Final report on "Comparative analysis of paternal protein's transmission to zygotes" OTKA /NKFI NN 108921 contract

Background of the project

The laboratory rabbit is the third most often used experimental mammal (2.78 %) within the EU (EU report 2010: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2010:0511:REV1:EN:PDF). The laboratory rabbit is prolific with short generation time and can be raised under specific pathogen-free conditions. The rabbit is the primary source of polyclonal antibodies and recent transgenic rabbit models significantly increased the antibody production potential of this platform [1,2]. Transgenic rabbit is also used as bioreactor for the production of pharmaceutical proteins. Ruconest®, the second milk-born recombinant product worldwide, produced and purified from transgenic rabbit's milk has already reached the EU market (http://www.pharming.nl/index.php?act=prod).

Rodents, rabbits and other mammals all have particular limitations and strengths as animal models in biomedical research and are best regarded as complementary. With the advent of novel transgenic methods the renaissance of laboratory rabbit as a reproductive model is also expected, due to its advantages in early embryogenesis compared to rodents, such as high cell numbers, yield of blastocysts and late implantation time. As we summarized in a recent review publication among the already existing transgenic rabbit strains the models of cardiovascular diseases are one of the most important [3]. There is a *continuing need* for developing simple, effective and reproducible technologies for genome manipulation in animal models. The Rabbit Genome and Biomodel group at NARIC-Agricultural Biotechnology Institute has been working on developing and adapting novel technologies to rabbit [4,5] and creating rabbit models for enhanced antibody production [6] or as cardiac arrhythmia model [7] among others.

Results

I. In the frame of a German- Hungarian collaboration (OTKA NN 108921), we described a detailed protocol for high-efficiency germline transgenesis and sustained transgene expression in rabbits by using the *Sleeping Beauty* transposon system [8]*. The protocol is based on co-injection of synthetic mRNA encoding the SB100X hyperactive transposase together with circular plasmid DNA carrying a transgene construct flanked by binding sites for the transposase into the pronuclei of fertilized oocytes. Upon translation of the transposase mRNA, enzyme-mediated excision of the transgene cassettes from the plasmids followed by permanent genomic insertion, produces stable transgenic animals. Transposition-mediated gene delivery is a method that can easily be implemented by any laboratory, thereby providing investigators with an attractive method to genetically modify animals at a more cost-effective manner for biomedical, agricultural and pharmaceutical research. SB transposon-based gene delivery combines the advantages of viral vectors with those of naked DNA molecules (simple, safe and inexpensive manufacture of plasmid vectors). SB transposition is a cut-and-paste mechanism that only involves DNA and transposon vectors can tolerate larger and more complex transgenes, therefore SB system is not strictly limited by the size of expression cassettes [9].

Because the transposase is only transiently present in the cell, the integrated transposon is stable (will not undergo further rounds of transposition). This feature makes transposons easily controllable DNA delivery vectors that can be used for versatile applications, including germline gene transfer. A hyperactive variant of the SB transposase, called SB100X, was recently developed by *in vitro* evolution[10], and shown to support efficient germline transgenesis in mice[5,10], rats[5], rabbits[5] and pigs[11] with the coauthorships of the participants of this research project.
Transposition results in precise (the ends of the integrating DNA are well defined) genomic integration of monomeric transgene units within a short timeframe following administration, thereby minimizing mosaicism. Furthermore, unlike retroviral vectors [12-15], SB100X transposase-catalyzed transgene integration does not seem to trigger transcriptional silencing [5,16].
The adaption of the *Sleeping Beauty* (SB) transposon system to rabbit as described by ourselves will significantly enhance the rabbit genomic toolbox.

