MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

Hungarian University of Agriculture and Life Sciences

Animal Husbandry Doctoral School

Production of Myostatin knockout rabbits by genome editing

Thesis

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1. BACKGROUND AND AIMS OF THE STUDY

In recent days, applications of biotechnology help us to understand different biological tasks. In this process transgenic animal production has played a major role since its emergence in the 1980's thanks to the expanding methodological background. Gene modification has become relatively simple due to the genome editing techniques that have been developed in the past decade.

During my research, a myostatin knocked out (KO) domestic rabbit line was generated using the bacterial derived methods CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) and TALEN (transcription activator-like effector nuclease).

The size and the physiological parameters of rabbit made this animal a popular organism in modelling different human diseases and gene functions.

Through my research I was supposed to investigate the effects of myostatin absence in two different genetic backgrounds. Several artificially created or naturally occurred myostatin mutants have been reported in different species. Based on these studies it turned out that myostatin is the negative regulatory factor of the skeletal muscle development. The lack of myostatin results in the so called "double muscled" phenotype. Myostatin neuronal expression was also measured in rats, but the physiological roles of these patterns need further investigation. Beside muscle formation, myostatin also effects adipose tissue and bone development. Moreover, the role of myostatin can be assumed in placental metabolism.

Throughout our experiments other research groups have published scientific papers about myostatin KO rabbit production. In one of these papers the major observations were increased body mass and tongue parameters. While in the other publication increased body mass and perinatal implications were reported as the primary consequences of myostatin KO.

Detailed understanding of myostatin's biological function can facilitate the development of different therapies when it comes to diseases where the major symptoms are somehow related to muscle mass loss. In addition, myostatin may be utilized also in sport sciences. Furthermore, different breeds that can be raised quickly to reach a certain body mass would be highly appreciated in agriculture. In the near future myostatin KO animals can serve as important model organisms of muscular, neuronal diseases or even can provide insight into diseases related with abnormal placental metabolism.

Beside the production of myostatin KO rabbit line, our research group is highly engaged in the investigation of the tissue specific roles of myostatin due to placental-specific gene inactivation. During my doctoral research, one of my goals was to generate a green fluorescent protein (GFP)

expressing placental-specific model system since placental gene transfer was not well established in rabbits. This system can facilitate the investigation of myostatin mediated extraembryonic metabolism.

1.1. Primary goals

- Production of a myostatin KO domesticated rabbit lines using genome editing techniques.
- Generating myostatin mutation in the breeds Hycole and Pannon White. Study the observed differences between the two breeds and review the effects of different genetic backgrounds on KO generation.
- Creating a placental-specific GFP expressing model system in rabbits that can contribute to the study of tissue specific gene expression and placental abnormalities.

2. MATERIALS AND METHODS

TALEN and CRISPR/Cas9 mediated genome editing were carried out using Hycole and Pannon White breeds which are selected to be quick in reaching a certain body mass. The lentiviral experiments were conducted using New Zealand White breeds. Experimental animals were kept in the animal facility of the MATE Intitute of Genetics and Biotechnology. All the corresponding requirements that are involved in the Hungarian legislation (40/2013) about the regulation of animal experiments were fulfilled. Animals were kept individually within 18±3 °C temperature range, were fed *ad libitum* and continuous water supply was provided. The animal facility had half day lighting. Animal experiments were conducted with the corresponding permission (permission id: PEI/001/329-4/2013).

The donor does were at least 18 weeks in age and 3,5 kg. These rabbits were intramuscularly injected with 120 IU PMSG. For facilitating the ovulation, after PMSG treatment the donors were intravenously injected with 180 IU hCG. Following the hCG treatment in the next 10 minutes, the donors were artificially inseminated.

The next day after insemination, the oviducts were removed, and zygotes were flushed using 20% PBS FCS medium flow.

The mRNAs of TALEN and CRISPR/Cas9 constructs were microinjected into the cytoplasm of a one cell stage zygotes, while the GFP containing lentiviral vector was microinjected into the perivitelline space. The lentiviral vector was constructed by Krisztián Kvell et al..

After performing embryo tests, the microinjected embryos were transferred into the oviducts of pseudopregnant does.

Mutant newborns were selected using T7 endonuclease assay and sequencing. Two founders were selected for further breeding with different genetic backgrounds.

Phenotypic changes in body mass and muscle fat ratio were investigated using Computed Tomography. Histological analysis was performed to study hypertrophy and hyperplasia. The expression pattern analysis of myostatin was carried out by qPCR. Potential off-targets sites were amplified by PCR and analysed by sequencing.

In case of lentiviral transgenesis, after the efficiency and promoter activity studies, 8-16 cells stage embryos were microinjected and transferred into the oviducts of pseudopregnant recipient does. Foetuses and related tissues were studied with RT PCR and fluorescent microscopy.

