blood samples were collected before and 3-4 hours and 4, 7, 8, 10, 14, and 15 days after the injection. The enrichment of $^{2}H$ and $^{18}O$ in plasma samples were analyzed with an isotope ratio mass spectrometry. Carbon dioxide production rate was calculated by measuring the difference between the elimination rates of $^{2}H$ and $^{18}O$, and converted into the energy expenditure. Body composition was also estimated by isotope dilution space of $^{2}H$ and $^{18}O$. The contribution ratio ($R^2$) of the linear regression, between natural logarithm transformed enrichments and days after DLW injection, was 0.998 ± 0.002 (mean ± SD) for $^{2}H$ and 0.999 ± 0.001 for $^{18}O$. The energy expenditure was 340.1 ± 9.3 kcal/day. Total body water and body fat percentage were estimated to be 2.3 ± 0.1kg and 16.0 ± 1.4%. The present results showed that usage of the current dose (0.92g/kg) and 15 days of experimental period can be used to precisely access the energy metabolism in rabbits.

## GENERATION AND QUALITY ASSESSMENT OF PLURIPOTENT STEM CELLS IN RABBITS

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Human induced pluripotent stem (iPS) cells have the potential to establish a new field of promising regenerative medicine. Therefore, there are increasing demands on suitable as well as easily accessible animal models for clinical trials on iPS cells. Here, we report the establishment of rabbit iPS cells, the first human-like iPS cells generated from an easily accessible small laboratory animal species. Using lentiviral vectors, four human reprogramming genes (c-MYC, KLF4, SOX2 and OCT3/4) were introduced successfully into adult rabbit liver and stomach cells. The resulting rabbit iPS cells closely resembled human iPS cells; they formed flattened colonies with sharp edges and proliferated indefinitely in the presence of bFGF. They expressed the endogenous pluripotency markers c-MYC, KLF4, SOX2, OCT3/4 and NANOG, while the introduced human genes were completely silenced. Using in vitro differentiating conditions, rabbit iPS cells readily differentiated into ectoderm, mesoderm and endoderm. They also formed teratomas containing a variety of tissues of all three germ layers in immunodeficient mice. Thus, we conclude that rabbit somatic cells can be reprogrammed successfully to generate stable iPS cells, which are very similar to their embryonic stem (ES) cell counterparts. On the other hand, although the global gene expression profiles became closer to those of the rabbit ES cells with cell passage proceeded, a slight but rigid difference between the two types of rabbit pluripotent stem cells was revealed. Recently, we are establishing the systems for assessing the quality of the rabbit ES/iPS cell lines using the in vitro differentiation ability as an indicator. Actually, several rabbit iPS cell lines showed limited ability in differentiation to neural lineage. Next our objective is to improve rabbit ES/iPS cell lines to appropriate quality for applying translational research model and for producing genetically engineered rabbits. The rabbit model should enable us to compare iPS cells and ES cells under the same standardized conditions in evaluating their ultimate feasibility for cell-based regenerative medicine in humans.

## NAIVE AND PRIMED PLURIPOTENT STEM CELLS IN THE RABBIT

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Recent advances in stem cell research have revealed a puzzling diversity of pluripotent stem cells in mammals. In mice, pluripotent stem cell lines can be obtained, (i) from the early epiblast of the blastocyst in the form of Embryonic Stem Cells (ESC) by exploiting LIF signaling to self-renew in the so-called naive state of pluripotency, and (ii) from the late epiblast of the pre-gastrulation embryo in the form of Epiblast Stem Cells (EpiSC) by exploiting FGF2 signaling to self-renew in the so-called primed state of pluripotency. It is usually assumed that only the pluripotent stem cells in the naive state of pluripotency can colonize the embryo and participate in fetal development. In human and non-human primates, ESC lines exhibit most, but not all, the features of mouse EpiSCs, albeit they are derived from pre-implantation embryos. The situation observed in the rabbit seems to add some further complexity to the present situation. First, rabbit ESCs can be generated from pre-implantation embryos by exploiting either FGF2 or LIF-signaling to allow them to self-renew in the undifferentiated state. Second, both FGF2- and LIF-dependent ESC lines exhibit a mosaic population of SSEA-1 and SSEA-4 cell surface antigens, whereas these antigens are expressed in mutually exclusive patterns in mouse (SSEA-1) and human (SSEA-4). Third, contrary to both mouse and human ESCs, rabbit ESCs fail to activate the ICM-specific distal enhancer of Oct4. Fourth, rabbit ESCs exhibit a dramatically longer G1 phase of the cell cycle, as compared to mouse and human ESCs. Strikingly, some FGF2-dependent rabbit ESC lines exhibit a DNA damage checkpoint in G1 like somatic cells, whereas such a checkpoint is absent from all mouse, human, and monkey ESC lines studied so far. Together, these results indicate that rabbit ESCs are far more heterogeneous than their mouse and primate counterparts regarding their molecular and growth characteristics.

