Establishment of a guinea pig platform to study disease models in vivo

Identifier: NN113162

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Introduction

Guinea pigs are widely used as laboratory animals to study the background of human diseases. Guinea pigs have some special biological similarities to humans. That is the reason why these animals are extremely important on some special research fields.

Allergy and respiratory diseases are the most important research areas where guinea pig models are used. They are the best models for extreme allergic reaction called anaphylactic shock as other species can not show strong signs. The respiratory tract of these animals are sensitive to allergens so asthma studies can be investigated using inhaled medications (1).

Guinea pigs differently to other laboratory animals but similarly to human require Vitamin C in their diet and high levels of folic acid, thiamine and arginine (2).

Most of their plasma cholesterol is carried in low density lipoprotein so guinea pigs are also good models to study atherosclerosis. This phenomenon can be found in laboratory rabbits also but as they are more easy to breed rabbits used more frequently in atherosclerosis as model animals.

Innate and adaptive immune system, their complement system, infection induced IFN-γ response and expression patterns of immune system genes (p35 and p40, CD8, and Leukocyte Antigen, CD1, IL-8 and CXCR1) are also more close to human than other models which make these animals valuable in immunology.

These animals are preferred animal model for studying auditory systems as their ear is similar to that of humans while their hearing range is also similar.

Transgenic technology has been accelerated in the last decade. Completely new techniques appeared like transposon mediated transgenesis and genome editing technologies. These new technologies are relatively cheap, easy to perform and effective enough to change model animal studies.

The ultimate aim of our study was to produce the first transgenic guinea pig using the new technologies. There were several attempts to produce transgenic guinea pigs using traditional microinjection but all of them failed.

Our idea was that the high efficiency of transposon based transgenesis can produce transgenic newborns. The project has been fallen in the high gain-high risk category.
Producing the first transgenic guinea pig would be an important step in animal biotechnology. Widespread expectance appeared in animal model community to get transgenic guinea pigs. Several patented technology containing claims of transgenic guinea pigs however nobody was able to produce them (3).

Preliminary studies

Our project started as an international collaboration project (NN). Our collaboration with Zoltán Ivics has started in 2010 at the Transgenic technology meeting in Berlin where we introduced our lentiviral results in rabbits and we were informed about the efficiency of the Sleeping Beauty technology.
Later we used their sleeping beauty technology to produce transgenic rabbits and rodents with high efficiency. Using a non viral technology we were able to establish transgenic founders which all expressed the fluorescent venus protein. Transgenes were integrated randomly but never hit coding regions of known genes. Transgenes were integrated with low copy numbers. We published our results with very high impacts in four different papers (4-7).

Results

Animal room for guinea pigs

As a first result we have established a Guinea pig colony and a guinea pig room in the NAIK-ABC. All equipments suit the latest EU requirements (valid from 2017). This room was the first animal room in the institute which fulfills all EU directives. We can keep maximum 100 animals at once. The room and the equipment can be used later for other guinea pig studies our even for other rodent experiments (fig 1).


Fig1. The new animal room for guinea pigs and the new EU cages for guinea pigs. The cages later can be converted to keep different rodents.

Superovulation protocol

One of the first but most important basic technique in transgenesis is superovulation. Guinea pigs have relatively long oestrus cycle. The first step was to determine the exact oesrus cycle of our animals. We used Dunkin hartley guinea pigs in our laboratory. These animals are albino ones and currently the only available laboratory strain in Hungary. The oestrus cycle of our animals was found to be between 16 and 23 days. The first protocol we used was the PMSG/HCG protocol which is frequently used in laboratory animals. A Chinese publication indicated that it was useable in guinea pigs. We failed to reproduce their results. Instead we modified a superovulation protocol from Japan. They used Human Menopausal Gonadotropin (hMG)(8). As our oestrus cycle was a little bit shorter than Suzuki reported the hMG was administrated on days 15, 16 and 17 post ovulation intraperitonealy. We used 5-6 IU/kg/day for superovulation in the first year which resulted 5,6 zygotes averages per donor animals. Later we reduced the amount of hormones to 3 IU/kg/day. Decreasing superovulation hormones resulted only 3,1 zygotes per donor animals but in vitro development of these embryos were elevated.

Zygote or embryo collection was similar to other laboratory animals. Zygotes or early embryonic stages were collected from oviducts while morula and blastocyst stage embryos were collected from uterus horns.

