SZENT ISTVÁN UNIVERSITY
Animal Husbandry Doctoral School

CREATION AND CHARACTERIZATION OF A TRANSGENIC MODEL FOR LONG QT SYNDROME TESTING

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The doctoral school's name:	Animal Husbandry Doctoral School
Field of science:	Animal Husbandry

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1. ANTECEDENTS OF THE WORK, OBJECTIVES TO BE MET

1.1. ANTECEDENTS OF THE DISSERTATION

Animal experiments have been an indispensable part of the advancement of medicine for long centuries. Vaccination, antibiotics, organ transplants and chemotherapy would not exist without preceding animal experiments, and our knowledge on diseases like malaria and smallpox would be much smaller. Animal experiments can contribute not only to saving human lives, but to the advancement of our fundamental biological and genetical knowledge, and thus to improving the life expectancy and life quality of farm animals and pets alike. The development of genetic and biotechnological methods has recently enabled us to create purposefully modified animals to model human diseases. This revolutionized medicine, as there are experiment animals that can be specifically used to find answers for a question of human biology. Depending on their size, internal constitution, etc. the most frequently used experiment animals are suitable for the modeling of differing human diseases. Another advantage of mutant animals is that their molecular characteristics are precisely known, thus more detailed results can be achieved through a lesser number of specimens. This technological advancement also enables experiment designs to efficiently applying the 3R rules and regulations of animal protection, which include the recommendation to minimize the number of experimental animals (Russel et Burch, 1959). Sudden cardiac death (SCD) is a major cause of mortality worldwide, with over 4 million casualties annually (Silvia et al 2015). A cause factor for SCD is the abnormal heart rhythm called *torsade de pointes*, which can be caused by the so-called long QT syndrome. The long QT syndrome is an anomaly indicated by the prolongation of the QT interval on ECG. Each form of the long QT syndrome features an abnormal repolarization of the heart. Hereditary long QT syndromes each originate from a mutation of an ion channel gene; these mutations can extend the action potential duration (APD) and elongate the QT interval. 15 distinct types of long QT syndromes have been identified, each caused by different ion channel mutations.

To prevent sudden cardiac death caused by hereditary long QT syndrome, predictive methods necessitate animal disease models where ion channel anomalies are known and their effect is measurable. The LQT1 and LQT2 transgenic rabbit models, developed in the frame of a US and German research collaboration to model mutations of two ion channels in 2008, since than have been proved to be useful in multiple researches. *Oryctolagus cuniculus*, the European rabbit has major advantages to the laboratory mouse, as the electro-physiological features of the rabbit heart are closer to those of the human.

My PhD work addresses the slow delayed-rectifier K(+) current (IKs). Within the myocytes of the human heart, the IKs channel consists of an α-subunit (KCNQ1; Gene ID: 3784) and a modulating β subunit (KCNE1; Gene ID: 3753). The gene KCNE1, as the β subunit of the IKS codes a transmembrane protein that associates to the α subunit and together they constitute the IKs channel. Mutations of the KCNE1 gene contribute to the elongation of the action potential and atrial arrhythmogenesis. KCNE1, also known as minK, was one of the first ancillary subunits of the Kv channel that were cloned from a human heart. The missense mutation of the KCNE1 in our rabbit model is based on the point mutation amino acid swap G52R. This mutation was first identified in a Chinese family suffering from LQT5 syndrome (Ma et al 2003). Cardiac muscle tissue has several types of ion channels that are able to substitute each other, this is called repolarization reserve. In our transgenic rabbit, the anomalous function of the mutant human KCNE1 protein decreases the repolarization reserve of the heart and increases the tendency of arrhythmia. In certain cases, the elongation of the QT interval does not predict the onset of arrhythmia. However, we found the variability of QT to be a method of greater precision, which can help the prediction of lowered repolarization reserve.

1.2. OBJECTIVES

1. To create the transgenic constructs that contain the rabbit beta-myosin promoter and the variants of the human G52R, carrying both a mutant and a non-mutant version of KCNE1 in cDNA.
2. To create a transgenic mouse model suitable for monitoring the expression of mutant KCNE1 protein.
3. To create a transgenic rabbit model to investigate the LQT5 syndrome, to examine transgenic inheritance, establish a strain.
4. To examine the tissue specific expression of the integrated transgene using methods of molecular biology, moreover toidentify the transcribed transgenic protein and assess its amount.
5. Together with our collaborators, to characterize the transgenic rabbit model of LQT5 syndrome with electrophysiological methods.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL PERMITS AND ANIMALS

Laboratory rabbits in the National Agricultural Research and Innovation Center (NARIC), the Agricultural Biotechnology Institute (ABC), the animal house of the Animal Biotechnology Department were properly placed, housed and maintained in accordance with the relevant European Union rules and the Act XXVIII of 1998 ON THE PROTECTION AND SAVING OF ANIMALS, Government Decree 243/1998 (31 December) on animal testing and FVM-KÖM-GM Decree 36/1999 (02 April) on the rules of managing, keeping, transporting etc. test animals. We have the following permissions to conduct our experiments: PEI/001/275-4/2013.

