

ABSTRACTS OF 2<sup>nd</sup> INTERNATIONAL MEETING ON RABBIT BIOTECHNOLOGY,  
JOUY EN JOSAS, FRANCE. JUNE 2007.

Rabbit is more and more used as an experimental animal for the study of biological functions and of human diseases. The reasons of this development are multiple. Rabbits are closer to human than rodents. Rabbits are larger than mice and even rats thus more appropriate for some surgical operations. Cloning in rabbits is now possible and its complete genome is about to be sequenced. Moreover, rabbits are being used to produce pharmaceutical proteins at an industrial scale. A specific domain of rabbit biotechnology has become a reality. Expectedly, the techniques of biotechnology should be implemented in future to solve some specific problems of rabbit breeding and particularly for the struggle against diseases.

This urged Jianglin Fan to organize in Tsukuba the first meeting on rabbit biotechnology in 2005. This first success inclined to pursue the venture. The 2<sup>nd</sup> meeting thus took place in Jouy en Josas in June 2007. About 20 speakers and 40 participants attended the two days meeting. A major part of the presentations referred to models, transgenic or not, for the study of human diseases. The state of the art in the use of genetic markers, cloning and use of ES cells was depicted. The techniques for breed conservation (semen, embryo and ovary freezing) were shown. The summary of the presentations and posters are reported in this issue.

An important point is that a community involved in the development of rabbit biotechnology emerged during the meeting. It was decided to organize other meetings on rabbit biotechnology in future with the idea of not competing with the World Rabbit Meetings but to be complementary. The next rabbit biotechnology meeting should take place in China in 2009.

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## RABBIT SELECTION AND GENOME

### FROM WILD RABBITS TO INRA STRAINS: GENETIC DIVERSITY OF *ORYCTOLAGUS CUNICULUS*

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The history of the European rabbit (*Oryctolagus cuniculus*) is well-documented. Fossil data and archaeological remains suggest that the species arose in the southern half of the Iberian Peninsula and was able to extend its range through the Pyrenean barrier into Mediterranean France ca. 500,000 years ago. Several studies based on mitochondrial DNA polymorphism within the rabbit's native range reveal two highly divergent maternal lineages, each with a well-defined geographical distribution: one lineage occurs in south-western Iberia and the other in north-eastern Spain, France, and in domestic breeds. Domestic rabbit is the only domesticated mammal of Western European origin. Nowadays, wild and domestic populations still coexist. The first step after the antic period lied in hunting or keeping wild rabbits in warrens, from the Middle Ages to the 17<sup>th</sup> century. Domestication of rabbits took place during the 18<sup>th</sup> and mainly the 19<sup>th</sup> centuries. The most important step of creation of breeds occurred during the first half of the 20<sup>th</sup> century. Meanwhile, societies of breeders were created to maintain and improve these breeds. Nowadays, national associations of rabbit breeders exist in many European countries; they are

in charge of the making and updating of the standard book of rabbit breeds. Rabbit meat production is realised using specialised herds and is mainly based on crossbred rabbits obtained from very few commercial strains disseminated through pyramidal systems. These commercial strains were created from some middle-sized breeds (mainly New Zealand White, Californian) and few heavy breeds for paternal lines. In a few cases, they were crossbred with other breeds or local populations to produce synthetic lines. The INRA undertook in 1972 the creation and the selection of strains intended to be used in crossbreeding according to a pyramidal device. Two strains (INRA1077 and INRA2066) were more specifically selected on their prolificacy, in the presence of a control strain (INRA9077). Now, the INRA is released gradually from this role and concentrates on the search for new goals and new methods of selection, benefiting from progress in statistical models and knowledge of the genome of rabbit. Nowadays, the current objectives of selection focus less on the increase in the production than on the improvement of maternal qualities and adaptation. The new INRA1777 strain is selected with an index combining litter size at birth and young weight at weaning, with a mating plan optimized to control the increase of consanguinity. INRA selects also new strains for the longevity of the females (using survival analysis tools), the homogeneity of the weight of the young rabbits at birth (using canalising selection), the residual feed consumption, ... The existence of a microsatellite-based genetic map and a first contig database opens new prospects for exploration of genetic variability, detection of QTL and selection assisted by marker or introgression. The evolution of genetic diversity from the wild populations to the patrimonial breeds and the selected strains has been estimated with microsatellites and other markers.

There was no important genetic bottleneck, including in the event of strong reduction of genetic number. This genetic diversity has to be conserved and exploited: a national germplasm cryobank was created. It includes 13500 rabbit embryos belonging to 49 populations: 9 patrimonial breeds, 9 lines with particular genotype and 31 selected populations or strains.

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## RABBIT GENOME MAPPING TOOLS

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The importance of European rabbit (*Oryctolagus cuniculus*) as a production, pet and laboratory animal and the serious lack of genetic and genomic tools for this species led the INRA institute to launch a genome-wide mapping project in 2001. An integrated genetic and cytogenetic map was built directly using a strategy based on cytogenetically localized microsatellites. Rabbit gene-containing BAC clones were isolated using similarities between rabbit cDNAs and human genomic sequences to design intra-exonic primers suitable for BAC library screening. To date, over 250 new genes have been mapped by fluorescent in situ hybridisation (FISH) along the 23 chromosomes, thus increasing twelve-fold the number of genes mapped in rabbit. 305 microsatellites were isolated either from FISH-mapped BAC clones or from a plasmidic genomic library, of which 183 were anchored on all the chromosomes except OCU21. The microsatellites were used to genotype 187 animals chosen from three-generation rabbit families in which angora and albino characters segregated and the number of alleles found for polymorphic markers ranged from 2 to 7 (on average 3,3). This genetic map comprises 20 linkage groups spanning 2766.6 cM over all chromosomes except OCU20, 21 and X and contains 111 markers, including 104 INRA microsatellites, five other microsatellites and the two morphological markers angora and albino. Despite its low density, it allowed us to map the albino and angora characters on OCU1 and 15, respectively. The 2X rabbit genome sequence available since 2005 ([http://www.ensembl.org/Oryctolagus\\_cuniculus/index.html](http://www.ensembl.org/Oryctolagus_cuniculus/index.html)) will be valuable to rapidly increase the map density in targeted genomic regions. In conclusion, the genomic tools that are now available for rabbit include an integrated genetic and cytogenetic map, large DNA fragment libraries, a number of cDNAs and EST in public databases, a rabbit-man comparative map based on reciprocal chromosomal painting refined by cytogenetically-mapped genes and a first assembly of the genome sequence. Altogether, these tools are suitable for genetic, cytogenetic and physical mapping as well as sequencing and polymorphism studies. It is anticipated that new projects on QTL mapping and positional cloning of candidate genes in rabbit will soon be developed in both biomedical and zootechnical research fields.