Embryo culture

Flushed zygotes/embryos were cultured in vitro in different medias. We tested three different medias (M16, KSOM, RDH) and several conditions. In our hand RDH media was best media. We reached 75% embryo survival rate till morula stage using RDH media. Interestingly the best results were obtained using elevated level of CO2 (5,5%). Reduced O2 level with increased N2 level has not increased the survival rate. We always used rabbit and mouse zygotes as positive controls in all cases. Later we realized that that supportive rabbit embryos are helpful. We cocultured rabbit and guinea pig embryos and were able to reach 45% blastocyst stage (fig 2). The ratio of supportive (healthy) rabbit embryos- guinea pig embryos were 2:1 in culture media droplets

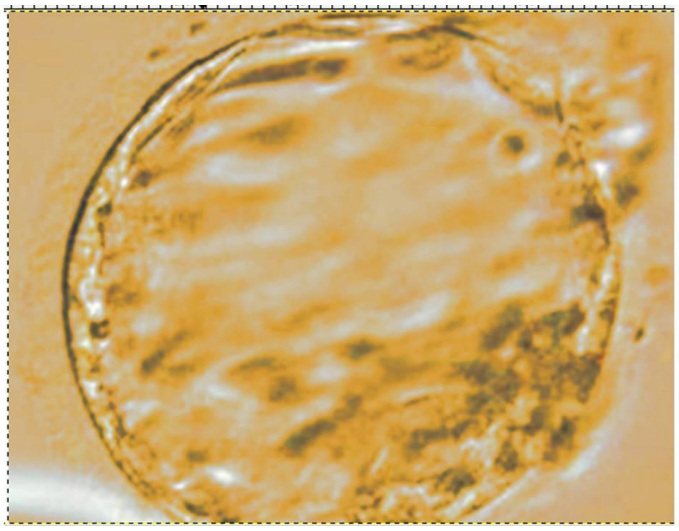

Fig 2: In vitro cultured guinea pig blastocyst

Anesthesia

To perform a successful surgery method for embryo transfer an efficient anesthetic protocol should be used. We have developed a new protocol which is safe, efficient and does not require special equipments and/or drug permits from Narcotic Drugs Control Department. A combination of three different anesthetics was useful for a complete anesthesia. Our mix was the following:250 mg zoletil powder was diluted in 10 ml 20 mg/ml xylazine +0.75 ml 10 mg/ml butorphanol. 0.15 ml/ kg of the mix was used as a subcutaneous injection

Sleeping Beauty transposon system for efficient transgenesis

DNA transposons can integrate into the chromosomes of the host cells. This feature forms the basis of their use as vectors in transgenesis. DNA transposons harbor a gene encoding a transposase with inverted terminal repeats carrying transposase binding sites. During transposition the transposase excise and integrates the DNA sequence found between the terminal repeats. Transposon systems are usually used as bi component systems in biotechnological applications. DNA sequences of interest are cloned between the transposon ITRs and supplementing transposase enzyme is added (expression plasmid or RNA). The first transposon which was capable to perform efficient transposition in vertebrate cells and was molecularly reconstructed from a fish named Sleeping Beauty in Zoltan Ivics's laboratory.

Our bi-component transposon vector system for delivering transgenes are maintained in plasmids. One component contains a gene of interest (GOI) cloned between the transposon inverted terminal repeats carried by a plasmid vector. Our GOI was a fluorecent reporter gene called Venus. The other component was an mRNA encoding the transposase. The transposon carrying a GOI is excised from the donor plasmid and can be integrated at a chromosomal site by the transposase.

The whole SB transposon system we used was developed and produced in our collaborators laboratory. In vitro molecular evolution experiments resulted a hyperactive variant of the Sleeping Beauty system called SB100X. This system has been shown to produce efficiently germline transgenic rodents and rabbits in our collaboration earlier (4-7).

Our collaborator prepared and sent the pcGlobin2-SB100X plasmid coding the transposase and the pT2venus plasmid as a reporter gene construct. They have purified the donor plasmid for microinjection (pT2venus) and in vitro transcribed the SB100X transposase mRNA using the original plasmid (pcGlobin2-SB100X). We have determined the halflife of the in vitro transcript which was more than 4 hours at 37 °C. It means that this purity is absolutely enough for microinjection into one cell stage zygotes as microinjection lasts max one hour. They have optimised the microinjection mixes and found to be most efficient in a concentration of 0.4ng/ul circular reporter plasmid and 5ng/ul SB100X mRNA.

We have successfully tested our transposon based construct in guinea pig cells. Fibroblast cells were isolated from guinea pigs and the transposase/reporter gene constructs were transfected to guinea pig fibroblasts. Reporter gene expression was detected even 7 days following transfection which represent stable transfected guinea pig cells (fig 3.). Cell transfection efficiency was less effective than in mouse fibroblast cells.