II. The generation of transgenic mice, rats, rabbits and swine carrying an identical Venus reporter construct delivered by transposon-mediated gene transfer enables comparative studies of gene expression in these lines of mammalian models. Whereas comparable expression patterns of the Venus reporter were found in somatic tissues, preliminary studies suggested that a striking difference in reporter expression may exist in mature spermatozoa of these species.
In the frame of this research project our laboratory could show the differential expression of Venus reporter protein during spermatogenesis of the two compared species, the laboratory rabbit and mice [17]*. We provided the first evidence for the functionality of intercellular bridges in the male germline and genotype-independent transgenic phenotype of rabbit spermatids. Our data suggest that the reporter rabbit line may be a *suitable tool to identify molecular mechanisms in testicular development*, and may contribute to develop *better non-mouse animal models for male infertility in men*.
Previously, Venus fluorophore expression was analysed in detail in SB transgenic boars and genotype-independent expression was detected in the mature spermatozoa [18]. Surprisingly, preliminary observations indicated that SB-transgenic mouse spermatozoa did not express Venus. Those independent observations suggested that the terminally differentiated spermatozoa may represent an exception from the comparable expression pattern of the Venus reporter seen in somatic tissues of the SB transgenic species (mouse, rat, rabbit and swine). Those observations lead us to examine by identical methods Venus expression in spermatozoa and testis of SB-transgenic rabbits and mice. Spermatozoa obtained from the epididymis of homozygous males of *three different transgenic mouse lines were Venus-negative* [17] contrary to the transgenic pig's and rabbit's, although those transgenic animals were created with the exact same transgene and technique.

The potential influence of an ectopic protein expression on sperm quality and the SB transgenic buck's litter size was also evaluated [17]*. Transgenic rabbits expressing fluorescent reporters (EGFP) under control of the cytomegalovirus enhancer, chicken beta-actin (CAG) promoter were already created and characterized earlier e.g. for organ transplantation, however transgene expression in the ejaculated spermatozoa have not been analysed before [19-21]. In the frame of this project we could clearly show the *differential expression* of Venus reporter protein during spermatogenesis of the three compared species, the swine, laboratory rabbit and mice.

SB transgenesis with the CAG-Venus transgene resulted in fluorophore-expressing spermatocytes, spermatids and mature spermatozoa in the laboratory rabbit. The intensity of expression correlated with the transgene copy number, lining up with the observations made in the SB-transgenic pig lines [18].

Our observation that haploid spermatids of a hemizygote transgenic buck containing a single copy of the Venus transgene at 50% ratio (that are therefore genetically distinct) *all expressed the Venus protein suggests that either the transgenic mRNA or protein moved between the syncytial intercellular bridges*.

Formation of intercellular bridges in the rabbit male germline were published [22,23], however evidence for the functionality of intercellular bridges in this species has not been reported to date. The testis-expressed gene 14 (TEX-14) is a marker for sperm cell intercellular bridges.

The presence of TEX-14 protein in the testes intercellular bridges of transgenic rabbits was shown, and as outlined above we provided the first evidence of their function. *We also showed that hemi- and homozygote SB-transgenic spermatozoa carrying the ectopic Venus protein fulfilled the standard semen quality requirements*. Indeed, these semen samples were used for artificial insemination through four generations, and *neither reduced fertility nor smaller litter sizes were experienced [17]**.

Human male infertility is a regrettably common and complex problem. Male infertility may be caused by genetic, epigenetic, environmental, and nutritional factors, but the background of the male infertility often remains unknown. The important periods of pre-spermatogenesis and timing of testicular development of the laboratory rabbit are closer to human than to rodents. Thus, our results indicate that the Venus-transgenic rabbit could be used as a model for detailed analysis of spermatogenesis. Alternatively, it might be interesting to create novel SB-transgenic rabbit lines, in which the fluorophore protein is combined with expression of a mutant human gene causing sterility.

III. The use of transgenic animals, particularly rabbit, swine, small ruminants and dairy cow, as bioreactors ("biopharming") is a cost-effective alternative to cell culture methods. Animals automatically supplement their body fluids with fresh nutrients, remove waste products, reliably regulate their internal temperature and pH and resist pathogens. By directing (*or targeting)* the expression of the transgene product so that it is produced by the secretory cells of the liver, lactating mammary gland or kidney, "pharmers" may collect and process body fluids with minimal effort. The mammary gland is probably the most promising target tissue because it produces large amounts of protein in a temperature-regulated fluid that may be collected daily in a non-invasive fashion.