In a given species, the induced pluripotent stem cells (iPSC) exhibit virtually all the features of the embryonic stem cells observed in that species. Again, rabbits seem to not strictly follow such rule. Contrary to ESCs, FGF2-dependent iPSCs can be regularly passaged by gentle trypsinisation after dissociation into a single cell suspension, they activate the ICM-specific distal enhancer of Oct4, they exhibit a significantly shorter G1 phase, and they all lack the DNA damage checkpoint in G1. Most importantly, as opposed to ESCs, iPSCs seem capable of colonizing the rabbit pre-implantation embryo, albeit with low efficiency. Together, these results suggest that rabbit iPSCs display growth and functional characteristics that place them closer to the naive state of pluripotency.

## PROGRESS AND BOTTLENECKS TOWARDS GENERATING GERMLINE CHIMERA FORMING INDUCED PLURIPOTENT STEM CELLS IN RABBIT

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[2] *Biotalentum Ltd, Gödöllő, Hungary*

Recently discovered induced pluripotent stem cells (iPSCs) technology which allows direct reprogramming of somatic cells to a pluripotent state, substantiated only four years prior, is one of the most rapidly developing areas of stem cell research. iPSCs are very similar to embryonic stem cells (ESCs) with respect, that they have the capacity to divide indefinitely and to differentiate to all the somatic tissues and they can be genetically manipulated in vitro. iPSCs are also a promising tool for gene-function studies and generation of patient-derived pluripotent cells applicable to disease modeling, drug screening, toxicology tests and, ultimately, to autologous cell-based therapies. Importantly, they overcome ethical concerns about creating and sacrificing human embryos.

Since 2006, when Takahashi and Yamanaka first reported the generation of induced pluripotent stem cells from murine fibroblasts via retroviral transduction of a defined set of transcription factors, various new methods have been developed to refine and improve reprogramming technology in mouse, human, pig and other species. Many teams have worked on developing non-retroviral gene delivery systems to produce iPS cells. However, the process of slow (several weeks) reprogramming in certain species is rather inefficient and often incomplete, therefore needs further investigations.

Our team has successfully created the first mouse iPSC lines and the first human iPSC cells in Hungary in 2009/2010, and similar strategy can be employed to generate rabbit iPS cells as well. Given similar cocktails of transcription factors (e.g. oct4, sox2, c-myc, klf4), we have developed three systems in order to reprogram somatic cells. These are: (1) polycistronic, deletable lentiviral (LeV); (2) non-viral sleeping beauty transposon (SB) and (3) by recombinant protein (ReP) mediated technology. The advantage of these systems that the reprogramming casettes can be removed therefore the end product is insertion free. Thus, these systems can significantly reduce the risk of disruption of gene functions and malignant transformations and currently being applied in rabbit.

Cloning and characterisation of the panel of pluripotency genes (e.g.: oct4, sox2, c-myc, klf4) in rabbit is also in progress in our laboratory. By using rabbit specific pluripotency genes we might be also able to reprogram somatic cells and generate iPSCs more efficiently. Future validation of rabbit pluripotent stem cells would benefit greatly from a reliable panel of molecular markers specific to pluripotent cells of the developing rabbit embryo. Our further goal is to produce chimeric rabbit embryos with iPS cells injected into host embryos.

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## PLURIPOTENCY MARKERS IN EARLY RABBIT DEVELOPMENT AND EMBRYONIC STEM CELLS

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The aim of our work was to analyze the expression pattern of embryonic stage specific factors and microRNAs at preimplantation stages of rabbit embryonic development. The molecular regulation of early rabbit embryonic development is not fully understood. Unlike human and mouse blastocysts, the ungulate blastocyst does not implant immediately after hatching. Around the third day of development, blastocyst formation begins and the embryo reaches the uterine horn. In the following three days, the embryo hatches and expands (6–7 d.p.c.). The polar trophoblast (the Rauber's layer of embryos) is an intact epithelium, overlying the epiblast of the embryo disk, exposing the cells of the embryonic disc to the maternal milieu (Hoffman et al., 1998).

There are well-known genes that direct pluripotency in the mouse, such as POU5F1 (Oct4), NANOG, but their role in the rabbit is unclear. The Oct4 transcription factor is considered a pluripotency factor; however in rabbit, like in bovine, Oct4 is also expressed in the trophoblast cells (Berg et al., 2011).