3. RESULTS

First, we intended to generate myostatin mutants with TALEN, but modifications could not be detected in the offspring nor in the *in vitro* cultured blastocysts. So we decided to change our protocol to the CRISPR/Cas9 system, which started to become popular back in these days.

After the embryonic assessment we picked up the sgRNA with 41% efficiency for producing myostatin KO mutants. Twenty percent of the newborns were proved to carry a CRISPR/Cas9 induced monoallelic mutation. In the two founders selected for further studies the myostatin expression was blocked on the targeted allele due to genome editing induced deletion. In heterozygous individuals the mRNA expression has dropped by half compared to wild type animals. We proved that no modification has happened at the predicted off-target sites.

None of our genome edited animals had a specific phenotype (muscle hypertrophy, increased tongue parameters) like the ones reported by other research groups.

One of the founder animals had 11bp deletion in the myostatin gene with a Pannon White genetic background. This breed was selected to have an increased muscularity. In this case increased adipose tissue density and decreased fat/muscle ratio were observed with CT. In addition, fertility abnormalities were observed presumably due to the decreased fitness effected by the density of the adipose tissue.

We reported a new myostatin linked phenotype, which in the age of 3.5 months the hind limbs started to develop paralytic which proved to become even more severe as time went by.

The background of this phenomenon was clarified by results observed by the NÉBIH laboratories where neurodegenerative abnormalities in the hind limbs were reported.

Due to this unfortunate fact we could not establish a transgenic line from this founder, hence the comparison of the effect of myostatin KO in different genetic backgrounds could not be performed.

One strain was established using a Hycole individual with a 14bp deletion, but we did not find any homozygous offspring in the F2-F3 generations. Decrease in the density of the adipose tissue was not observed like we did in the case of Pannon White breed.

Some of the individuals with decreased myostatin expression levels had the degradation symptoms that have been mentioned previously. Hind limbs were the firsts to be paralyzed and then as the time went by, different body parts and forelimbs also developed paralysis. Fertility, pregnancy or parental care related evidence were not detected.

The exact role of myostatin in placental glucose metabolism still needs further investigation. In the modelling of placental diseases, using rabbit as a model organism is still a better option than using the traditional rodent models (rat and mouse). Hence, we developed a rabbit model using a lentiviral extraembryonic tissue-specific gene transfer to be able to investigate placental myostatin related processes.

The used lentiviral system contained a GFP driven by a constitutive promoter. Extraembryonic tissue specific GFP expression was detected in eight tissues related to a 14,5-day old foetus (four of the placentas and two of the yolk sacs). No GFP expression was detected in any of the embryonic tissues of the foetuses. In addition, the levels of mosaicism were different through the samples.

3.1. New scientific results

1. Generation of Hycole and Pannon White myostatin mutants using CRISPR/Cas9 system.
2. First ever detection of myostatin related *nervus tibialis* degeneration in rabbit which effected the behaviour of the mutant animals. Hence, I proved that myostatin has an important role in the peripheral nervous system.
3. Proof of that myostatin deficiency in rabbit causes decrease in body fat and promote changes in muscle-fat ratio in female animals. These conditions can lead to abnormalities in fertility.
4. First ever proof that placental tissue-specific gene expression using lentiviral systems can be achieved in rabbits.

4. CONCLUSIONS AND RECOMMENDATIONS

During my doctoral research, my primary goals were to study the function of myostatin and to investigate the consequences of myostatin KO. We were intended to conduct our research using TALEN and CRISPR/Cas9 genome editing techniques in Hycole and Pannon White breeds.

Even though these methods are way more precise, efficient and faster than the previously used genome editing techniques, they still have some drawbacks and they do not have 100% efficiency. There are some possible explanations for the failure of TALEN experiments. On one hand it is possible that the constructs were not diluted appropriately, and these altered concentrations resulted in a decreased number of new-borns and mutations. On the other hand, it is also possible that the construct itself was not designed or assembled properly. Obviously, the mixture of the two mentioned explanations is also possible. After the unsuccessful TALEN experiments, we used CRISPR/Cas9 to produce KO rabbits.

CRISPR/Cas9 experiments in embryos resulted 21-41% efficiency depending on guide RNAs in case of the rabbit myostatin. The most efficient sgRNA/Cas9 complex had 20% KO efficiency in the second exon of myostatin in case of newborns. In the future works of our laboratory, I would recommend the simultaneous microinjection of two combined sgRNAs since the higher efficiency of this approach is proven. At the same time, the appearance of off-target mutations can be increased, which is disadvantageous.