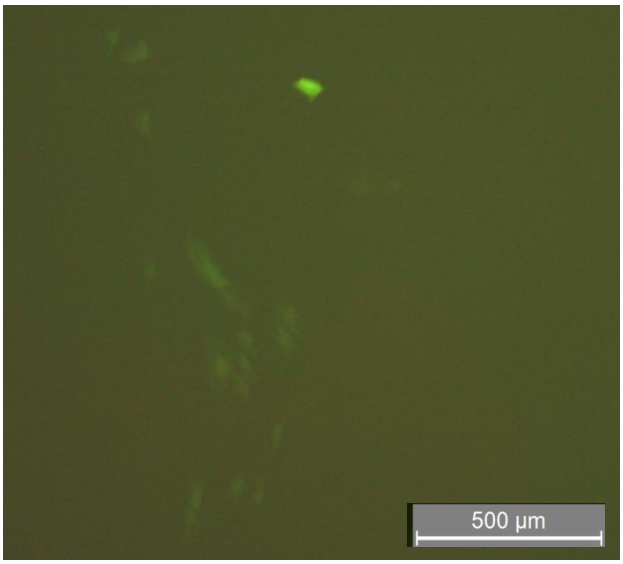

Fig 3. Transfected guinea pig fibroblasts. Green cells expressing the venus fluorophore seven days after transfection. 20% of the cells expressing the transgene.

Microinjection

We have optimized the microinjection of the guinea pig embryos. We have developed a new microinjection capillary adapted to guinea pig zygotes. Most modern transgenic techniques (transposon based techniques and genome editing) are all using cytoplasmic microinjection of the construct. It seems that cytoplasmic microinjection is easier to perform in Guinea pig embryos than in mice. We tested the amount of the microinjection construct and ascertained that 3 pl can be microinjected at a survival rate of 85% while 1.5 pl injection resulted in 91% survival rate.

Later we have also shown that the microinjected reporter gene construct was integrated into the genome of the embryo.

We have microinjected 14 guinea pig embryos from 6 donors. 12 survived zygotes were cultured and five were developed till blastocyst stage using supportive rabbit embryos. Genomic DNA was isolated from the embryos and were tested for the presence of the transgene by PCR. We were able to show the presence of the transgene in one blastocyst (fig 4).

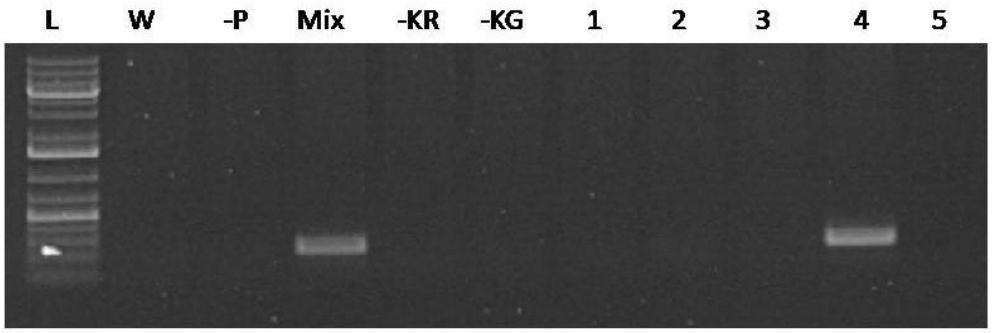

Fig 4: PCR detection of the transgene in a guinea pig blastocyste. L-DNA ladder; W-water control, -P-negative plasmid control; Mix-microinjection mix; -KR- negative genomic rabbit DNA control; -KG- negative genomic guinea pig DNA control; 1-5 injected guinea pig blastocysts.

Genomic PCR test was only an indirect evidence as plasmids can survive in living cells as episomes.

The DNA from the positive blastocyst was used in further experiments. We designed oligonucleotide primers to determine the integration site of the transgene using inverse PCR technique. According to the inverse PCR the transgene was supposed to integrate into two different genomic regions. We were able to isolate and sequence one of the two integration sites. The transgene was integrated into a TA target site as usual. The integration site was found to be in an intergenic region, similarly to rodents (fig 5.). This is the first time where experimentally justified transgene integration was shown in guinea pigs. The integration site was not joined to an exact chromosome as guinea pig genome is sequenced but not fully annotated. The integration site was found to be on scaffold_44.