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## CRYOPRESERVATION OF RABBIT GENETIC RESOURCES BY THE FEMALE WAY

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The cryopreservation of rabbit genetic resources can be achieved as well as by the male way and the female way. Presently, methods of semen and embryos freezing are well described and can be routinely used to preserve different populations or genotypes by cryobanking. In addition to semen freezing, methods of embryo preservation allow the saving of mitochondria genome and the storing of this biological material with a higher level of sanitary status. Moreover, pups can be directly obtained one month after embryo transfer without any additional backcrossing and an easier genotyping. Most of the time, superovulation treatments with pFSH or eCG are used to improve the embryo production. For the last 15 years, we have implemented a 3 d standard protocol using pFSH. With this protocol, 2/3 to 3/4 of treated females produced 20 to 30 frozen embryos according to their genotype and physiological status. After embryo thawing and transfer into synchronized recipients, around 80% of females delivered at least one alive pups. The embryo survival rate (number of pups/number of embryos transferred) is of about 40% with an important variation between 17% to 53% according to genotype of the recipients and the rearing environment. Presently, the French national cryobank contains more than 15000 frozen embryos from 50 different genotypes (endangered breeds, commercial strains, transgenic lines...). Nowadays, a new method of cryopreservation: the ovarian tissue freezing, is being developed to save genotypes with a high scientific or genetic value, under emergency situation. Today, pups can be obtained using recipients grafted with frozen-thawed ovarian fragments.

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## RABBIT EMBRYO PRODUCTION AND CONSERVATION

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Embryo production, conservation and transfer are basic tools in both embryo micromanipulation and cryopreservation programmes. Nowadays, the production of rabbit mature oocytes and embryos *in vivo* are most favourable than *in vitro* production, the superovulatory responses (20 to 40 ovulating follicles) allow to obtain a high number of mature oocytes and embryos of sufficient quality. In addition, the development of oviductal *in vivo* recovery and transfer techniques during the last decade offers new opportunities to study the individual response to hormonal treatments, for example, and this technique improves also the preservation of valuable animals. The embryos can be cryopreserved efficiently reaching a survival rate about 50% after transfer. However, the efficacy of the program depends on several factors, such

as stage of embryos, genotype of donors and recipients. Embryo cryopreservation can be used as a tool for the establishment of genetic resource banks for preservation of biodiversity in both, animal breeding or laboratory products (transgenic, clones), protecting against loss through diseases or hazards. From a genetic point of view, the cryopreservation of inbred strains is useful for the establishment of control populations to study the genetic drift and gain when selection programmes are applied.

## RABBIT SEMEN CONSERVATION

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We have been developing several transgenic rabbits as human disease models. At Saga University, we currently maintain 15 lines from 8 transgenic rabbits. For maintenance and conservation of laboratory animals as a bioresource, especially mutant and genetically modified animals, cryopreservation of sperm and embryos is becoming an important technique. In the rabbits, several methods of cryopreservation of sperm and embryos have been reported. Cryopreservation of sperm is more attractive to us than embryos, because of the lower cost and simpler techniques of collection, freezing and artificial insemination (AI) compared to the comparative techniques needed for embryos. Moreover, to maintain transgenic rabbit lines, we need only to pay attention to single transgene transmission. Therefore, the combination of cryopreservation of semen and AI appears suited to the maintenance of transgenic rabbits. On the other hand, in cases where polygenes contribute to the expression of phenotype, embryos can be chosen for cryopreservation because the phenotype might disappear with just a combination of cryopreservation of semen and AI to wild type females. In our facility, cryopreservation of sperm has been carried out using egg yolk-acetamide extender. We are currently keeping in liquid nitrogen more than 800 straws of sperm from our transgenic rabbit lines. The motility of post-thawed sperm shows  $38.4 \pm 8.9\%$ . Utilizing our system of semen freezing, using  $20 \times 10^6$  motile sperms of post-thawed spermatozoa for AI, we achieved a pregnancy rate of 60-80% with a mean litter size of 4-5 pups. If we use more than  $40 \times 10^6$  motile sperm, we can expect performance similar to that of fresh semen. Our system, with its combination of cryopreservation of sperm and AI appears a useful method to maintain transgenic rabbit lines.

## VIABILITY OF RABBIT EMBRYOS AFTER 15 YEARS STORAGE IN LIQUID NITROGEN

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Embryos freezing and transfer represent interesting tools for ex-situ conservation of animal genetic resources in rabbit species and are also used to produce specific pathogens free populations or to store transgenic lines producing therapeutic proteins. Actually, no study has evaluated the viability of frozen rabbit embryos after a long period of storage and therefore the long term efficacy of this method. In 1992, embryos of one endangered population of rabbit called "Brun Marron de Lorraine" were recovered, slowly frozen and stored for 14 years in liquid nitrogen before assess their viability. In September 2006, a total of 134 morulae were thawed and transferred on 14 synchronized recipients does of which ovulation was induced 50-60 h before transfer. After thawing, embryos are mounted in a capillary glass and around 10 embryos was transferred into each uterine horns by midventral laparotomy way. The *in vivo* development viability of the thawed embryos was assessed with the parturition rate and the embryo survival rate. Overall, 100% of the synchronized recipient does transferred have delivered at least one pup. A total of 69 live pups were born and the embryo survival rate is 51.5%, similar to those usually obtained after transfer of fresh embryos. So, this study has highlighted that the *in vivo* development capacity of slowly frozen embryos is not altered by a long period of storage in liquid nitrogen. These techniques of embryos freezing and transfer can be efficacy used in French National Cryobanking programs to preserve the genetic diversity in rabbit species and like an interesting tool for breeds' management.