Using culture medium supplemented with two inhibitors (2i: Mek inhibitor PD032590 (PD) and Gsk3 inhibitor CHIR99021 (CH)), embryonic stem cell (ESC) lines could be derived efficiently from all mouse and rat strains tested to date (Nichols et al., 2009). We cultured the rabbit embryos from eight-cell-stage (1.5 d.p.c.) in RDH, or RDH+CH+PD culture medium until blastocyst stage (4.5 d.p.c.). The expression level of epiblast specific Oct4 and Nanog, the trophectoderm specific, Cdx2 and hypoblast specific Gata4 and Gata6 were examined in 4.5 d.p.c. rabbit embryos. The expression level of Oct4 and Nanog was significantly higher in RDH+PD+CH culture medium, but there was no significant difference in CDX2, GATA4, GATA6 expression compared to embryos cultured in RDH medium. According to the data of the SOLiD™ System sequencing we could identify rabbit embryo and embryonic stem cell specific miRNAs. The next step will be an analysis of the expression pattern of these miRNAs in cultured rabbit embryos. We hope that we can maintain the pluripotency of the epiblast-derived stem cells in RDH+PD+CH containing medium during long-term culture.

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## STEM CELL - SPECIFIC miRNA EXPRESSION IN RABBIT EMBRYOS AND EMBRYONIC STEM CELLS

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MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, small non-coding RNA molecules of about 22-nt long, which regulate gene expression through translational repression or cleavage of the target mRNAs by binding to complementary sites within 3' UTR of their targets. These regulatory RNAs play important role in multiple biological process including embryonic development and the maintenance of stem cell self-renewal and pluripotency. Embryonic stem (ES) cell-specific miRNAs are abundant in the pluripotent stem cells, but rapidly down regulated during differentiation. It is well known that ES-specific miRNA promoters are regulated by pluripotency-associated transcription factors Oct4, Sox2 and Nanog (Bar et al., 2008; Barroso-delJesus et al., 2008). The best studied ES cell-specific miRNAs are: miR302-367, miR371-373 and miR512-519 clusters in human ESCs; and miR290-295 cluster in mice.

Initially, our experiments were focused on the functional characterization of miR290-295 cluster and demonstrated that the miR290-295 cluster regulates cell cycle at multiple points and multiple targets promoting self-renewal and pluripotency of mouse ESCs, thereby representing a master regulator in the maintenance of pluripotency (Lichner et al., 2010).

The SOLiD™ System Small RNA Analysis allowed us to identify the rabbit embryonic stem cell specific ones, among the numerous rabbit miRNAs. Subsequently, we analyzed the expression of miR290-295 and miR302-367 clusters during rabbit early embryonic development and ES-like cells maintenance. Preliminary data shows that expression of miR290-295 cluster is higher in early embryonic stages. Contrary, the members of the miR302-367 clusters had higher expression in the later embryonic stages and early passages of ES-like cells.

Our experiments are the first step toward characterization of the stem-cell specific miRNAs, which are essential and specific for the earliest stages of rabbit embryo development and ESC self-renewal and proliferation

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## TARGETED GENOME MODIFICATION IN RATS, MICE AND RABBITS USING ZINC FINGER NUCLEASES

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The mouse has long been an important experiment model for scientific research, largely due to the ability to create genetic changes in mouse embryonic stem cells in order to model human disease. Other animal models hold great potential, such as the rat for studies of toxicology and neurodegenerative disorders or the rabbit for cardiovascular and ocular disease. Unlike the situation with the mouse, efficient gene targeting in the rat and rabbit has remained a near impossibility, with researchers forced to rely on random methods of mutagenesis, such as ENU and transposon-based manipulation. The zinc finger nuclease technology is a powerful tool for the targeted manipulation of genomes and has been utilized successfully in vitro for a broad array of applications. We have now taken this technology and applied it to an in vivo setting. We will present data on the creation of targeted knockout rats, mice, and rabbits where key genes involved in human disease, such as Parkinson's Disease and Alzheimer's, have been removed from the genome and demonstrate the characterization of these animals. Furthermore, we will present data on the addition ("knock-in") of using this technology and discuss the potential far-reaching importance.

