Final report

Functional analysis of a mutation in the cattle motilin gene, OTKA PD 111964

1. Introduction

Left-sided displacement of the abomasum (Left Displaced Abomasum, LDA) is a common dairy cattle disease where abomasum starts bloating and displaces from the dexter abdominal floor to the sinistral abdominal wall. The etiology of the LDA is multifactorial, the two main factors are the abomasal hypomotility and the disfunction of the intrinsic nervous system. The prevalence is usually 1-7% in German Holstein-Friesian populations, 5% of newly calved dairy cows will develop LDA in the Netherlands (Kelton, 1998). In some cases, 8% presence was also reported. Unfortunately, in current dairy practice the incidence of LDA is rising. Disease can be found in intensive beef production as well. The condition is commonly associated with high yielding, intensively fed dairy cows in late or early gestation lactation. The LDA can occur any time of the lifespan, however 80% of the displacements are seen within 1 month of parturition. LDA can be treated surgically by fixing the abomasum into its correct position. Alternative treatment methods usually resulted in redisplace of the abomasum to the left side. However, even if the treatment succeeded without any incidents, significantly reduced milk performance and increase in the culling risk was reported. Occasionally LDA leads to death of the animal. Left displaced abomasum is often associated with metabolic or hepatic disease (Wolf, 2001; Rohn, 2004). LDA is a multifactorial problem and the following risk factors have been identified: high yielding cows (Ricken, 2004), intensive concentrate feeding, late gestation or early lactation, changes in weather conditions (da Silva, 2004), marked body condition score loss in the periparturient period (Kim, 2003), and feeding (Grymer, 1981; Constable, 1992; Rohrbach, 1999). LDA increases veterinary costs for dairy farms and decreases life expectancy for dairy cows with restricted milk production. The estimated economic loss is 250- 450 dollars per case. Geishauser in 2000 have calculated the annual loss in North America due to LDA as up to 220 million dollars. The frequency of the disease is also increasing in Hungarian dairy industry.

The heritability of LDA reported in various studies ranged between 20% and 50% (Wolf, 2001; Hamman, 2004). This heritability index is much higher than in any other common cattle disease. However genetic factors play very important role in LDA (Mömke, 2013), the genetic background of the disease is still unclear. Significant QTLs were located on bovine chromosomes 21, 24 (Mömke, 2004), and a proximal QTL on bovine chromosome 23. A recent study found motilin (MLN) as a candidate gene on for LDA in bovine (Mömke, 2012). Motilin is a gastrointestinal hormone synthetized and secreted by specific endocrine cells in the epithelia of small intestine. The role of this 22 aminoacid

peptide is to regulate the contractions of the digestive system. This hormone is encoded in two exons with no untranslated exon (Sanger, 2011). A single nucleotide polymorphism as a regulatory SNP in the first non-coding exon of motilin gene may affect a NKX2-5 transcription factor binding site and showed significant associations with the disease.

Expression study showed that significantly decreased MLN expression can be found in cows carrying the mutant allele. This study pointed to the possible role of the motilin in the LDA disease but there are no functional studies for this mutation.

The main aim of the project is to understand if an SNP in the promoter of motilin gene is affecting the emergence of the common bovine disease, LDA. To find out the role of this SNP we investigate on the genetic background of LDA with RNA-seq method and examine the promoter both in vitro assays and in vivo animal models. The ultimate goal is to determine the genetic factor behind the LDA and to establish a genetic marker, which can significantly reduce the possibility of the disease.