It was eighteen years ago, when six generations of transgenic rabbits with stable integration and production of biologically active IGF-1 in their milk was reported [24]. The lack of any negative effect on their physiological or reproductive performance was found [24]. Since then, more than 200 foreign proteins have been produced experimentally from different organs and in several animal species, though milk remained the main source of recombinant protein production [25].

Alternatives for recombinant protein production in biological fluids:

Urine: Urine is an abundant biological fluid already used to prepare proteins such as gonadotropins for pharmaceutical use. The system allowed expression in urothelium may be useful if it happens that the foreign protein is maturated in a more appropriate manner in urothelium than in the mammary gland or if the side-effects of the protein are less deleterious for the animals.

Seminal plasma: Seminal plasma is relatively abundant biological fluid in some species and it can be easily collected. This system, as urine, may have specific advantages. In both cases, it is not known how complex proteins are matured and secreted.

Together with the German partner, we described a *radically different approach to achieve high levels of recombinant proteins in the milk* of transgenic pigs [26]*. Scientists at the German laboratory analyzed the milk of lactating transposon sows, and found high level Venus protein expression, albeit the design of the transgenic construct did not include a signal peptide for the secretory pathway, which is thought to be critical for the transport of recombinant proteins into the milk.

Based on the unexpected results in the German laboratory, we have analysed the expression of the Venus reporter protein in the biological fluids of the transposon transgenic rabbit line. Our results show that the SB-CAG-Venus transgenic rabbits secrete recombinant protein into milk, tear oral saliva, urine and seminal fluid, despite the lack of gland specific promoters and signal peptide cDNA for the secretory pathway (*Figure 1*).

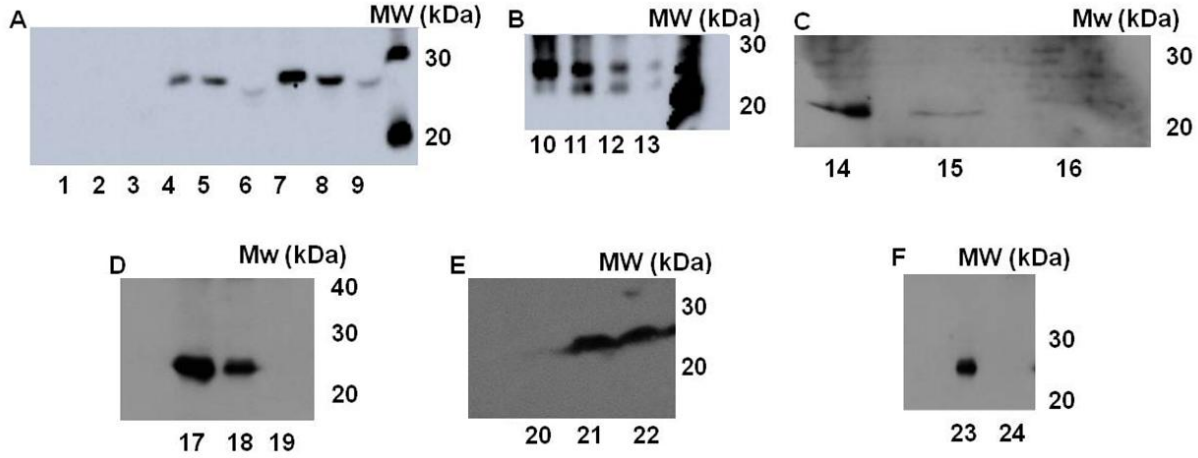

Figure 1. Western analysis of Venus recombinant protein production. A: Milk fractions 1,4,7 whey, 2,5,8 fat, 3,6,9 somatic cells; 1-3 control milk, 4-6 heterozgote doe milk, 7-9 homozygote doe milk; B: concentration curve, 10:250ng;11:125ng;12:62,5ng;13:31,25ng C: Saliva 14:homozygote;15: heterozygote;16:control; D: Tear 17:homozygote;18: heterozygote;19: control; E: Seminal plasma 20:control;21: heterozygote;22 homozygote; F Urine 23: homozygote;24 control. The Mw of the Venus protein is 27kDa.