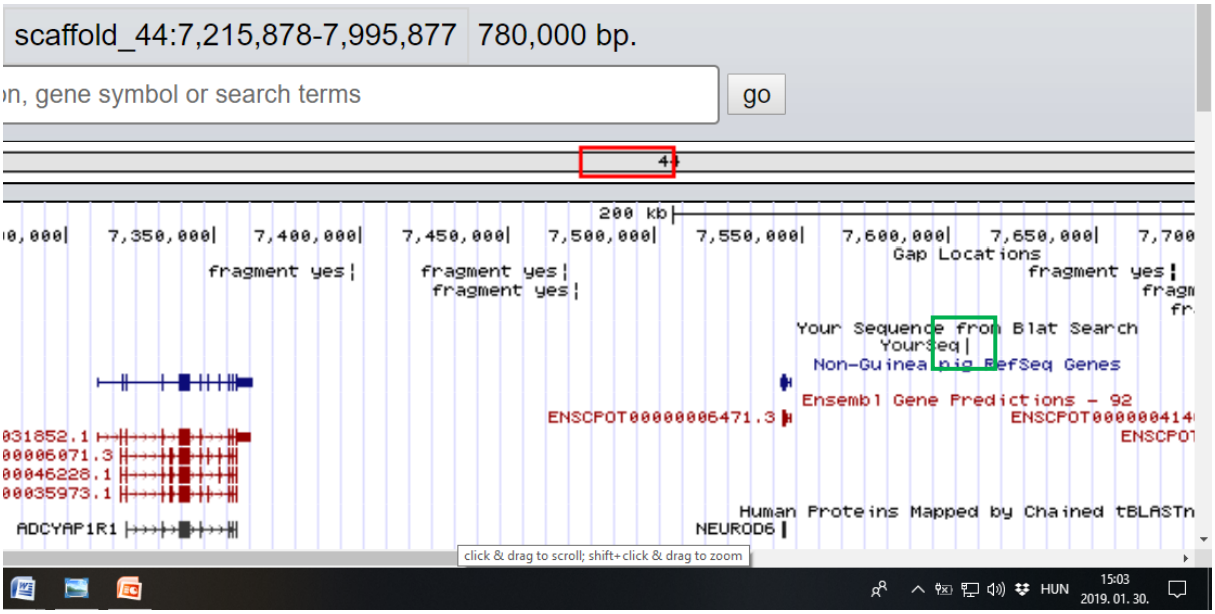

Fig 5. Screenshot of the integration site of the transgene. Green rectangular shows our integration site. Red lines symbolize guinea pig genes. Blue lines are non- guinea pig reference genes. The integration occurred far from known genes.

We have also built a lentiviral construct with GFP as a marker gene under the control of EF-1 (elongation factor 1) promoter. Lentiviral constructs usually work with very high efficiency in different species. This construct was built as a “B” plan if transposon based manipulation would not be efficient enough.

According to our results transposon based constructs are efficient in guinea pig cells (both fibroblasts and embryo). So ee have not used this lentiviral construct in guinea pig micromanipulation experiments later.

Genome editing experiments

We have also performed some initial steps to produce a knock-out guinea pig. It was not an original plan but in the meantime CRISPR/CAS9 system developed rapidly. As this technique is also very effective we have designed and produced a CRISPR/CAS-9 construct to knock-out the Myostatin (MSTN) gene of the guinea pig. This gene is an easy target and the lack of it would not produce any lethal effect not in heterozygous nor in homozygous animals.

We have microinjected 9 guinea pig embryos from 3 donor animals. All 9 survived zygotes were cultured and four were developed till morula-blastocyst stage. Genomic DNA was isolated from the embryos and were tested for any events produced by CRISPR/CAS9 by T7 assay (Fig 6.) In two cases we were able to detect indels produced by the CAS9.

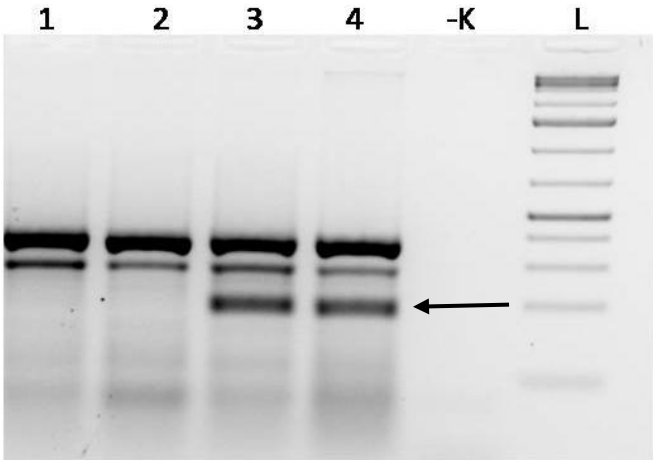

Fig 6: T7 assay of DNA samples from microinjected guinea pig embryos. In embryos 3 and 4 an extra band show the nuclease activity of the T7 enzyme. These genomes harbor indel mutations as we planned. Arrowhead shows the extra band

The exact mutation from embryo number 3 was also determined. Primers were designed to amplify a 300 bp region containing the CAS9 cutting site. The resulted DNA fragment was purified and cloned into pGEM T-easy vector and sequenced.
The mutation in embryo no.3 was a heterozygous mutation and found to be a four base pairs deletion (fig 7).