2. Investigation on genetic background of LDA disease:

The most important aim of the project was to find other major and minor genetic factors influencing the development of the LDA. The recent developments in high throughput sequencing allow the analysis of transcriptomes at high resolution. The 'next generation' RNA sequencing technology (RNA-seq) can detect expression differences at genomic level, unannotated transcriptional activity, distinguish between different transcriptional or splicing isoforms, and can provide new SNP data from expressing genes. Using this technology, it is possible to explore new candidate genes and pathways in different diseases.

milestones:

-sample collection from abomasum of healthy and diseased animals

After the symptoms are detected in the LDA affected animals, an immediate surgical intervention is needed. Mainly, there are two types of the surgery, endoscopic and normal surgery. Dr. András Horváth identified several diseased animals and attended the sampling from the wall of the abomasum. In the first trials the sampling proved to be difficult and endangers the success of the endoscopic surgery, thus we revised the sampling procedure and decided to find another LDA animals which were cured by the normal, open surgery method. The sampling was implemented in 6 diseased and 3 healthy Holstein-Friesian animals.

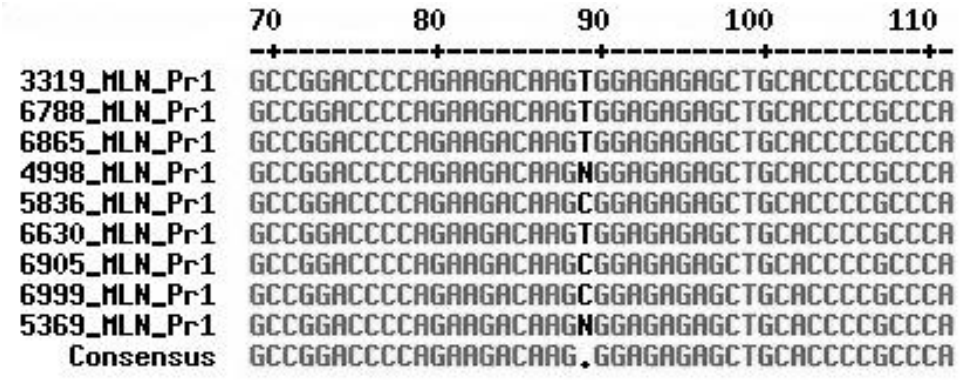
#	ID	sex	LDA	Sampling	DNA	SNP
						90:T/C
1	3319	cow	-	2016.02.24	2016.02.25	T/C
2	4998	cow	diseased	2016.02.24	2016.02.25	T/C
3	5369	cow	-	2016.02.24	2016.02.25	T/C
4	5836	cow	diseased	2016.02.24	2016.02.25	T/C
5	6630	cow	-	2016.02.24	2016.02.25	T/C
6	6788	heifer	diseased	2016.02.24	2016.02.25	T/C
7	6865	heifer	diseased	2016.02.24	2016.02.25	T
8	6905	heifer	diseased	2016.02.24	2016.02.25	C
9	6999	heifer	diseased	2016.02.24	2016.02.25	C

-Nucleic acid purification, sequencing

DNA was purified from the samples with phenol-chloroform method and the DNA samples were sequenced by Sanger method to inspect the SNP genotype. RNA was also purified from the samples with RNAsol RT solution and they passed the first quality control.

Unique primers were used for the PCR amplification and sequencing of bovine motilin gene (AT, annealing temperature, PS, product size):

Primer ID	5' > 3' sequence	AT	PS
Bt_MLNpr1_R	GTCCGGCTCTTTTGTTAACTT		
Bt_MLNpr1_F	CAGGAATCCAGACTCCTCAC	60°C	521
Bt_MLNpr2_F	CCACAAAAGGTTTCCTGCAT	60°C	1015
Bt_MLNpr4_F	ATAACACGTGGGCTTCCTTG	60°C	1248
Bt_MLNpr5_F	TGGAGCAACAACTGAGCAAC	62°C	1495



-RNA library preparation and sequencing (TruSeq RNA Sample Prep Kit, Illumina HiScan SQ)