## OVARIAN TISSUE CRYOPRESERVATION IN THE DOE RABBIT: FROM FREEZING TO BIRTH

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Ovarian tissue cryopreservation aims to preserve simultaneously thousands of immature follicles from the ovarian stock. This recent technique may allow preserving the animal genetic resources. The objectives of this study were (1) to determine the influence of different freezing parameters and (2) to validate a slow freezing process for the cryopreservation of the ovarian tissue in the doe rabbit. A two-level fractional experimental design was used to discriminate 5 freezing parameters: the nature (DMSO vs. 1,2-PROH) and the concentration (1.5M vs. 2M) of the cryoprotectant, the nonpenetrating cryoprotectant (sucrose vs. trehalose), the equilibration process (1 vs. 3 steps) and the post-seeding freezing rate (0.3 vs. 2°C/min) were evaluated. The morphological analysis of the preantral follicles was performed before (control) and after freezing. The best combination of freezing parameters was finally challenged by orthotopic autograft and *in*

*vivo* resumption of folliculogenesis. Cryopreserved fragments from one ovary were grafted to the contralateral ovarian pedicle of 16 females. Then females were inseminated along 9 months. The experimental design showed that 1,2-PROH ( $P=0.08$ ) and trehalose ( $P=0.07$ ) tends to improve the morphology of preantral follicles. The freezing rate seems to have the greatest impact on the follicular morphology which was improved by the use of a freezing rate of  $0.3^{\circ}\text{C}/\text{min}$  ( $P<0.01$ ). No significant effect was observed for the other freezing parameters. Thus, transplantations were performed with ovarian tissues frozen in a medium containing 1.5M 1,2-PROH and 0.2M trehalose (3 step-equilibration process, seeding then  $0.3^{\circ}\text{C}/\text{min}$ ). The proportion of morphologically normal follicles was significantly decreased after freezing as compared to fresh control ( $65.8\pm 4.8\%$  vs.  $82.6\pm 2.8\%$ ;  $P<0.001$ ). Overall, pups were born from females grafted with cryopreserved ovarian tissue. The doe rabbit ovarian tissue seems to be successfully preserved by slow freezing process. Pups born after grafting from cryopreserved ovarian tissue enhance the efficiency of such protocol. (Grant from region Rhône-Alpes).

## NORMAL RABBITS AS MODELS TO STUDY HUMAN DISEASES

### WHHL RABBIT FAMILY IS SUITABLE FOR STUDIES OF HYPERLIPIDEMIA AND THE RELATED DISEASES IN HUMAN.

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According to WHO the World Health Report 2002, 16.7 million people around the world die of cardiovascular diseases each year and this is about one-third of all deaths globally. Principally, atherosclerosis and lipid disorders are the main causes in cardiovascular diseases. In atherosclerosis and lipid disorders, the characteristics of rodents including genetically altering mice are largely different from humans. Therefore, it is difficult to translate the results from mice experiments to humans (e.g., effects of hypolipidemic drugs, lipoprotein metabolism, atherosclerotic lesions, and others). In contrast to rodents, the features of rabbits for those parameters closely resemble humans. There are several rabbit models for human hyperlipidemia, atherosclerosis, and myocardial infarction; e.g. the WHHL rabbit family (original WHHL, coronary atherosclerosis-prone WHHLCA, and myocardial infarction-prone WHHLMI rabbits), St. Thomas' mixed hyperlipidemic rabbit, postprandial hypertriglyceridemic rabbit, and others. We have developed the WHHL rabbit family. WHHL rabbit family shows hypercholesterolemia due to deficiency of low-density lipoprotein (LDL) receptors and is an animal model for human familial hypercholesterolemia. In this strain, LDL accumulates in the plasma and the high-density lipoprotein levels are extremely low. As a result, atherosclerosis is developed spontaneously in the aorta, coronary arteries,

and other main arteries. In WHHLCA and WHHLMI rabbits, the coronary atheromatous plaques closely resemble human coronary plaques. In WHHLMI rabbits, myocardial infarction develops spontaneously after maturation due to coronary occlusion with atheromatous plaque. In addition, WHHLCA and WHHLMI rabbits show metabolic syndrome-like features. WHHL rabbit family has been used in studies of lipid metabolism, atherosclerosis, and development of hypocholesterolemic and/or anti-atherosclerotic compounds to translate the results into humans. It is necessary for translational researches to use animal models, which have closely similar characteristics to humans about the mechanisms and features of diseases, and the effects of drugs, in addition to appropriate body size. In conclusion, WHHL rabbit family is useful for translational researches about human cardiovascular diseases.

### METABOLIC SYNDROME-LIKE FEATURES OF WHHLMI RABBITS

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WHHLMI rabbits, which suffer from severe coronary atherosclerosis and myocardial infarction despite feeding of normal chow, also show metabolic syndrome-like features. Therefore, we examined the metabolic syndrome-like features of WHHLMI rabbits. After 15 h fasting, glucose tolerance test was carried out using WHHLMI rabbits aged 9-18 months old. The plasma was prepared periodically and measured glucose and immunoreactive insulin (IRI) levels. The homeostasis assessment insulin resistance (HOMA-IR) was calculated using fasting blood glucose and IRI levels. Plasma lipoprotein levels were examined by ultracentrifugation. Small and dense LDL cholesterol (sdLDL) levels were assayed with a kit (sdLDL-C SEIKEN). Serum C-reactive protein (CRP) levels were measured with an ELISA kit. After euthanasia by intravascular injection of pentobarbital sodium, weight of the visceral fat was measured and surface lesion areas of the aorta were measured with a computer assisted image analysis. In glucose tolerance test, WHHLMI rabbits showed high fasting IRI levels despite normal of the plasma glucose levels. Consequently, the HOMA-IR levels were increased and insulin resistance was observed. In WHHLMI rabbits, the HDL cholesterol levels were low and the serum triglyceride and sdLDL levels were elevated markedly. Fat accumulation was marked at the visceral area. Accumulation of visceral fat was correlated to fasting blood glucose, VLDL-triglyceride, serum free-fatty acids, serum CRP, and aortic atherosclerosis. Correlations between accumulation of visceral fat and fasting insulin, HOMA-IR, serum triglyceride, or systolic blood pressure measured at the carotid artery under anesthesia were suggested. Aortic lesion area was correlated to visceral fat accumulation, fasting insulin level, HOMA-IR, and serum free fatty acids. Correlations between aortic lesion area and abdominal circumference, insulin index of glucose tolerance test, or serum CRP level were suggested. Present results suggest that the WHHLMI



rabbit is a suitable animal model for human metabolic syndrome.