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Reference sequence atgaaagacggtacaaggtata
                   ||||||||||||||||||||
Embryo no3         at----gacggtacaaggtata
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Fig 7a. Side by side alignment of the reference sequence and sequence from embryo no 3. We detected a four bp deletion.

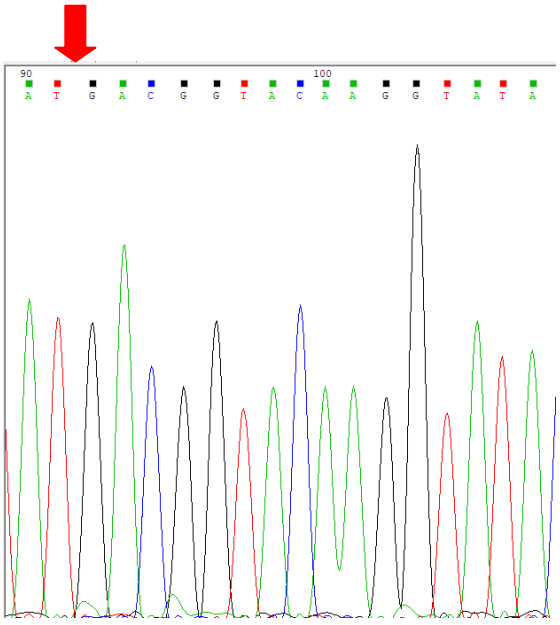

Fig 7b. The electroforetogram of the mutant MSTN allele. Red arrowhead shows the place of the mutation.

These results verified that we have produced the first knock out transgenic guinea pig embryos in the world.

Embryo transfer

The first surgery method we tested was the uterine transfer. Uterine transfer can be applicable for late morula or blastocyst stage embryos. First we tested only the surgery method, so we used vital stains only instead embryos. We performed five surgeries. All recipients survived. Similarly we have also performed the initial steps of oviduct transfer. This microsurgery method should be the ideal method for zygote transfer as it requires less in vitro culture however we found this method is more difficult to carry out. 4 of the first 6 recipient survived the embryo transfer.

The next step was to repeat the oviduct transfer using flushed zygotes. We performed four zygote transfer. Two- two zygotes were transferred to both sides of the oviducts. Three animals survived but we got no newborns.

In the second year of the project we performed 22 different transfers without newborns. In these experiments we used microinjected zygotes. Females were sacrificed and investigated 60 days following embryo transfer. We could not detect any successful implantation sites in the females. (The length of pregnancy in guine pigs is 58-65 days).

Next we established a new surgery protocol where early embryos without any manipulation were transferred to the infundibular part of the oviduct. Several trials (8 recipients) have not resulted successful pregnancy.

We have also adopted a technique where embryos were transferred to the ampullary or to the isthmic part of the oviduct. In this experiments females were sacrificed within 96 hours and embryos were reflushed from the oviduct-uterus. Interestingly further embryo development was only detected when embryos were transferred to the isthmic part of the oviduct (in 2 recipients from 9). This phenomenon is very unusual and none of the laboratory animals show this strange effect.

In 2017 we refined the isthmic embryo transfer. We were able to show that after reflushing, 23% of the embryos were able to develop to early blastocyst stage in the uterus (3/13 transfer). We have also performed refined isthmic transfers without reflushing the embryos from the uterus. Unfortunately we did not get any progeny however we were able to detect terminated early pregnancies two times. Terminated implantation sites in the uterus suggest that pregnancies were lost before 12th day.

Genetic background can be a very important issue related to embryo transfer. It is not unique for guinea pig but also for mice. Embryo transfer efficiency is very low in some inbreed mouse lines. Until now only one “half successful” guinea pig embryo transfer was reported in a conference from Peru where blastocyst stage embryos were transferred which resulted stillborn animals. In this experiment they did not used the laboratory version of guinea pigs (Dunkin-Hartley Guinea Pig). Researchers used native animals. Unfortunately European rules exclude the possibility to use non laboratory origin of animals and the only available laboratory strain in Hungary is Dunkin-Hartley. Our hypothesis is that embryo transfer could be highly variable between guinea pig strains.