FVB/N mice were used as donors in the *in vivo* pre-experiment, as their pronuclei are well visible, making them suitable for pronucleus microinjection. The recipient mice were strain CD1, having high numbers of offspring and a good preservation rate of their young. We used adult New-Zealand white rabbits weighing 3.5 kg to create transgenic rabbits. Both the mice and rabbits were kept in a conventional enclosure, with a 12-hour daylight lighting pattern, fed *ad libitum.* The electrophysiological examinations were conducted on 3.5-4-month-old adult rabbits of either G3 or G4 generation, providing wild type specimens for control. There were no sex-based differences found, thus the measurements done on male and female specimens were summed.

2.2. CLONING AND EXPRESSION VECTORS

For the mutagenesis of human KCNE1 cDNA, a 4270 bp, pSC-A-amp/kan vector was used, from a Stratagene StrataClone PCR Cloning Kit. The β−MHC promoter from the rabbit genome and the mutant KCNE1 cDNA were combined in an Invitrogen: pCRW-Blunt II-TOPO vector sold by Thermo Fischer Scientific. As an expression vector, Invitrogen's pcDNA™3.1 (+) Mammalian Expression Vector was used. Cloning was done in E. coli DH5α.

2.3. HUMAN CDNA LIBRARY

The human KCNE1 cDNA was isolated from a Human Heart cDNA library (Cat no: SC6204) ordered from 3h Biomedical AB, Sweden. According to the vendor's description, the sample is from adult human atria, negative for HIV, Hepatitis B (HBV), Hepatitis C (HCV), mycoplasm bacteria, yeast or fungal infections. Concentration: min. $1x10^6$ cells/ml.

2.4. CREATING A TRANSGENIC ANIMAL

I created two transgenic constructs, both containing the genes governed by the rabbit β- myosin (MYH1; Gene ID: 100125991) promoter and either human wild-type KCNE1 cDNA or the human KCNE1 G52 mutant cDNA and a cattle polyA signal. The position-specific nucleotide swap in the human KCNE1 cDNA was conducted with a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technology).

First, *in vitro* testing was done with the wild-type or the G52R-KCNE1 mutant constructs in the presence of KCNQ1 polus forming protein in transfected CHO cells. Then, pronucleus microinjection was used to produce transgenic mice, followed by transgenic rabbits with the mutant transgenic construct. The 8-10 weeks old FVB/N donor mice received 5 NE PMSG intramuscularly, followed by 5 NE hCG 48 hours later. The embryos were collected from the artificially ovulated FVB/N mice and microinjected, then transferred into pseudo-pregnant CD1 mice. Then genomic PCR was used to identify the transgenic individuals in the offspring. One transgenic mice were born, passing on the transgene to the next generation.

The donor rabbits were female New-Zealand rabbits weighing minimum 3.5 kg, receiving 120 NE PMSG intramuscularly at the age of 3-4 months, then 72 hours later they received 180 NE hCG and were artificially fertilized. The following day the embryos were collected from the superovulated rabbit and after microinjection were inserted into the oviducts of pseudo-pregnant recipient animals via endoscopic method. The rabbits born from the embryo transfer were screened with transgene-specific PCR. The tissue-specificity of the transgene expression in the new generation was assessed with RT-PCR, then the amount of endogenous and G52R-KCNE1 was determined through western blot and isoelectric focusing. The position of the integrated transgene was determined via inverse nested PCR. The heredity of the transgene was observed in both transgenic species. Tissue-specific transgene expression was measured on different expression levels in both transgenic species via molecular biological methods. In addition, the electrophysiological traits of the transgenic rabbit model were analysed in the Pharmacology Institute of the University of Szeged, Faculty of Medicine. The electrophysiological evaluation of the LQT2-5 dual transgenic rabbit model is ongoing, in cooperation with the University of Freiburg.