## RABBIT MODEL TO STUDY IMMUNITY TO PAPILLOMAVIRUS INFECTION

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Papillomaviruses are important human pathogens that cause epithelial lesions including skin and mucosal warts and associated malignancies. HPV infections result in more than 250,000 deaths from cervical cancer in women world-wide. The strict species restriction of these viruses provides a challenge to research into the mechanisms of natural and vaccine-induced immunity to these infections *in vivo*. Several animal models have been used to study papillomavirus immunity including the domestic rabbit. Our research has focused on two tissue-tropic papillomaviruses including cottontail rabbit papillomavirus (CRPV) a skin-tropic PV; and rabbit oral papillomavirus (ROPV) a mucosotropic PV. We have used the rabbit model to induce protective neutralizing antibodies upon vaccination with virus-like particle (VLP) and other capsid proteins. In addition, we have assessed immunity induced by cell-mediated responses upon gene-gun-based DNA vaccinations in both protective and therapeutic scenarios. Some recent model developments include the production of an HLA-A2.1 transgenic rabbit line to more accurately measure the role of epitope-specific CD8 T-cells in host immunity to papillomavirus infections. The transgenic rabbits expressed the HLA protein at high levels, and HLA-A2.1 restricted rabbit CD8 cells were induced in these rabbits. These latter T-cells were detectable using tetramer staining technology and specifically lysed epitope-pulsed A2-positive target cell lines. We conclude that the rabbit is an excellent model to assess both natural and induced immunity to papillomavirus infections. Improvements to the model are ongoing and include a transgenic model to measure CD8 responses to papillomavirus infections. We envisage that these transgenic rabbits will have utility for assessment of immunity to other human pathogens that are permissive in rabbits.

## THE RABBIT MODEL OF TUBERCULOSIS

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The global epidemic of tuberculosis claims over 2 million lives yearly. Mycobacterium tuberculosis (Mtb) latently infects one-third of the world's population. An improved understanding of the immunopathogenesis of latency and reactivation is urgently needed as co-infection with human immunodeficiency virus (HIV) becomes more prevalent in tuberculosis endemic regions of the world and multi-drug resistant strains emerge. After aerosol infection with Mtb, rabbits form caseous lung granulomas which are strikingly similar to tuberculous lung lesions in humans. Unlike guinea pigs and mice (commonly used animal species) that ultimately die after aerosol infection due to multibacillary chronic infection, rabbits are relatively resistant to infection with Mtb. Using the muzzle-only aerosol system

developed at USAMRIID, New Zealand white rabbits are aerosol-infected with MtbH37Rv. The inhaled bacilli initially replicate in the lung. At 5 weeks after infection, they formed grossly visible lung granulomas. Following the acquisition of specific immunity, both the granuloma size and the culturable number of bacilli decrease so that by 20-25 weeks after infection only a few lesions are visible. This paucibacillary long-term infection is consistent with human latency and occurs predictably. 60% of the infected rabbits with "latent" infection intramuscularly treated with dexamethasone will have culturable bacteria in the lungs 5 weeks later. After the corticosteroid treatment is discontinued, those rabbits with the highest burden of bacteria are at the highest risk for an immune reconstitution inflammatory syndrome (IRIS) with the return of CD4 T cells. This is characterized by large multi-centric caseous lesions in the lung. Using flow cytometry, lymphocyte proliferation and TNF-alpha intracellular cytokine staining, rabbit cellular responses to Mtb antigens can be measured. The aerosol model of tuberculosis in rabbits accurately recapitulates many of the stages of the human disease. Because rabbits are relatively resistant to aerosol infection with Mtb compared to mice, the rabbit is an attractive model to study latency and reactivation. Effective utilization of this resistant animal model could lead to new tuberculosis diagnostics as well as the elucidation of important correlates of protective immunity.

## ES CELLS AND CLONING

### ESTABLISHMENT OF RABBIT EMBRYONIC STEM CELLS

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The present use of transgenic rabbits is limited by the low efficiency of microinjection and the absence of proven embryonic stem cell (ES) lines. We have developed a new method for the derivation of a rabbit ES like cell line. Rabbit ES like cells grow as tightly packed, flat colonies similar to those of human ES cell lines and express several markers characteristic of human and/or mouse ES cell lines. We have succeeded short-term cultivation of alkaline phosphatase positive rabbit ES-like cell lines and shown that they express Oct4, SSEA-1, Nanog, CD9 and LIFR. Expression of LIFR is not detected in rabbit blastocysts. LIFR cDNA was isolated from the genital ridge of 13.5 d old rabbit embryos and partially sequenced to prepare a probe to screen a rabbit BAC library. Two BAC clones were identified and used as probes to FISH-map the LIFR gene on OCU 11p11.1. We have also demonstrated that the rabbit ES like cells could differentiate into beating cardiomyocytes by inducing cardiomyocyte differentiation with standard rabbit ES cell culturing medium without LIF but in the presence of 20%

fetal calf serum. Towards the aim of creating second-generation transgenic rabbits, another essential step was to develop an efficient method to create rabbit chimeras from preimplantation stage embryos. It remains to be tested whether these putative embryonic stem cells are truly pluripotent and can give rise to viable chimera progeny when we introduce them into recipient rabbit blastocysts.

## GENE TARGETING USING SOMATIC CELL NUCLEAR TRANSFER

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Cardiovascular disease (CVD) is the single most common cause of death in the United States and most Western countries. Although the study of the CVD has greatly benefited from the use of gene-targeted transgenic mouse models, these small rodents do not accurately reflect human cardiovascular physiology. The Rabbit would be an excellent animal model for CVD research, considering its many similarities to humans in cardiovascular anatomy, physiology, and lipid metabolism. However, the production of transgenic/knock-out (KO) rabbits is hindered by low efficiency of traditional DNA microinjection and the unavailability of embryonic stem cell lines. We proposed to use somatic cell nuclear transfer (NT) and gene-targeting technology to produce transgenic KO rabbits as models for the study of human CVD and other diseases. Our first objective of the study was to prove the feasibility of cloning rabbits since rabbit was a difficult species to clone. Rabbit oocytes were flushed from the oviducts of superovulated donors treated with FSH and hCG. Oocyte enucleation was conducted in 10% FBS M199 and confirmed by fluorescent microscopy. Cumulus cells used for nuclear donors were prepared from fresh cumulus-oocytes complexes. Donor nucleus was transferred into a recipient oocyte by cell fusion. Briefly, a small donor cell with the diameter approximately 15-19  $\mu\text{m}$  was transferred into the perivitelline space of an enucleated oocyte; subsequently the somatic cell-cytoplasm pair was fused by applying three direct current pulses at 3.2 kV/cm for a duration of 20  $\mu\text{sec}$ /pulse. Fused embryos were activated by electrical stimulation regime, and subsequently cultured in M199 + 10% FBS containing 2.0 mM 6-dimethylaminopurine (DMAP), 5  $\mu\text{g}/\text{mL}$  cycloheximide for 2 h. Cloned embryos were cultured for 20-22 h *in vitro* before transferred into pseudopregnant rabbit recipients. Pregnancy was monitored by palpation and/or ultrasound on Day 14-16 post embryo transfer (ET). One full-term but stillborn, and one live and healthy clone rabbit was delivered on Day 33, and Day 31 post ET, respectively. In the following study, we developed a working transfection protocol to target the CRP gene in rabbit fibroblast cells. Fetal fibroblast cells were chosen because these cells were shown to be successfully transfected and used to produce transgenic animals in cattle, sheep and pigs. We further demonstrated that CRP<sup>-/-</sup> fibroblasts supported a competent preimplantational embryo development

*in vitro*. A rate of 21.6% blastocyst development was achieved after NT using CRP<sup>-/-</sup> fibroblast cells. In all embryos that were examined, 100% showed the same genotype with the donor cells (CRP<sup>-/-</sup>). Our further study will focus on the production of CRP knockout rabbits. CRP<sup>-/-</sup> fibroblast cells will be used for nuclear transfer to produce founder clones (CRP<sup>-/-</sup>). Subsequently, homozygous CRP knockout rabbits (CRP<sup>-/-</sup>) will be produced by routine breeding strategy.