## PLURIPOTENT AND MULTIPOTENT STEM CELLS FOR CELL- MEDIATED TRANSGENESIS IN RABBITS: CHIMERIC AND NUCLEAR TRANSFER ANIMALS

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The ability to perform precise genetic engineering such as gene targeting in rabbits would benefit biomedical research, enabling for exampling the generation of genetically defined rabbit models of human diseases. This has so far not been possible because of the lack of functional rabbit embryonic stem cells and the high fetal and perinatal mortality associated with rabbit somatic cell nuclear transfer. We examined cultured pluripotent and multipotent cells for their ability to support the production of viable animals. Rabbit embryonic stem (ES) cells were derived and shown capable of in vitro and in vivo pluripotent differentiation. We report the first live born ES derived rabbit chimera. Rabbit mesenchymal stem cells (MSCs) were derived from bone marrow and multipotent differentiation was demonstrated in vitro. Nuclear transfer was carried out with both cell types and embryo development assessed in vitro and in vivo. Rabbit MSCs were markedly more successful than ES cells as nuclear donors. MSCs were transfected with fluorescent reporter gene constructs and assessed for nuclear transfer competence. Transfected MSCs supported development with similar efficiency as normal MSCs and resulted in the first live cloned rabbits from genetically manipulated MSCs. Reactivation of fluorescence reporter gene expression in reconstructed embryos was investigated as a means of identifying viable embryos in vitro, but was not a reliable predictor. We also examined serial nuclear transfer as a means of rescuing dead animals.

## IgG BINDING FCRN TRANSGENIC RABBITS CREATED THROUGH BAC TRANSGENSIS

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Bacterial artificial chromosome (BAC) transgenes can be used to generate transgenic animal models that express genes at physiological levels with the same developmental timing and expression pattern as endogenous genes. BACs contain long segments of genomic DNA that often contain regulatory information necessary to provide normal expression patterns. Adaptation of BAC transgenesis for expressing economically important genes in livestock species was suggested (Zuelke, 1998). Since then position independent, copy number related and mammary gland specific expression of goat and porcine milk protein genes have been described (Rival-Gervier et al., 2002; Stinnakre et al., 1999). The neonatal Fc receptor (FcRn) plays a crucial role in the maternal IgG transport, regulates the IgG and albumin homeostasis, takes active parts in phagocytosis and delivers antigen for presentation. We reported position independent and copy-number-related expression of the bovine FcRn in transgenic mice carrying a 102 kb BAC genomic fragment and shown that overexpression of FcRn extends half-life of mouse IgG by reducing its clearance (Bender et al. 2007) and significantly augments humoral immune response (Cervenak et al, 2011; Vegh et al, 2011; Schneider et al, 2011). To study if FcRn overexpression results in similar immuno-phenotype in rabbits we created six independent transgenic rabbit lines; three of them carry the bovine FcRn, while three others carry rabbit FcRn genes. Experiments are in progress to characterize the phenotypic consequences.

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## SLEEPING BEAUTY MEDIATED TRANSGENESIS IN RABBIT

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Transgenic rabbit is an important model of atherosclerosis, lipoprotein metabolism and cardiovascular diseases because physiology and pathology reflect the human situation closer than rodents. Since conventional rabbit transgenesis is still inefficient we assessed the suitability of the Sleeping Beauty (SB) transposon system for enzyme mediated transgenesis in rabbit. A new hyperactive version (SB100X) of the vertebrate specific Sleeping Beauty transposase - which is over 100-fold more active in mammalian cells than the prototype - was used to generate transgenic rabbits harbouring YFP reporter gene construct.
Fertilized rabbit zygotes were injected with circular reporter plasmid driven by CAGGS promoter and SB100X mRNA injection cocktail. 15% of rabbits born from embryo transfer showed the expression of the fluorescent protein. Transposon mediated transgenesis is far more efficient than any other method applied earlier in rabbits. All SB100X founders showed some degree of mosaicism in the skin but germline transmisson was successful in all cases with high proportion of expressing F1 pups. Low copy transgene integration introduced by the SB transposon system was observed without any transgene silencing. Hence Sleeping Beauty transposon system can also offer better control of transgene expression levels and patterns.

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## NUCLEAR TRANSFER TECHNOLOGY IN RABBITS

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Somatic cell nuclear transfer (SCNT) is a powerful tool of research and considerable possibilities exist for its biomedical and agriculture applications. Most of the current interest for this technology in rabbit is from the biomedical field. However, the efficiency of SCNT animal production is low, despite numerous efforts to develop this technique. SCNT combined with transgenic and/or stem cell technology can offer new biomedical animal models for human diseases or to create bioreactor animals.

Successful nuclear reprogramming from a somatic to an embryonic state is the key event in SCNT, and largely depends on the epigenetic state of the nuclei. Histone acetylation is one of the major epigenetic events. Studies have shown that elevated levels of histone acetylation in donor cells or cloned embryos could improve their development, thus improving the efficiency of this technology. The treatments of a histone deacetylase (HDAC) inhibitors (sodium butyrate (NaBu), Trichostatin A (TSA)), increased the developmental rate of cloned blastocysts. Also the histone acetylation pattern of the TSA-treated rabbit SCNT embryos appeared to be more similar compared to those of the normal embryos.