3. RESULTS

Many transgenic mouse models have been created in order to model the long QT syndrome; but did not show the same phenotypes as humans. Ours is the third transgenic rabbit model to study the electrophysiological symptoms caused by the ion channel mutations resulting in long QT syndrome. During my doctoral candidacy, I established two transgene constructs. Both contained the rabbit β –myozin promoter, they differ in the cDNA as either human wild-type KCNE1 or G52R mutant KCNE1 was placed in the transgene.. The β-MHC-G52R-KCNE1-bGHpolyA construct was microinjected into mouse and rabbit embryos; while the wild-type transgene was used in experiments with *in vitro* CHO cells.

First, *in vitro* experiments were done on CHO cells to compare how the wild-type and mutant transgene constructs function. When the mutant subunit was associated with KCNQ1, then the alpha subunit with mutant KCNE1 resulted in a current that did not show the characteristic slow activation of IKs, and current density also differed from the results obtained with wild-type KCNE1. Thus it was proved that the G52R mutation indeed causes ion current problems. The *in vivo* testing of the transgene construct was done on FVB/N mice. Superovulated FVB/N females were bred with FVB/N males; after mating, 305 embryos were collected from the oviducts of the animals. 268 were found to be suitable for pronucleus microinjection, and 224 embryos were transfected into pseudopregnant CD1 mice. 14 young was born, one being transgenic founder.

In the case of rabbits, 466 of the 497 injected embryos were inserted into 21 pseudo-pregnant recipients. 38 (8%) was born, of which 4 (10%) displayed transgenic integration when assessed via genomic PCR. I characterized the tissue-specific transgene expression in both species with molecular biological methods and it was shown that the mRNA expression of the alpha IKs subunit increased significantly, along with the expression of rabbit KCNE1 mRNA in the transgenic rabbit heart. The mutant human and rabbit KCNE1 proteins were separated based on their isoelectric points and their ratio was determined. I also found that certainly homozygote rabbits did not survive. During the breeding of several years, we intended to create homozygote offspring, but there was no one born alive rabbit, which in our two-phase detection method confirmed to be homozygote. In the two-phase system, homozygote rabbits were identified by first selecting potential homozygote offspring with transgene-specific qPCR primer. Those rabbits where the relative expression of the transgenewas at least 1.6x greater than the certainly heterozygotic individuals were considered as homozygoties. When those rabbits reached sexual maturity, they were mated with wild-type animals and the produced offspring were examined for the presence

of the transgene with PCR- If the parent was homozygotic, all offspring should be heterozygote. However, we were not able to accomplish the second phase. Potentially homozygotes did not live to maturity or no offspring was produced from the wild-type crossbreeding. In addition, among the heterozygotic offspring a significantly smaller proportion of females were identified than theoretically expected. As to the transgenic-wild-type ratio, I found that the proportion of transgenic offspring was significantly smaller than expected as well.

The electrophysiology experiments on LQT5 rabbits used addition of IKr blocker dofelitide to show that the repolarization reserve of transgenic rabbits is lower than wild-type siblings, consequently they are more susceptible to arrhythmia. Dofelitide decreased heart rates in transgenic animals only. In addition, dofelitide significantly increased the short-term QT variability (STVQT) of the transgenic animals. Dofetilid triggered a similar QT elongation in both groups, implying that its STVQT-increasing effect was more pronounced and arrhythmias were more prevalent in transgenic animals.

4. NEW SCIENTIFIC RESULTS

1. I created the transgene constructs to study the long QT5 syndrome: the vectors containing the G52R mutation and the wild-type variant of the human KCNE1 cDNA.

2. I managed to create a transgenic mouse model by the long QT5 transgene construct, where its function was demonstrated on RNA and protein level as well.

3. I created the third transgenic rabbit model in the world that can be used to study the long QT syndrome, its effectiveness was demonstrated through the electrophysiological results.

4. The tissue-specific expression in the LQT5 transgenic rabbit model was demonstrated by molecular biological methods, the results of the immune-histochemical painting revealed its localization within the cellular membrane.

5. We managed to determine the amount of human in channel protein expressed in the LQT5 transgenic rabbit model, and its separation via its isoelectric point.

6. In the case of a transgenic heterozygotic LQT5 strain that a sexual imbalance is among the heterozygotic transgenic offspring, with significantly less females born. Homozygotic offspring could not be created.

5. CONCLUSIONS AND RECOMMENDATIONS

Animal models have always been important part of basic research. Their use in medical research has increased exceptionally during recent decades, due to the fast advancement of biotechnology. The rabbit, as a laboratory animal, has been useful for researchers, as suitable models of certain human diseases, and even beneficial recombined proteins can be produced in its various tissues, after genetic modification (Bősze et al, 2003). Physiologically and phylogenetically, rabbits are closer to humans, than mousse, rats, or other laboratory animals.