## ISOLATION OF RABBIT ES-LIKE CELLS WITH HUMAN ES CELL FEATURES

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Our research project aims at isolating and characterizing embryonic stem (ES) cell lines from rabbit embryos. ES cells are derived from the pluripotent epiblast of the peri-implantation embryo. They can self-renew, i.e. proliferate indefinitely *in vitro* while retaining pluripotency. Isolation of ES cell lines relies on the adaptation of epiblast stem cells to *in vitro* conditions supporting self-renewal. This has only been achieved in a limited number of species including mouse, primates and chicken. We have isolated and cultivated inner cell mass cells of rabbit blastocysts using optimized procedures for derivation of primate ES cell lines. These cultures are performed in presence of FGF2 and inactivated mouse embryonic fibroblasts. We routinely obtain flat colonies of compact cells with a high nucleus/cytoplasm ratio and prominent nucleoli. These cells are similar to human ES cells. They are phosphatase alkaline positives and express the pluripotency Oct4 gene. They proliferate very rapidly and, therefore, require passaging every two days. However, they differentiate spontaneously after six passages, a phenomenon associated with the loss of Oct4 expression. To prevent differentiation, we now aim to overexpress transcription factors involved in sustaining pluripotency in mouse and human ES cells, namely Oct4, Sox2 and Nanog. The first strategy makes use of SIV-derived lentiviral vectors to overexpress the human cDNAs in the inner cell mass cells. The second strategy is based on Tat-mediated protein transduction which allows reversible penetration of transcription factors into blastocyst cells. It makes use of the membrane penetration property of the Tat protein of HIV and has been shown to allow the entry of biologically active proteins into mammalian cells, including mouse ES cells, with high efficiency. Either strategies should prevent spontaneous differentiation of inner cell mass cells in culture and, therefore, increase the derivation of self-renewing ES cells.

## TRANSGENIC MODELS TO STUDY HUMAN AND ANIMAL DISEASES

### RABBIT AS A MODEL FOR EMBRYO DEVELOPMENT

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After having played a leading part in physiological analyses of early development few decades ago, the rabbit embryo was replaced by the mouse model and its associated genetic tools in most developmental biology studies. In the mouse however an abrupt embryonic genome activation (EGA) occurs already at the 2 cell stage concomitantly with an extensive epigenetic genome remodelling. This remodelling takes place several cell cycles ahead of the first cellular differentiation events at the blastocyst stage. In most other mammals including human, cattle and rabbit, EGA spans over several cell cycles and epigenetic remodelling is much less pronounced than in the mouse. To analyse the molecular mechanisms underlying EGA, and to study the consequences of ART (Assisted Reproductive Technologies) associated practices on embryo transcriptome, we decided to use the rabbit embryo as a model. The metabolic needs of this embryo are considered to be much closer to the human embryos than the mouse ones. Finally rabbit embryos can easily be manipulated *in vitro* and *in vivo* developed embryos are easily available. To analyse regulation of gene expression at EGA and during the first differentiation events, we developed a "first generation" rabbit cDNA array dedicated to early embryogenesis. This array displays 2000 ESTs corresponding to unique contigs expressed between EGA and blastocyst stage. Screening this array with *in vivo* developed embryos, we obtained a first representation of transcriptome variations at EGA and first differentiation in this species and identified seven clusters of commonly regulated genes. We then analysed the molecular consequences of procedures associated with ART: *in vitro* culture, and ICSI. Comparing *in vivo* and *in vitro* cultured embryos we evaluated the effect of *in vitro* culture in three distinct culture media on embryo transcriptome at two key developmental stages: EGA and first differentiation. The functional consequences of the perturbations we observed will now be analysed by following the foetal development of these cultured embryos.

### PROLONGED QT AND SUDDEN DEATH IN TRANSGENIC RABBIT MODELS FOR LONG QT SYNDROME TYPES 1 AND 2.

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The human Long-QT syndrome (LQTS) is characterized by delayed ventricular repolarization, prolonged QT-interval, ventricular arrhythmia and sudden death. Current murine transgenic animal models of LQTS are limited by substantial differences in murine and human cardiac electrophysiology. We have created the first two transgenic rabbit models for LQTS by overexpressing KvLQT1-Y315S (for LQT1) and HERG-G628S (for LQT2) transgenes in the rabbit heart under the control of the rabbit- $\beta$ -MyHC promoter to drive high-level expression in the ventricle. Injection of these constructs into embryos resulted in two founders; the presence of the transgenes and the proteins was confirmed by Southern-blot and Western-blot analyses. To establish a heart-rate (HR) correction for the QT-interval, we analysed 12-lead ECGs of 14 wild-type littermates at the age of  $113.3 \pm 24.7$  d and used linear regression to derive an equation that expresses QTc as a % of expected QT at any given HR. The age-matched offspring of both founders had markedly prolonged QTc (WT:  $99.8 \pm 8.9\%$ , LQT1:  $119.5 \pm 9.2\%$ , LQT2:  $115.0 \pm 6.2\%$ , both  $P < 0.01$  vs. WT). Spontaneous ventricular arrhythmias were detectable in LQT2 rabbits. Kaplan Meier curves revealed a markedly reduced survival of LQT2 rabbits, with a high incidence of sudden death (SD): 14 of 34 LQT2-rabbits had SD at an average age of  $255 \pm 62$  d compared to 1 of 51 littermates ( $P < 0.0001$ ). By contrast, survival of LQT1 rabbits was similar to that of WT rabbits. We observed a gender difference in QTc of LQT1 rabbits. In females we observed a gradual increase in the mean QTc during development as follows: month 2: 108.5%; month 3: 109.9%; month 4: 114.2%; month 6: 120.7%; month 7: 122.7%;  $P < 0.001$ ), while no significant change of the prolonged QTc after month 2 was seen in males. Telemetric monitoring confirmed ventricular tachycardia as the cause of death in 2 animals, and differences in the RR/QT relationship with a stepwise increase in the slope of linear regression (WT < LQT1 < LQT2). These first transgenic rabbit models of LQT1 and LQT2 resemble the human phenotype with a prolonged QTc, gender differences in QTc intervals, ventricular arrhythmias and a high incidence of SD in LQT2.