In rabbit, similarly to SCNT in other species, only 1-3% of the transferred embryos gave rise to live pups, despite the fact that nearly 50% of the rabbit SCNT embryos could have developed to blastocyst stage in vitro. It is possible that many of the defects in SCNT embryos occurring during the reprogramming stage might attribute to disorders during placenta development. Previous reports in pig indicated that the co-transfer of parthenogenetic embryos was efficient for the SCNT embryo pregnancy maintenance and improved the efficiency of SCNT technology.

In our studies we have improved several steps of rabbit embryo production and micromanipulation, and investigated the effect of TSA treatment and co-transfer of parthenogenetic embryos on the term development of the SCNT rabbit embryos. In vitro developmental data shown no differences in the cleavage and blastocyst rates between the TSA treated or non-treated cloned embryos. Blastocyst cell numbers did not differ among the groups. After ET in the TSA group, two pregnant female delivered 7 live and 4 stillbirth pups but all the live pups died within 1 h-10 days after birth. In the non treated group, four pregnant female gave births to 6 live and 8 stillbirth pups. Most interestingly, finally four does from the untreated group reached adulthood and three of them gave birth of healthy progenies while none of the TSA treated clones reached adulthood. This indicates that the epigenetic effects of the TSA treatment on the overall health condition of the clones need further investigation and potential scenarios for DOHAD (developmental origin of adulthood diseases) need to be taken into account while applying SCNT. Furthermore, the co-transfer of additional parthenogenetic embryos might have benefited the pregnancy of cloned embryos in rabbit.

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POSTER

## MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF INDUCED PLURIPOTENT STEM CELLS IN THE RABBIT

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In order to develop the induced Pluripotent Stem Cell (iPSC) technology in rabbits, we generated four rabbit iPSC lines, making use of MoMuLV-based retroviral vector that express human Oct4, Sox2, Klf4 and c-Myc to reprogram ear adult fibroblasts. The overall efficiency of iPSC derivation was estimated to $5.10^{-4}$%. All four lines express the cardinal markers of pluripotent stem cells: (i) they are positive for alkaline phosphatase activity; (ii) they express the pluripotency-associated Oct4 and Nanog transcription factors, as well as the SSEA-1, SSEA-4, Tra1-60 and E-Cadherin cell surface markers; (iii) they display a normal karyotype (44XX), and (iv) they can form teratomas containing tissues of ectodermal, mesodermal and endodermal origin upon injection under the kidney capsule in SCID mice. After 25 passages, expression of all four transgenes was fully repressed in three lines out of the four analyzed, indicating complete reprogramming of fibroblasts into iPSCs. Self-renewal of rabbit iPSCs is dependent on FGF2 signaling. Upon infection with EOS – a lentiviral vector expressing the Green Fluorescent Protein (GFP) under the control of the ICM-specific distal enhancer of the mouse Pou5f1 (Oct4) gene – rabbit iPSCs show extensive fluorescence. Moreover, rabbit iPSCs display cell-cycle features that are characteristics of pluripotent stem cells, including a short G1 phase, and the lack of DNA damage checkpoint in G1 phase. The capacity of rabbit iPSCs to colonize the preimplantation embryo was explored by microinjection of GFP-expressing iPSCs into 8-cell stage rabbit embryos, and subsequent culture to the blastocyst stage. Two blastocysts, out of 65 analyzed, displayed a GFP fluorescence in the ICM.
Altogether, these results indicate that rabbit iPSCs, albeit dependent on FGF2 signaling for self-renewal, display some features of rodent iPS cells including the capacity to colonize the pre-implantation embryo.

## MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF RABBIT EMBRYONIC STEM CELL LINES

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We have derived four rabbit Embryonic Stem Cell (ESC) lines from New Zealand GFP-transgenic or wild-type embryos. Inner cell masses (ICMs) were isolated from 25 rabbit blastocysts by immunosurgery, and plated onto growth-inactivated murine embryonic fibroblasts in a medium supplemented with 4 ng/ml FGF2. Fifty percents were able to form an outgrowth, and 16% produced a population of highly proliferating cells that could be regularly passaged. Among the four lines obtained, one expresses the Green Fluorescent Protein (GFP) and three do not. All four Rabbit ESC lines express the pluripotency markers Oct4 and Nanog. They also express both SSEA1 and E-cadherin cell surface antigens that characterize mouse ES cells, and SSEA4, Tra1-60 and N-cadherin that characterize primate ES cells and mouse EpiSCs. Noteworthy, the percentage of SSEA1-, SSEA4-, and TRA1-60-positive cells varies considerably between ESC lines. Contrary to mouse ESCs, rabbit ESCs derived from wild-type embryos do not express the GFP after infection with the EOS lentiviral vector. EOS carries the Green Fluorescent Protein (GFP) under the control of the ICM-specific distal enhancer of the mouse Pou5f1 (Oct4) gene. To eliminate the possibility that the Pou5f1 distal enhancer is not active in the rabbit, early cleavage stage rabbit embryos were infected with EOS, and subsequently cultured until the blastocyst stage. Confocal microscopy analysis revealed the presence of fluorescent cells within the ICM. Furthermore, after ICM isolation, infection with EOS, and subsequent plating, GFP-positive cells were visible in the resulting outgrowths, but fluorescence disappeared after 48 hours. Therefore, we conclude that the Pou5f1 distal enhancer is active in rabbit embryonic stem cells in vivo, but its activity is rapidly lost upon in vitro culture. All ESC lines tested display the capacity to make teratomas after injection beneath the kidney capsule in SCID mice. All teratomas contain derivatives of the three embryonic germ layers, demonstrating the pluripotent nature of these cell lines. Contrary to mouse and primate ESCs, rabbit ESCs exhibit a long G1 phase in that as much as 50% of the SSEA1-positive cell fraction displays a 2n DNA content. Surprisingly, of the four ESC lines analyzed, two exhibited a DNA damage checkpoint in G1 like somatic cells, whereas the other two, like mouse ESCs and EpiSCs, did not. We can conclude from these studies that rabbit ESC lines are heterogeneous in nature, with only some lines showing the cell-cycle cardinal features of pluripotent stem cells.

We also explored the capacity of rabbit ESCs to colonize the pre-implantation embryo. To this aim, we used the GFP-expressing ESC line that does not exhibit a DNA damage checkpoint in G1. Dually GFP/SSEA1-positive cells were FACS-sorted, subsequently micro-injected into 8-cell stage embryos, and the resulting embryos cultured to the blastocyst stage. No evidence of GFP-positive cells in the ICM was found from 58 embryos analysed.

Altogether, these results indicate that rabbit ESCs do not all exhibit the cell-cycle cardinal features of pluripotent stem cells. Moreover, they are unable to participate in embryo development in vivo.

## EFFICIENCY OF THE MACS TECHNIQUE PROVED ON THE BASIS OF MOTILITY AND MEMBRANE STATUS OF SEPARATED RABBIT SPERMATOZOA

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The aim of this study was to verify the efficiency of the rabbit apoptotic spermatozoa elimination by the use of the MACS technique (Said et al., 2006; Vasicek et al., 2011) on the basis of the spermatozoa quality assessment according to their motility and membrane status. Ejaculates from 11 New Zealand White (NZW) bucks were collected using an artificial vagina. They were mixed to make heterospermic pool, routinely diluted in a commercial insemination diluent (MiniTüb, Tiefenbach, Germany) at the ratio of 1:6 and divided into two portions. One portion was filtered through a Sartorius filter to wash out of seminal plasma, whereas the second portion remained unfiltered. The non-filtered spermatozoa were re-diluted in a commercial insemination diluent (1:3.66), whereas the filtered spermatozoa were re-diluted in a binding buffer (Annexin V Microbead Kit, Miltenyi Biotec, Germany) at the ratio 1:3.66 and both portions were subdivided into two groups: the experimental group intended for MACS separation and the control group without MACS separation. The semen samples from the control group and $AnV^-$ fraction, both filtered and non-filtered, were evaluated using a CASA system (Sperm Vision™). Morphology of sperm head membranes (membrane status) was determined using transmission electron microscopy (TEM). Obtained data were analyzed using Chi-square test.

Following the MACS treatment the semen concentration was insignificantly increased in the $AnV^-$ fraction compared to the control samples. Similarly, the passage through the column slightly increased percentage of motile (non-filtered 90.28 % vs. 86.59 %; filtered 82.07 % vs. 74.92 %) and progressively motile spermatozoa (non-filtered 85 % vs. 79.2 %; filtered 73.34 % vs. 62.96 %) in the $AnV^-$ fractions compared to the control samples, although the differences were not statistically significant. Moreover, the MACS procedure caused a higher distribution of non-filtered spermatozoa into first two categories: I and II (94 %) in comparison to the control sample (88.25 %), whereas a lower distribution into last categories: III and IV (6 % vs. 11.75 %). Similarly, the MACS treatment caused also higher distribution of filtered spermatozoa into first two categories: I and II (89.5 %) in comparison to the control sample (86.25 %), whereas a lower distribution into last categories: III and IV (10.5 % vs. 13.75 %), although the differences were not statistically significant.

In conclusion, the CASA analysis strongly correlated with the membrane status of observed rabbit spermatozoa that could potentially prove the efficiency of the MACS technique.