After the microinjection, ten newborns proved to be carriers of a monoallelic modification at the targeted site of myostatin. The two founder individuals (selected for further breeding) were checked whether CRISPR/Cas9 modified any other site then myostatin, but no evidence was found for off-target effects at the nine most probable off-target sites. We suppose, that the observed alterations in phenotype are purely due to the targeted genome modifications in the myostatin gene as they occurred in two different independent transgenic line

In case of Pannon White doe and in some individuals of Hycole strain, hind leg paralysis was observed. We presume, that the downregulated myostatin expression affects the myelination of the motor neurons hence the saltatory conduction can be altered. This hypothesis has been confirmed by the fact that the individuals with this phenotype showed signs of neuronal degradation in *nervus tibialis,* but thigh and skeletal muscle were not affected. Based on these observations, we concluded that the downregulated myostatin related abnormalities in movement coordination has purely neuronal origin. There is publication about myostatin expression in the central nervous system of rats, but the exact role of the myostatin protein is still unclear.

Based on our CT studies, the Pannon White doe had decreased adipose tissue density. It is possible that the fertility related abnormalities originate from this phenomenon. No alteration in adipose tissue density, fertility or parental care was observed in the case of the genome edited Hycole breed. On one hand, this is probably because the intensively selected Pannon White has a very sensitive lipid metabolism which was affected by decreased myostatin protein level. On the other hand, it is also a possible explanation that the CT selected population had that much of a muscle mass that it affected the fertility. Based on the observed phenotypes, it is obvious that myostatin KO rabbit line generation is not a good idea in the case of breeds which was previously selected for muscle mass generation.

Strain establishment was not successful in the case of Pannon White due to the mentioned observations. Fertility related alteration was not reported in the case of Hycole buck, but homozygous offspring could not be generated. This phenomenon is probably originating from the pre- or perinatal lethality of homozygous myostatin KO individuals. Other laboratories reported that homozygous myostatin KO breeds could be generated. These experiments were conducted using the laboratory breed New Zealand White which is not selected for muscle mass production. The lack of homozygous animals raises the question when and why these individuals die during embryonic development. This topic needs further investigation which can help us to understand the embryonic role of myostatin.

In the case of Pannon White the lack of myostatin resulted in higher body mass compared to the control group until the unexpected phenotype was observed. Heterozygous Hycole myostatin mutant animals did not show significant increase in body mass. Even though, the founder buck had the highest muscle volume, this observation could not be confirmed by studying offspring. Myostatin KO related muscle hypertrophy could not be observed in any heterozygous animals. It is possible that myostatin mutation in a homozygous form would result in a muscle hypertrophy phenotype as reported previously.

In some articles the lack of myostatin contributes to different abnormalities like the increased tongue parameters, which can lead to lethal phenotypes. In our cases we did not observe similar feature.

It seems that breed selection can affect the consequences of myostatin absence. In case of Pannon White which undergone a selection to fast body mass development, decreased myostatin protein level resulted in neuronal inflammation and altered adipogenesis. Neuronal degradation related phenotype was show typically in adult individuals, while juveniles did not develop it. The background of this neuronal phenomenon is still unclear.

These observations confirm that quantity traits that were developed during a long breeding and selection process, cannot be upgraded forever. Similar phenomenon was reported in case of transgenic trouts. Body mass of trouts from non-selected wild type were highly increased by overexpressing growth hormone compared to the control group. But, there was no body mass differences found when domesticated breed were used in the same transgenic experiment.

To study the placental role of myostatin, we developed an extraembryonic-specific model using lentiviral based method in rabbits. Using this method, transgene expression was only detected in extraembryonic tissues. Beside the tissue-specific myostatin inactivation, there is a possibility to create a transgene cassette which contains myostatin to overexpress it in extraembryonic-tissues, which would make it possible to investigate the myostatin mediated placental processes.

Utilizing the myostatin KO rabbit line production in food industry is not recommended. In my honest opinion, myostatin KO individuals did not outperform significantly the breeds currently used, while from the breeding point of view myostatin mutation resulted in some undesired phenotypes (fertility and neuronal abnormalities). If the myostatin absence would be performed in a strain that was previously selected to parental care, then the observed abnormalities in fertility possibly can be compensated. This assumption needs further investigation.

5. PUBLICATIONS

5.1. First author paper

Skoda Gabriella, Hoffmann Orsolya Ivett, Gócza Elen, Bodrogi Lilla, Kerekes Andrea, Bősze Zsuzsanna, Hiripi László (2017) „Placenta-specific gene manipulation in rabbits." *Journal of Biotechnology 259 pp. 86-90, 5 p.*
IF=3,163

Skoda Gabriella, Kerekes Andrea, Hoffmann Orsolya Ivett, Lipták Nándor, Tatiana Flisikowska, Angelika E. Schnieke, Donkó Tamás, Meinrad Odermatt, Atkári Tamás, Bősze Zsuzsanna, Hiripi László (2021) „Investigation of a new phenotype in myostatin mutated rabbit created by CRISPR/Cas9 method." *Gene.* Submitted for publication
IF=2,984