### TRANSGENIC RABBITS TO STUDY ATHEROSCLEROSIS

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Atherosclerosis development is a complex process which involves at least two key steps: 1) the recruitment of circulating monocytes which enter the vascular wall and transform into macrophages, and 2) the oxidation of low density lipoproteins (LDL) which can accumulate in macrophages, leading to foam cell formation. Over the last decades, a number of transgenic animal models have been proposed to study atherosclerosis, raising an important, yet unanswered question: what is the most relevant model to study atherosclerosis? The choice of animal models is tightly dependent on the question to be



answered, and the selection of one given animal model should be mainly governed by the following criteria:

- does it reflect the human situation in terms of lipid metabolism and lesion formation?
- what is the initial expression level(s) of studied gene(s) as compared to humans, and should it be turned up or down?
- what is the tissue specificity of gene expression in animals and humans?

For a number of reasons, rabbit has longer been recognized as a highly relevant model to study atherosclerosis:

- unlike rodents in which circulating cholesterol is mainly localized in the antiatherogenic high density lipoproteins (HDL), rabbit, as human, displays elevated concentrations of proatherogenic apolipoprotein B-containing lipoproteins when fed a high fat/high cholesterol diet
- rabbits are highly susceptible to diet-induced atherosclerosis
- as in humans, rabbit plasma contains high levels of cholesteryl ester transfer protein (CETP), whereas rats and mice are CETP-deficient species.

However, the rabbit still remains perfectible as a model to study dyslipidemia and atherosclerosis. In particular, it lacks an analogue of apolipoprotein AII and it is an hepatic lipase (HL)-deficient species. Moreover, in earlier studies, we reported that the rabbit is one of the vertebrate species with the lowest level of the plasma phospholipid transfer protein (PLTP) which in contrast is highly expressed in both rodent and human plasmas. PLTP is a secreted protein which has the ability to bind and transfer a number of amphipathic compounds, including phospholipids, unesterified cholesterol, diacylglycerides, vitamin E and lipopolysaccharides. Through its interaction with multiple molecules, PLTP was found in our laboratory to modulate lipoprotein structure, liver metabolism, vascular biology, brain physiology and reproductive biology. Whereas the overexpression of human PLTP in the mouse with naturally high PLTP activity led to contrasting observations in terms of lipoprotein metabolism and atherosclerosis, PLTP deficiency in PLTP-knocked out homozygotes crossbred with hyperlipidemic mouse lines came in clear support of a proatherogenic property of circulating PLTP. Complementary experiments with tissue-specific knocked out of PLTP expression revealed that while systemic overexpression of plasma PLTP is proatherogenic, macrophage-derived PLTP is anti-atherogenic, indicating clearly that the precise localisation of PLTP is a major determinant of its pro- or anti-atherogenicity. In an attempt to further establish the underlying molecular mechanism of the relationship between PLTP and atherosclerosis (through its ability to transfer cholesterol and vitamin antioxidants), and between PLTP and innate immunity (through its ability to transfer lipopolysaccharides) in a human-like situation (i.e. with naturally high plasma CETP activity and elevated levels of apoB-containing lipoproteins), we set up the first model of human PLTP transgenic (HuPLTPTg) rabbits in collaborative studies with the group of Professor Louis-Marie Houdebine (INRA, Jouy-en-Josas). Ubiquitous PLTP expression, leading to a controlled increase in plasma PLTP

activity, was found to produce a significant increase in circulating levels of apoB-containing lipoproteins, together with a significant decrease in their vitamin E content and antioxidant status. A marked increase in aorta atherosclerotic lesions in HuPLTPTg rabbits was observed. Overall, recent observations from our laboratory contributed to indicate that the nature of PLTP action and the predominance of its beneficial versus deleterious effects is actually determined by the metabolic context.

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## APOBEC-1 AS A POTENTIAL TARGET FOR OBESITY AND TYPE II DIABETES TREATMENT

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Many obesity related genes have been proposed as targets for the treatment of obesity. However, these obesity genes do not provide efficient drug therapy for obesity treatment. This is mainly due to the redundancy of the biochemical pathway involved in obesity and the lack of specificity of the gene targets. Obesity genes may have conferred some evolutionary advantages in times of shortage of nutrition through efficient energy exploitation as proposed by the "thrifty genome" theory. Nevertheless, when food is abundant and life become sedentary, the same genes yield obesity, type II diabetes and other obesity-related diseases. It is therefore a challenge to identify crucial gene(s) involved in energy absorption. Here we suggest apobec-1 as a potential target for obesity treatment. The advantage of this anti-obesity target; the gene is expressed exclusively in the intestine (tissue specific), it is not redundant and has a unique deaminase activity responsible for fatty acids absorption, contrary to other obesity targets which are not specific and usually are redundant. Preliminary results: Generation of transgenic rabbits expressing human apobec-1 results in obese phenotype, a work done in collaboration with INRA. Shut down of apobec-1 using RNAi technology should validated this target and open new avenues for both obesity treatment and target validation in rabbit.

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## TRANSGENIC RABBIT AS A MODEL TO STUDY PRION DISEASES

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Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of neurodegenerative diseases, including Creutzfeldt-Jakob disease (CJD) or kuru in humans, bovine spongiform encephalopathy (BSE) or scrapie in ruminants or transmissible mink encephalopathy (TME). They are characterized by the accumulation in the nervous tissue of PrP<sup>Sc</sup>, an abnormal isoform of a host encoded cellular protein, PrP<sup>C</sup> or prion protein. Interspecies TSE transmission depends mainly upon a species barrier closely linked to the sequence homology between the host PrP<sup>C</sup> and the PrP<sup>Sc</sup> in the inoculum. Mouse transgenesis has been extensively used with various mammalian PRNP genes (encoding PrP protein), either on a mouse Prnp<sup>7+/-</sup> wild type- or on a Prnp<sup>0/0</sup>- background, to overcome this barrier and analyse its molecular bases. Rabbit is one of the species that exhibit an apparent universal resistance to TSEs. None of the attempts to infect rabbit with CJD, kuru, ovine scrapie, TME or mouse-adapted-scrapie have ever been successful. We show here that transgenic rabbits harbouring the scrapie V<sup>136</sup>R<sup>154</sup>Q<sup>171</sup> allele of the ovine PRNP gene became susceptible to scrapie. This new animal model, co-expressing a totally resistant and a highly susceptible prion proteins, could be of great interest for studying TSE pathogenesis and particularly the species barrier phenomenon.