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## EFFECT OF DBCAMP ON RABBIT REPRODUCTIVE FUNCTIONS AND FOLLICULAR ATRESIA

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The aim of our study was to examine the influence of administration of $N^6$,2'-dibutyryladenosine 3'5'-cyclic monophosphate (dbcAMP), a cAMP analogue, on reproductive efficiency, ovarian folliculogenesis and atresia in rabbits, whose ovarian cycle and ovulation were induced by gonadotropins.

Ovarian cycle and ovulation of rabbits were induced by 80IU/female PMSG followed by 150IU/female hCG administration. Experimental animals received PMSG and hCG together with dbcAMP (at 50 µg/animal). After ovulation and insemination, the animals were sacrificed. The ovaries were weighted, histological sections of ovaries were prepared, and the presence of ovulated and non-ovulated follicles and different stages of atresia was evaluated by a light microscopy. The eggs were flushed from the oviducts after insemination and cultured up to the blastocyst cell stage. Numbers of ovarian Corpora lutea, ovulated oocytes and oocyte-derived zygotes and embryos reaching hatched blastocyst stage were determined.

It was shown, that administration of dbcAMP was able to increase the proportion of ovarian follicles with cystic atresia (16,5% vs. control 8,7%). Furthermore, dbcAMP (50 µg/female) increased the number of corpora lutea (38.34 ± 2.20 vs. control 27,92 ± 1,64 CL/female), number of harvested oocytes (38.34 ± 2.20 vs. control 27,92 ± 1,64/female), zygotes (38,12 ± 2,21 vs. control 27,92 ± 1,64/female) and embryos at blastocyst stage derived from these zygotes after culture (38.34 ± 2.20 vs. control 27,92 ± 1,64/female).

These data demonstrate that dbcAMP can induce rabbit ovarian follicle atresia, stimulate ovulation, oocyte, zygote and embryo yield and development. Furthermore, they confirm the involvement of cyclic nucleotide-dependent intracellular mechanisms in the up-regulation of rabbit reproductive functions, as well as potential practical usefulness of dbcAMP for improving farm animal reproduction and fertility.

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## RECOMBINANT HUMAN TISSUE NON-SPECIFIC ALKALINE PHOSPHATASE SUCCESSFULLY COUNTERACTS LIPOPOLYSACCHARIDE INDUCED INFLAMMATORY RESPONSE IN MICE

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Alkaline phosphatase is a promising therapeutic agent in the Gram negative bacterial lipopolysaccharide (LPS) mediated acute and chronic diseases. Contrary to other alkaline phosphatase isozymes, purified tissue-nonspecific alkaline phosphatase (TNAP) is not available in large quantities from tissue sources, which would enable to analyse its efficacy in animal sepsis models. Transgenic rabbit fills an important niche between laboratory mouse and dairy animals because the quantity of milk produced by lactating does fulfil the requirements not only in experimental scale but also for clinical stage trials.
A mouse model experiment was carried out to examine the effect of recombinant human tissue non-specific alkaline phosphatase. Three groups of 10 mice received 15 µg lipopolysaccharide. After 3 hours one group was injected with 9 units of recombinant human tissue non-specific alkaline phosphatase isolated from transgenic rabbit milk, one group was injected with 9 unit bIAP (Alloksys), one group was injected with non active TNAP. Administration of recombinant human tissue-nonspecific alkaline phosphatase increased the survival rate from 30% to 80%. The recombinant human tissue-nonspecific alkaline phosphatase fraction isolated from transgenic rabbit milk has proved to be physiologically effective in lipopolysaccharide induced systemic inflammatory response syndrome, in an in vivo mouse model of sepsis.

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## THE PHENOMENON OF PERIPHERAL BLOOD LEUKOCYTES APOPTOSIS IN RABBITS INFECTED WITH RHD (RABBIT HAEMORRHAGIC DISEASE) VIRUS STRAINS.

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Abstract. As it has been noted that the mechanism of pathogenic effect of RHDV on the organism of rabbits is connected with its affinity to the blood vessels (causing disseminated intravascular coagulations - DIC) and the immunity mechanisms (mainly of non-specific cell immunity), together with apoptosis of many cells, such as hepatocytes and cells of immune system, the aim of the study was to monitor the induction of apoptosis in granulocytes and lymphocytes in peripheral blood of rabbits infected with strains of RHDV from Hungary - 24V/89, 1147 V/96, 72V/2003, Austria – 0104, 237/04 and France – 05-01, that have not been studied in this matter before. The study has been performed with the use of flow cytometry and the total activity of caspases: 1,3,4,5,6,7,8 and 9 (MP Biomedicals, USA) was measured in granulocytes and lymphocytes of rabbits' peripheral blood, and the results were shown as the percentage of apoptotic cells in granulocytes and lymphocytes populations.
The study has shown that the phenomenon of apoptosis is registered in granulocytes and lymphocytes and the intensity of it is higher in the latter cells. The process increases from 4-8 till 36 h p.i. It can be stated that the fact of registration apoptosis in immune cells is a clear evidence of importance of apoptosis in pathogenesis of RHD.