We reused our firstly developed uterus transfer method again. 17 embryo transfers were produced using non-manipulated morula-early blastocyst stage embryos. None of the transfer resulted successful pregnancy.

We have performed limited number of embryo transfers (8 recipients) using guinea pig females with mixed genetic background (the male grandparent line was not Dunkin-Hartley). Unfortunately further progress was not achieved. No newborns were produced from embryo

transfer experiments. It seems that the limiting factor of transgenesis in guinea pigs is embryo transfer.

Low-cost method for time-lapse monitoring of embryo development

During our guinea pig embryo experiments we have realized that these embryos are more sensitive to light, and other environmental factors than other rodent embryos. Following embryo development requires methods (handling, temperature changes, strong light changes under microscope) which are stressful. Successful embryo manipulation techniques require efficient transfer of embryos that will implant and develop correctly. Time-lapse microscope technology enables continuous observation and selection of embryos with higher implantation potential from the fertilization to the point of embryo transfer. Time-lapse imaging can follow developmental markers such pronuclear formation cleavage patterns and timing, embryo fragmentation. Time-lapse microscopy is an effective and non invasive method for observation of embryo morphology and development.

To minimalize stress problems we decided to build a time lapse embryo monitoring system. These systems are very expensive so there was no possibility to buy one of them from the grant. We have built an ultra low-cost time lapse microscope (fig 8.) from a conventional stereo microscope with phototube and a commercially available sport action camera. This equipment would be useful for determining the optimal embryo transfer time in guinea pigs.

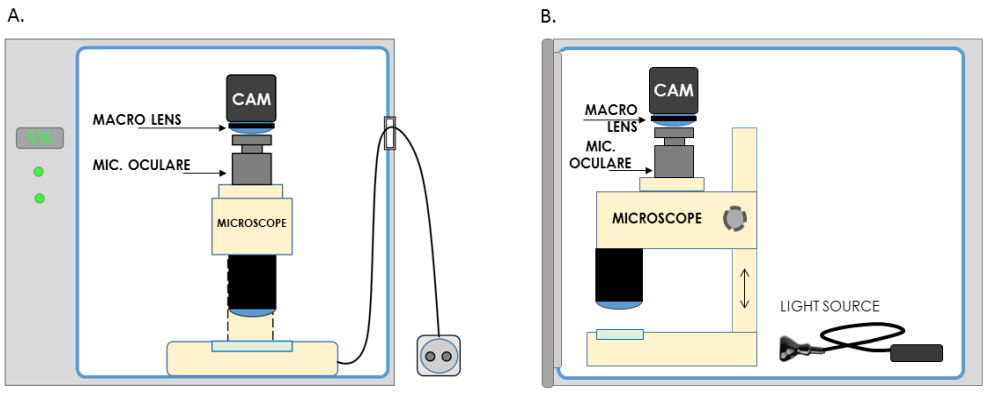

Fig 8. Laboratory built ultra low cost time lapse camera system

The system worked precisely. We have prepared a technological manuscript describing the equipment. The planned title would be: "A simple, low-cost method for time-lapse monitoring of embryo development by GoPro action camera" by Péter Major, Gergely Iski, Zsuzsanna Bősze, László Hiripi

Further improvements in transgenic techniques

Placenta specific gene manipulation

As I mentioned before we used rabbit embryos as supportive embryos in our experiments. Additionally we have produced a lentiviral construct with GFP as a marker gene under the control of EF-1 (elongation factor 1) promoter as a "B" plan if transposon based manipulation would not be efficient enough. Using these supportive embryos and GFP lentiviral construct we were able to develop a new transgenic technique in rabbit called placenta specific transgenesis.

Lentiviral gene constructs can be efficiently and specifically delivered to trophoblast cell lineages in rodents. In vivo genetic manipulation of trophoblast cell lines enables functional and developmental studies in the placenta. We have shown that genetic modification can be produced in the extraembryonic tissues of rabbits also by lentiviral gene constructs.

When 8–16 cell stage embryos were injected with lentiviral particles, strong reporter gene expression resulted in the rabbit placenta (fig 9). The expression pattern displayed some mosaicism. A strikingly high degree of mosaic GFP expression was detected in some parts of the yolk sac, which is a hypoblast-derived tissue. Whereas expression of the reporter gene construct was detected in placentas and yolk sacs, fetuses never expressed the transgene (fig 9.)

As rabbits are an ideal model for functional studies in the placenta, our method would open new possibilities in rabbit biotechnology and placentation studies. We have published our results in the Journal of Biotechnology.