## TRANSGENIC RABBIT MILK AS A SOURCE OF RECOMBINANT HUMAN TNAP

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Gram negative sepsis an LPS (lipopolysaccharide) mediated disease is a major cause of mortality in hospitals. APs (alkaline phosphatases) are promising therapeutic agents in the treatment of LPS mediated acute and chronic diseases. LPS is dephosphorylated and thereby detoxified by placental AP at physiological pH, suggesting that APs may be considered as important host defense enzymes and LPS is a natural substrate for APs. In our earlier experiments calf intestinal AP prevented 80% of mice from lethal *Escherichia coli* infection and attenuated LPS toxicity in piglets. More recently, bovine intestinal AP efficiently attenuated the inflammatory response in a polymicrobial secondary peritonitis mouse model. Contrary to other alkaline phosphatase isozymes, purified tissue non-specific AP (TNAP) is not available in sufficient quantities from tissue sources to enable the analyses of its efficacy in animal sepsis models. In order to investigate, whether TNAP can be used in the treatment of Gram negative sepsis, we created transgenic rabbits expressing TNAP in their milk under the control of the mammary gland specific whey acidic protein /WAP/ promoter. Two transgenic lines were created by pronuclear microinjection. High

TNAP activity was measured in the milk of the #932 founder /about 1000× of normal human serum levels/, while in the milk of #949 the activity was one order of magnitude lower, but still significantly increased. The molecular weight of recombinant protein was compliant with the authentic human form. Recombinant human TNA, purified from rabbit milk - if its effectiveness is confirmed in an animal sepsis model- may be a valuable option with important opportunities to treat LPS mediated inflammatory responses.

## PRODUCTION OF TWO VACCINATING RECOMBINANT ROTAVIRUS PROTEINS IN THE MILK OF TRANSGENIC RABBITS

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Rotaviruses are the main cause of infantile viral gastroenteritis worldwide leading to approximately 500,000 deaths each year mostly in the developing world. For unknown reasons, live attenuated viruses used in classical vaccine strategies were shown to be responsible for intussusception (a bowel obstruction). New strategies allowing production of safe recombinant non-replicating rotavirus candidate vaccine are thus clearly needed. In this study, we utilized transgenic rabbit milk as a source of rotavirus antigens. Individual transgenic rabbit lines were able to produce several hundreds of micrograms per mL of secreted recombinant VP2 and VP6 proteins in their milk. Viral proteins expressed in milk were immunogenic and induced a significant reduction in viral antigen shedding after muscular injections or gavage in the presence of Freund adjuvant and cholera toxin respectively followed by a challenge with virulent rotavirus in the adult mouse model. Rectal administration of a milk extract enriched in VP2 and VP6 in the presence of cholera toxin or detoxified LT(R192G) prevented almost completely fecal shedding induced by infectious challenge in mice. The vaccine generated rotavirus-specific fecal secretory IgA, systemic IgG and IgA and a rotavirus-specific Th1 response. To our knowledge, this is the first report of transgenic mammal bioreactors allowing the rapid co-production of two recombinant viral proteins in milk to be used as a vaccine.

## PRODUCTION OF PHARMACEUTICAL PROTEINS

### PRODUCTION OF RECOMBINANT HUMAN C1 INHIBITOR IN MILK

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Pharming Technologies B.V. is developing recombinant human C1 inhibitor (rhC1INH) produced in the milk of transgenic rabbits, as a treatment for acute attacks of Hereditary Angioedema. Transgenic rabbit founders were generated by microinjection of an expression cassette, containing the bovine  $\alpha_{s1}$  casein promoter and the C1-INH transgene into fertilized oocytes collected from FSH-stimulated New Zealand White donor rabbits. Embryos were re-implanted into recipient does. A total of 170 pups were born, 15 of which were transgenic. Presence of the transgene was verified by PCR. One animal was selected to be the founder of the production colonies and was genetically characterized. A sperm bank was established by collecting and freezing semen from the founder and his transgenic offspring. The sperm bank is used to generate genitors which are nested in the colonies and used to generate production does for replacement and expansion. All production animals are housed in SPF-controlled units with overpressure, HEPA-filtered air, decontaminated water, gamma-irradiated feed and gowning procedures for personnel. A health monitoring program is in place consisting of physical examination of individual animals and frequent screening of the colony for pathogenic agents. In principle, female transgenic production animals (F4 generation) are mated with non-transgenic males every 42 d on a continuous scheme of breeding and milking. The amount of milk obtained per animal during each milking session can vary from a few grams to over 300 g depending on the individual rabbit, the rabbit lactation stage, and frequency of milking. Milk is collected into plastic bags, stored at 2-8°C until milk is skimmed and frozen. A pool of milk is used in the purification process of rhC1INH. The pooling is aimed at reducing the batch-to-batch variation in glycosylation observed during various periods of lactation. Human C1 inhibitor is a glycoprotein with six predicted sites for N-glycosylation and at least seven sites for O-glycosylation. The structures of the N- and O-glycans on rhC1INH have been determined by NMR and mass spectrometry. RhC1INH contains a broad array of N-glycans, made-up of oligomannose-, hybrid- and complex-type structures. The complex-type structures are mono- or diantennary, mono- or disialylated, and some are core- or antenna- fucosylated (Lewis X). The O-glycans on rhC1INH are of the core 1-type, mono- or disialylated. The glycosylation analysis of 23 development batches of rhC1INH and three validation up-scaled batches as described above demonstrated a high level of batch-to-batch consistency, illustrative for a robust manufacturing process of this complex recombinant therapeutic glycoprotein in the milk of transgenic rabbits.