The aim of my work was to create a transgenic rabbit model that produces a mutant human ion channel protein in the cardiac ventricula, thus providing unprecedented details about the electrophysiological background of the long QT 5 syndrome. My PhD work demonstrated theintegration, heritability, and expression of the transgenic construct both on RNA and protein levels. Electrophysiology measurements at the Pharmacology Institute in Szeged presented the mutant human minK protein's effect on the IKs ion current in the heart of a transgenic rabbit. Though we used immunohistology to demonstrate the presence of the human minK protein in the membranes of the heart muscle cells of the transgenic rabbit, it would advisable to investigate the co-localization of the polus forming alpha subunits and their associated human minK beta subunits via immunhistology. Immune co-precipitation could be used to find out which ion channel proteins have interactions with the human transgene protein.

My work was started before the era of genome editing tools like TALEN, Zink Finger, CRISPR/Cas, so my results could be improved by applying such methods. Our existing LQT5 model could be used to create a novel transgenic model that has noendogenous KCNE1 because it knocked out with CRISPR/Cas therefore only the mutant human KCNE1 would be expressed in the transgenic rabbit heart. If there is surviving offspring, the transgenic model without endogenous KCNE1 would be suitable for further research: in what proportion does endogenous KCNE1 assist the function of an G52R mutant IKs channel; results of in vitro test could be verified or improved. Other physiological roles of the minK protein could be learned.

According to published data, mice afflicted with long QT2 type showed no difference in the development of heterozygotes and homozygotes until day 9.5. Another remarkable observation was the shift of the gender ratio among LQT5 transgenic offspring. An explanation can be the effect of female hormones, which increases the probability of cardiological anomalies and long QT syndrome. The individual effects of female hormones could be investigated on the LQT5 transgenic model, in an experiment similar to that was done on the LQT2 transgenic rabbits. The

ovaries of the transgenic LQT2 females were removed and the major sexual hormones were supplied later individually, thus being able to observe their separate effect in the organism of the hormone-depleted rabbits. This study demonstrated that females with the long QT2 syndrome had significantly longer QT intervals than those of the males, and that later complementation of female hormones (most importantly, östradiol) significantly increases the emergence of cardiological disturbances. Further, detailed hormone research on the transgenic rabbit model could provide vital information to understand the precise background of such electrophysiological diseases as sudden cardiac death, contributing to the development of new and more accurate prediction techniques and/or cure design. Among the 16 human long QT syndrome, only three have a transgenic rabbit model to date, including ours. Moreover, ff money and effort were of no concern, I would create the existing QT1, QT2 and QT5 rabbit models created by additive transgenesis, with one of the genome editing methods bí now adapted in our team. This could provide information on how results of the animals created by novel methods differ from the results of already existing transgenic models.

Moreover, we cross-bred our LQT5 model with LQT2 rabbit models in Freiburg, creating a double transgenic LQT2-5 model. The yet unpublished results of this double transgenic model shows that the LQT2-5 mutants are not only more sensitive to IKs blockers, but they have higher pro-arrhythmia biomarkers, the more severe arrhythmic symptoms tend to be longer and more prevalent.

These rabbit models could also be subject to basic research on the interactions of the NMD factors and ion channel proteins. We could understand much about the functioning of ion channels, but also about how the control of ion channel proteins are affected by altering the ratio of the proteins regulated by them. It would be useful to see whether the mRNA expression of NMD complex constituents is changed in the hearts of the transgenic rabbits.

Finally, the LQT5 transgenic rabbit model could be used in experiments where the electrophysiological characteristics of the most widely used species (dog, guinea pig, rabbit) would be compared. This could reveal which animal model is the most useable to model the human cardiac rhythm anomalies.

The developing transgenic techniques have been directly affecting the pharmaceutical and medical research projects in the recent years. New and new types of laboratory animals will be created in the forthcoming years, making so far less researchable metabolic processes easily modelable. The number of species that can be used to model human diseases will increase. Due to the exactly known or accurately characterized molecular biological background, the efficiency of the

experiments will increase, thus the number of animals required in one project can be decreased. Cardiology research uses hundreds of transgenic mouse models, but these are not fully reliable human disease models, due to their different ion current constitution. Human cardiology research has used minimal amount of genetically engineered rabbit models. The transgenic rabbit model created during my PhD work is an animal model which filled a gap,,.the results of our LQT5 rabbit has already provided important information to better understand repolarization ion currents of the human heart.

6. PUBLICATIONS

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