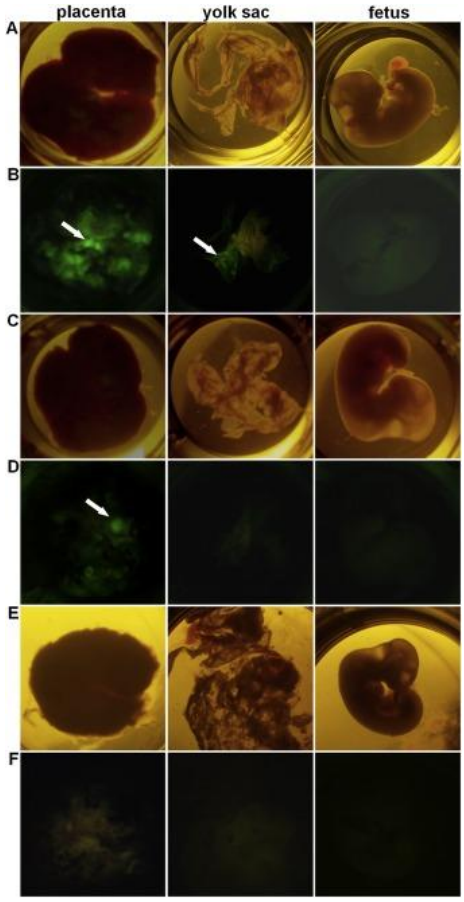

Fig 9. Microscopic analysis of placentas, yolk sacs and fetuses. A: No.3 placenta, yolk sac and fetus under bright field; B: No.3 placenta, yolk sac and fetus under dark field with UV light; C: No.5 placenta, yolk sac and fetus under bright field; D: No.5 placenta, yolk sac and fetus under dark field with UV light; E: Negative placenta, yolk sac and fetus under bright field; F: Negative placenta, yolk sac and fetus under dark field with UV light. White arrows show GFP expression. Only extraembryonic tissues expressing the GFP.

Secretion of a recombinant protein without a signal peptide by the exocrine glands of transgenic rabbits

We have also used Venus expressing rabbit embryos as supporting embryos for the guinea pig experiments, and found an interesting phenomenon in these animals Previously we generated a Venus expressing transposon transgenic rabbit line. The Sleeping Beauty (SB) transposon transgenic construct contains the Venus fluorophore cDNA, but without a signal peptide for the secretory pathway, driven by the ubiquitous CAG promoter. Despite the absence of the signal peptide, the fluorophore protein was readily detected in milk, tear, saliva

and seminal fluids (fig 9). Mammary gland epithelial cells of SB-CAG-Venus transgenic lactating does also showed Venus-specific expression by tissue histology and fluorescence microscopy. In summary, the SB-CAG-Venus transgenic rabbits secrete the recombinant protein by different glands. This finding has relevance not only for the understanding of the biological function of exocrine glands, but also for the design of constructs for expression of recombinant proteins in dairy animals.