## SPECIAL TOOLS

### GFP TRANSGENIC RABBIT AS A MODEL TO IMAGE ORGANS AND TISSUES IN SITU AND *IN VIVO*

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Advances in the understanding of animal development and physiology require the use of reliable long-term fluorescence *in vivo* imaging methods and technology. In recent years, the techniques of fluorescence, which use fluorophores and their excitation by laser, have contributed to the development of animal imaging *in vivo*. Confocal microscopes that use miniaturised optic fibres to deliver light and to measure either reflected or excited fluorescence light were used to image tissues in living animals. However, optic confocal imaging of organs and tissues has been limited by the difficulty of gaining access to these sites *in vivo*. Fibred confocal fluorescence microscopy (FCFM), also known as Cell-viZio (Mauna Kea Technologies, France), was recently conceived for *in vivo* imaging. The FCFM is built up on an optical principle of confocal microscopy, which is the ability to reject light from out-of-focus planes and to provide a clear in-focus image of a thin section within the sample. The excitation wavelength at 488 nm makes FCFM compatible with GFP transgenic animals. The other main barrier for *in vivo* imaging is that fluorescent dyes must be used to yield stable and sufficient signal without causing damage to biological tissues. FCFM technologies used to visualise different organs and tissues at the cellular level in living animals were based on the application of exogenous fluorescent dyes. However, removal of dyes by the vascular and the lymphatic networks, enzymatic degradation and the toxicity of the dyes constitute so many limiting factors that affect staining *in vivo* and do not allow reliable and continuous fluorescence imaging *in vivo*. The green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* has become the most widely used reporter protein in living organisms. The advantages of GFP are that its fluorescence is stable and species independent, and does not need exogenous substrates or cofactors. In the present study, the feasibility of FCFM to image *in vivo* and *in situ* cells expressing GFP in organs and tissues of transgenic rabbits was investigated. We also used non-transgenic female rabbits mated with transgenic males in order to visualise GFP-expressing cells in the foetal membranes. We choose a mutant form of the GFP (enhanced GFP), which fluoresces 35-fold more intensely than wild type GFP when excited at 488nm. The transgenic animals were obtained by the technique of pronuclear microinjection of DNA into fertilised rabbit eggs. The DNA construct contained the promoter of the ubiquitously expressed human EFl alpha gene, the eGFP gene, the P AC gene coding for a puromycin resistance protein under the control of an internal ribosome entry site and the insulator sequence 5'HS4 added before the promoter. eGFP gene expression during foetal development has been detected with excellent specificity and sensitivity

by FCFM. Thanks to eGFP expression in different organs and tissues, the structural morphologic feature of tongue fungiform and filiform papillae, the small intestine villi, the colon villi, pancreas, kidney cortex, bladder epithelium, lung alveoli, myometrium and adipose tissue were clearly identified by FCFM. FCFM images are close to those obtained by standard light microscopy after conventional staining. Cell structure, such as the shape, size and nuclear/ cytoplasmic ratio can be studied. This may allow histological evaluation under physio-pathological conditions in intact living animals. The transgenic rabbits used in the present study offer a potential source of tissues endogenously labelled with eGFP. This source of fluorescent tissues and the imaging technology described here open up new avenues to investigate graft localisation as well as investigations of graft morphology and function in recipient animals.

## TOWARDS THE CREATION OF A GLOBAL RABBIT RESEARCH CENTER

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In this post-genomic era, we need to elucidate physiological and pathophysiological functions of each gene using appropriate animal models. Rabbit, as emphasized in this symposium, is one of the best models for the study of human cardiovascular diseases such as atherosclerosis, diabetes, hypercholesterolemia, metabolic syndrome. They are not only valuable in studying basic science but also extremely important for the translational research, which can directly lead to the development of diagnostic tools and therapeutic strategies. However, compared to mice, the mostly common laboratory animal, rabbits suffer from several flaws that make them difficult to use. This includes (1) high cost for both purchasing and raising, (2) requirement of large space, (3) long time of generating genetically modified rabbits, (4) less genomic information and limited research reagents, etc. To overcome these problems, we need to make an effort to set up an international collaboration and make a full use of all rabbits (including transgenic rabbits). In Japan, we have organized such a "rabbit research center" supported by three universities (Saga, Kobe and Yamanashi). Through this collaboration, we can generate and breed transgenic rabbits, collect and stock sperms from different kinds of rabbits, and supply rabbit models for many researchers all over the world. To make this facility more efficient, we are applying for the government research grant for the support of this program called "National Bio-Resource Project". In this symposium, I will introduce our current commitments and hope to have a chance to get support from all rabbit researchers.

## STATE OF THE RABBIT TRANSGENIC MAMMARY GLAND OVEREXPRESSING HFVIII GENE

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The objective of this study was to compare the milk yield of transgenic and non-transgenic females, as well as histological structure, ultrastructural morphology and apoptosis occurrence of rabbit transgenic and non-transgenic mammary gland tissue during lactation and involution. New Zealand White transgenic rabbits (F3 generation) were obtained by breeding of transgenic rabbits bearing a whey acidic protein-human factor VIII (WAP-hFVIII) transgene integrated into their genome. The amount of rejected milk was measured by weight-suckle-weight method at 20<sup>th</sup> day of lactation, the content of milk protein and concentration of recombinant human factor VIII (rhFVIII) in transgenic and non-transgenic rabbits at three lactations were determined. Samples of mammary gland tissue from lactation and involution stages were isolated by surgical procedures. These samples were analysed for apoptosis (TUNEL), for histology (eosin-haematoxyline) and ultrastructure (electron microscopy). Significantly higher ( $t_{0.05}$ ) milk production at day 20<sup>th</sup> of third lactation was obtained in transgenic females compared to non-transgenic ones. Significant differences ( $t_{0.05}$ ) in content of milk protein (ranged from 10.24 to 10.79 g/100g in transgenic and from 9.64 to 9.67g/100g in non-transgenic females) at the first, second and third lactations were found. Concentrations of rhFVIII protein in the milk of transgenic rabbit females, determined by ELISA ranged between 4-9 µg/mL. No rhFVIII protein level was detected in non-transgenic rabbit milk samples. The percentage of apoptotic cells was significantly higher ( $t_{0.01}$ ) in non-transgenic comparing with transgenic mammary gland tissues (6.5 vs. 2.4%) taken at involution stage. No differences were found in the ultrastructural morphology of mammary tissue excepting the relative volume of mitochondria and vacuoles between transgenic and non-transgenic mammary gland epithelium were observed. Morphometric analysis of histological preparations at involution stage detected a significantly higher ( $t_{0.05}$ ) relative volume of lumen in transgenic animals compared with non-transgenic (60.00±10.35 vs 46.51±8.41). The transgenic mammary gland epithelium at lactation stage showed a significantly lower volume of mitochondria (11.8±1.30%) than the non-transgenic (19.8±1.40%) mammary gland. At the involution stage, a significant difference ( $t_{0.05}$ ) only in protein globules (5.0±0.60 vs. 2.0±0.60%) was found. Our results reveal no any negative effect of the mWAP-hFVIII transgene expression on the state of mammary gland of transgenic rabbit females.