## WHHLMI RABBITS WITH CORONARY LESIONS ARE A GOOD ANIMAL MODEL FOR CORONARY SPASTIC ANGINA

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**Background and Aims:** Coronary plaque rupture is considered as a direct trigger for the onset of acute coronary syndromes (ACS). However, the mechanism of coronary plaque rupture and the following thrombus formation has not been fully clarified yet. We examined the influence of coronary spasms on the coronary plaques of WHHLMI rabbits.

**Methods:** Coronary spasm was evoked in the WHHLMI rabbits by intravenous injection of ergonovine under norepinephrine infusion and the onset of coronary spasm was examined by ECG, echocardiography, and coronary angiography. After evoking of spasms, serum markers for myocardial ischemia (fatty acid binding protein (FABP), troponin-I and myoglobin) were assayed. The coronary lesions were examined histopathologically at 250 □m intervals.

**Results:** In ECG monitoring, ST depression, and an inversion of T-wave were observed after the treatment. In these rabbits, the wall motion of the left ventricle was reduced on echocardiogram and constriction of the epicardial coronary artery was detected on coronary angiography. These changes were normalized after the nitroglycerin administration. Serum levels of FABP, troponin-I, and myoglobin were markedly increased after the ischemic ECG changes. In histopathological examination, although disappearance of endothelial cells accompanied with macrophage flowing out from the plaque was observed in many rabbits, thrombus formation was not observed.

**Conclusions:** The WHHLMI rabbit can be used as an animal model for coronary spastic angina. Since simple plaque injury induced by coronary spasms was not sufficient for thrombus formation, some additional factors may play an important role in the onset of ACS.

## EFFECT OF FREEZING PROCEDURE ON RABBIT LATE BLASTOCYST TRANSCRIPTOME

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Embryo cryopreservation efficiency depends mainly on technical and embryo factors. While structural damages can be easily evaluated, physiological damages only can be estimated by analyzing their in vitro and in vivo development to later stages. In order to determine how freezing process affect embryo pre-implantory development, a transcriptional microarray study has been performed comparing gene expression of 6 days old rabbit embryos, previously frozen and transferred into recipients does, to their in vivo counterparts. A total of 75 morphological normal morulae were collected 70-72h post-insemination and frozen in 1.5 M dimethylsulfoxide solution at -1ºC/min from 20ºC to seeding temperature (-7ºC), and then -0.3ºC/min to -35ºC before plunging into nitrogen liquid. After thawing in a water bath at 20ºC, cryoprotectant was removed in three steps and thawed embryos were divided in transferable o non-transferable embryos according to morphological criteria based to damage embryo covers. Then, 54 (72.0%) thawed embryos were transferred via laparoscopy into oviducts of three synchronized recipient does. Three days later, 31 (57.4%) of them were recovered as late blastocyst. As control fresh group, thirty 6 days old blastocyst were recovered from uterine horns of inseminated females. For both groups, control and frozen late blastocysts, total RNA were extracted from 3 pools of approximately 10 embryos using traditional phenol/chloroform protocol with Trizol reagent. A specifically microarray designed to study rabbit gene expression profiling, the Rabbit 44K oligonucleotide array (Agilent Technologies), was used in this study. According to manufacturer's instructions, Dnase-treated total RNA of each sample was labeled with Cy3 or Cy5 dyes, employing Quick Amp labeling kit (Agilent Technologies). Then, three competitive hybridizations were carried out including one of them with a dye-swap to compensate dye-bias. Slides were scanned using a GenePix 4000B, and the GenePix 6.0 software was used for image acquisition. To identify differentially expressed genes, one class response significance analysis of microarray (SAM) was performed, and significant genes with an estimated false discovery rate (FDR) of less than 10% were identified. Functional annotation of 74 differentially expressed genes was obtained with Blast2GO tool, and the GO terms were assigned. Our results showed a downregulation in frozen embryos of 72 genes principally involved in signal transduction, transport and response to stress and to external stimulus. Only 2 genes were significantly upregulated, one related to calcium ion binding and the other one to glutathione transferase activity. Despite no morphological differences observed between control and treated embryos at 6 days of development, our results showed that effects of freezing procedure still remain in blastocyst pre-implantatory gene expression. Thus, further studies should be done to determine the importance of gene expression alterations on successful interaction between embryo and maternal endometrium or fetal development.

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