Our unpublished results (Kerekes et al. manuscript in preparation, 2016) will contribute with novel observations to the publication on Venus expression in sow milk at different points:

1. in the SB transgenic rabbit Venus protein expression is not restricted to milk, but present in other biological fluids;
2. our novel observation is, that beyond the milk, the seminal plasma or urine could be also an alternative to produce recombinant proteins;
3. transgene dose effect was detected on recombinant protein production with Western blot and densitometry analysis, when the homozygote and heterozygote milk fractions were quantified (*Table 1*);

Doe ID	**#4034 (SB-CAG-Venus heterozygote)**			**#4035 (SB-CAG-Venus homozygote)**		
Milk fractions	Whey	Fat	Milk cell	Whey	Fat	**Milk cell**
Venus protein (ng/20µg)	**108,59**	**125,26**	**19,55**	**357,48**	**238,79**	**49,57**

Table 1. Transgene dose effect on recombinant protein production in SB-rabbit milk fractions

In our previous findings, Venus protein was localized in the cytoplasm. The described transport of recombinant proteins in the transposon transgenic sow and rabbit milk, "trapped in the cytoplasm" of milk cells may be of interest *for proteins, which do not require glycosylation* (phosphorylation, lipidation) *for biological activity*, and for proteins, which can be glycosylated *in vitro*. One particular advantage of cytoplasmic expression of the recombinant protein in the mammary epithelia is that it is protected from degradation processes occurring in the lumen of the mammary gland, or from aggregation with fat micelles, which may complicate the purification.

IV. Investigation of the possible presence of transgenic (TG) cells or gene products in domestic animals is important due to the unpredictable impacts of TG animals on food chain. Fetal and maternal microchimerism could be a potential source of TG cells in non-TG animals. Microchimerism defined as the occurrence of small number of foreign cells (less than 1:100 cells) in the host animal. In the frame of this project, we also examined the fetal-maternal, maternal-fetal and fetal-fetal cell microchimerism in transgenic rabbits during pregnancy taking advantage of the Venus fluorophore reporter protein expression in all examined organs and tissues. Three different methods (fluorescence microscopy, flow cytometry and quantitative polymerase chain reaction) were employed to search for TG cells and gene products in blood and other tissues of non-TG rabbits *(Fig 2*). Significant difference was observed in percentage of Venus positive cells between adult and newborn TG rabbits by flow cytometry, suggesting the age-dependent expression of Venus protein in peripheral blood mononuclear cells PBMCs (*Fig 2 A and E*).

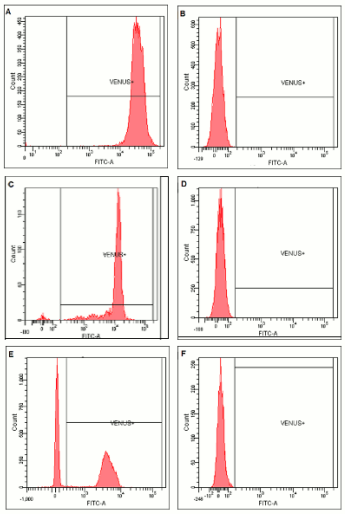

Fig. 2: Detection of TG cells in pregnant SB-TG rabbits by flow cytometry

A*: SB-CAG-Venus TG buck;* ***B****: non-TG buck;* ***C****: pregnant SB-CAG-Venus TG doe(inseminated with contol ♂);* ***D:*** *control (non TG) doe at 20th day of pregnancy inseminated with TG sperm;* ***E:*** *SB-CAG-Venus* TG newborn (TG♂ x non-TG♀ or non-TG♂ x TG♀); ***F:*** *non-TG newborn (TG♂ x non-TG♀)*

Our data were summarized in a manuscript which is under revision at Transgenic Research (Lipták et al. 2016). Monitoring of Venus transgenic cell migration during pregnancy in non-transgenic rabbits. TRAG-D-16-00063) and showed the lack of detectable cell transfer between TG and non-TG rabbits during gestation.

*Note: Our publications which were published with the OTKA/NKFI grant number 108921 are labelled with *asterisk in the report.*

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