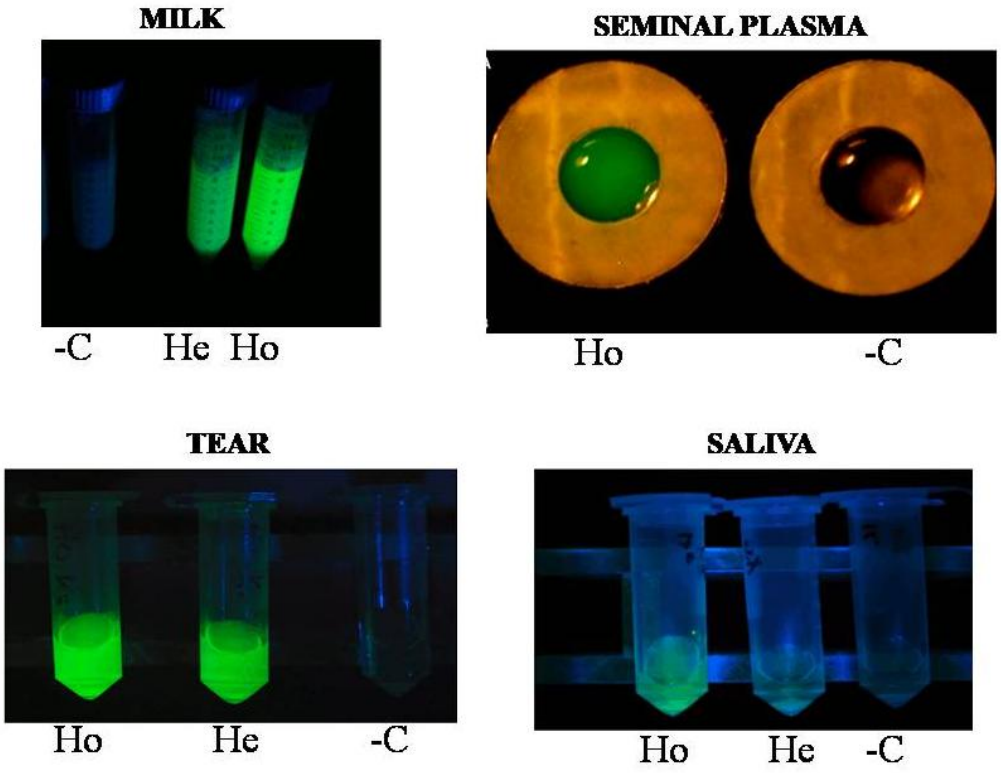

Fig 9. Expression of recombinant Venus protein in different body fluids. -C-negative control sample; Ho-sample from a homozygous animal; He-sample from a heterozygous animal.

We have published our results in PlosOne.

Summary

During this project we adapted some important basic transgenic techniques in guinea pigs. We successfully set out a superovulation protocol, embryo collection, embryo culture conditions, anesthetic protocol, microinjection protocol.

We have successfully prepared the necessary microinjection mixture which consists of a tranposase mRNA construction in combination with a donor plasmid construction harboring a fluorsescent marker gene bordered by inverted terminal repeats. We have shown that this bicomponent system can produce transgenic cells (fibroblast) and even transgenic guinea pig embryos.

We have produced the first knock out guinea pig embryos targeting its MSTN gene.

The ultimate aim of this study- to produce the first transgenic adult guinea pig was failed due to the lack of successful embryo transfer method.

We have developed a new transgenic technique in rabbits where extraembryonic gene manipulation manifested and we have verified that recombinant proteins can be expressed without a signal peptide by the exocrine glands of transgenic animals.

Publications where NKFIH-OTKA NN113162 was indicated as funding agent:

1. Secretion of a recombinant protein without a signal peptide by the exocrine glands of transgenic rabbits. Kerekes A, Hoffmann OI, Iski G, Lipták N, Gócza E, Kues WA, Bősze Z, Hiripi L. PLoS One. 2017 Oct 27;12(10):e0187214. doi: 10.1371/journal.pone.0187214. eCollection 2017.
IF: 2.766

2. Placenta-specific gene manipulation in rabbits. Skoda G, Hoffmann OI, Gócza E, Bodrogi L, Kerekes A, Bösze Z, Hiripi L. J Biotechnol. 2017 Oct 10;259:86-90. doi: 10.1016/j.jbiotec.2017.07.037. Epub 2017 Aug 1.
IF: 2.533

The PI was the corresponding author in both papers. The cumulative impact factor of these papers were 5.3 and both publications belong to Q1. Both publications are open access.

Manuscript in preparation: A simple, low-cost method for time-lapse monitoring of embryo development by GoPro action camera Péter Major, Gergely Iski, Zsuzsanna Bősze, László Hiripi

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4. Transposon-mediated transgenesis, transgenic rescue, and tissue-specific gene expression in rodents and rabbits. Katter K, Geurts AM, Hoffmann O, Mátés L, Landa V, Hiripi L, Moreno C, Lazar J, Bashir S, Zidek V, Popova E, Jerchow B, Becker K, Devaraj A, Walter I, Grzybowksi M, Corbett M, Filho AR, Hodges MR, Bader M, Ivics Z, Jacob HJ, Pravenec M, Bosze Z, Rülicke T, Izsvák Z. FASEB J. 2013 27(3):930-41.

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6. Germline transgenesis in rabbits by pronuclear microinjection of Sleeping Beauty transposons.Ivics Z, Hiripi L, Hoffmann OI, Mátés L, Yau TY, Bashir S, Zidek V, Landa V, Geurts A, Pravenec M, Rülicke T, Bösze Z, Izsvák Z. Nat Protoc. 2014 Apr;9(4):794-809. doi: 10.1038/nprot.2014.009. Epub 2014 Mar 13.

7. Transposon-Based Reporter Marking Provides Functional Evidence for Intercellular Bridges in the Male Germline of Rabbits.Hoffmann OI, Kerekes A, Lipták N, Hiripi L, Bodo S, Szaloki G, Klein S, Ivics Z, Kues WA, Bosze Z. PLoS One. 2016 May 5;11(5):e0154489. doi: 10.1371/journal.pone.0154489. eCollection 2016.

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