5.2. Lectured papers with impact factor

Pethető Gábor László, Kerekes Andrea, Mihálffy Máté, Donkó Ágnes, Bodrogi Lilla, **Skoda Gabriella**, Baráth Mónika, Hoffmann Orsolya Ivett, Szeles Zsolt, Balázs Bernadett, Sirokmány Gábor, Fábián Júlia R., Tóth, Zsuzsanna E., Baksa Ivett, Kacskovics Imre , Hunyady László, Hiripi László, Bősze Zsuzsanna, Geiszt Miklós (2021) „Disruption of the NADPH Oxidase 5 Gene Aggravates Atherosclerosis in Rabbits" *CIRCRESAHA. (2021)* 120.318611. Accepted
IF=15,211

Pintér Tímea, Geiszt Miklós, Pethető Gábor, Mihálffy Máté, **Skoda Gabriella**, Lipták Nándor, Kerekes Andrea, Bősze Zsuzsanna, Hiripi László, Bodrogi Lilla (2020) „The creation of a multiallele knockout genotype in rabbit using CRISPR/Cas9 and its application in translational medicine." *Applied Sciences-Basel. (2020) 10. 23 Paper: 8508, 14 p. IF=2,474*

5.3 Lectured paper without impact factor

Major Péter, Kerekes Andrea, **Skoda Gabriella**, Hiripi László, Bősze Zsuzsanna (2013) „Genetically modified animals as potential genetic resources." *Slovak Journal of Animal Sciences (2013) 46: 4 pp. 155-159., 5 p.*

5.4. Lectures in international conferences

Skoda Gabriella, Kerekes Andrea, Atkári Tamás, Meinrad Odermatt, Tatiana Flisikowska, Hiripi László (2017) „Myostatin knock-out rabbit line generation using CRISPR/Cas9 system.*"* Magyarország, Eger. In: *Heiszler Zsuzsanna, Hohol Róbert, Éles-Etele Nóra (szerk.) Hungarian Molecular Life Sciences (2017): Programme and Book of abstracts. Budapest, Magyarország: Diamond Congress Ltd., ISBN 978-615-5270-34-5*

Hiripi László, Hoffmann Orsolya Ivett, **Skoda Gabriella**, Bősze, Zsuzsanna (2014) „New generation transgenic techniques in rabbits.” In: *Bene Szabolcs (szerk.) 20th Youth Scientific Forum: University of Pannonia Georgikon Faculty Keszthely, Magyarország: Pannon Egyetem Georgikon Mezőgazdaságtudományi Kar, (2014) pp. 1-6., 6 p.*

5.5. Lectures in hungarian conferences

Skoda Gabriella, Kerekes Andrea, Hoffmann Orsolya Ivett, Bősze Zsuzsanna, Hiripi László (2016) „Célzott genom módosítás a nyúl miosztatin génjében.” In: *Gócza Elen; Kiss Erzsébet; Maráz Anna; Várallyay Éva (szerk.) Fiatal Biotechnológusok Országos Konferenciája, Gödöllő, Magyarország: Szent István Egyetemi Kiadó, (2016) pp. 26-26., 1 p.*

Skoda Gabriella, Kerekes Andrea, Hoffmann Orsolya Ivett, Bősze Zsuzsanna, Hiripi, László (2015) „Célzott genetikai módosítás nyúlban.” Magyarország, Gödöllő. *25. MBK Napok 2015.11.11.*

5.6. Posters in international conferences

Skoda Gabriella, Kerekes Andrea, Atkári Tamás, Meinrad Odermatt, Tatiana Flisikowska, Bősze Zsuzsanna, Hiripi László (2017) „The effect of genetic background on the phenotype of myostatin KO rabbits.” Németország, Halle (Saale): *SALAAM Final conference 2017.09.28-2017.09.29.*

Hiripi László, Gócza Elen, Bodó Szilárd, Hoffmann Orsolya Ivett, **Skoda Gabriella**, Kerekes Andrea, Bontovics Babett, Lázár Bence, Bősze Zsuzsanna (2014) „Rabbit Biotechnology in the NARIC-Agricultural Biotechnology Institute.*”* Horvátország, Zágráb, *3th RGB-Net Meeting 2014.05.06.*

5.7. Posters in hungarian conferences

Kerekes Andrea, **Skoda Gabriella**, Hoffmann Orsolya Ivett, Balogh Laura, Debnár Viktória Johanna, Bősze Zsuzsanna, Hiripi László, Bodó Szilárd (2014) „Transzgénikus állatvonalak *ex situ* genetikai megőrzése.” Magyarország, Herceghalom. In: *20. Szaporodásbiológiai Találkozó (2014) pp. 25-25., 1 p.*