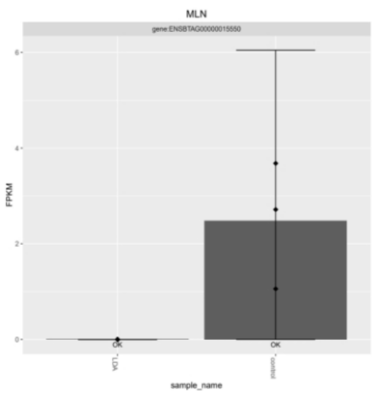
The samples were quality checked and send to UD Genomed Ltd for sequencing. The RNA sequencing service of UD Genomed Ltd contains a bioanalyzing of the RNA samples, preparing of the sample library and the sequencing of the RNA samples with Illumina platform. Total RNA was poli-A fractionated and libraries were prepared with Illumina TruSeq RNA library prep kit according to the

manufacture's recommendations. The libraries were sequenced on Illumina HiScanSQ. 50 bp long single end reads were obtained from the sequencing. 12 samples were sent, 6 LDA affected and 6 heathy animals. 11 samples were selected to proceed the RNA sequencing, above these samples 10 contained sufficient number of good quality reads, sample 3 was excluded from the further analysis.

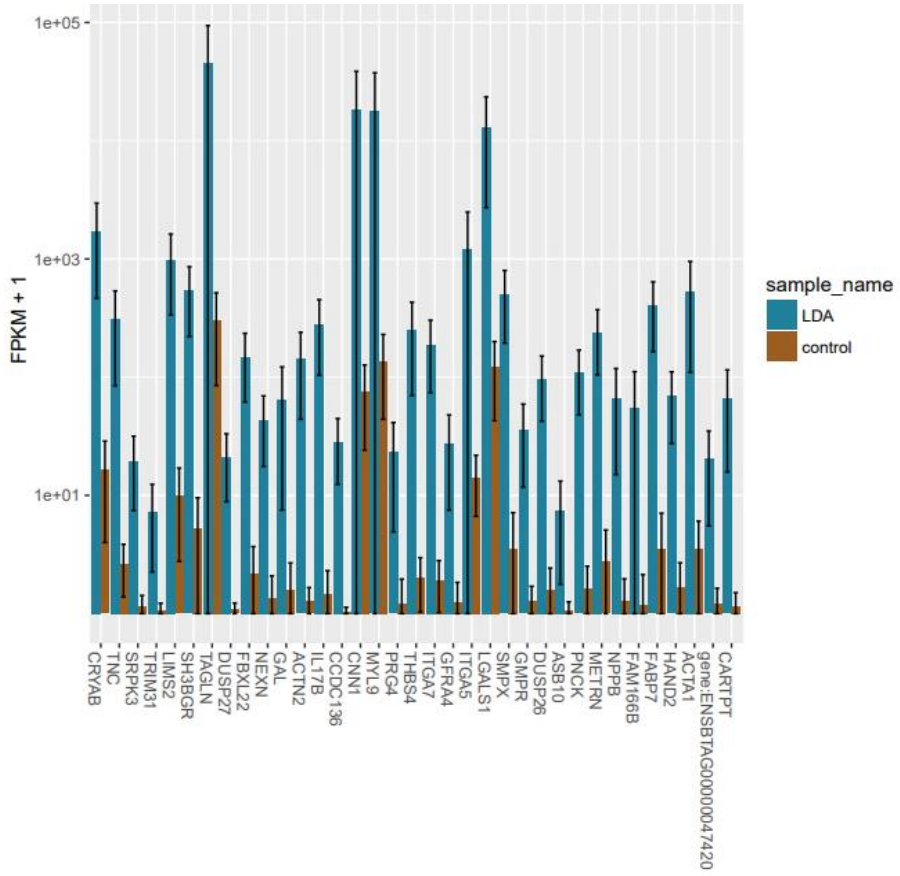
Sample ID	Raw read number	Post quality control
10.trimmed_fastq	40963939	38477391
11.trimmed_fastq	43664887	41496832
12.trimmed_fastq	35138608	33481287
1.trimmed_fastq	23298135	20745984
2.trimmed_fastq	28385258	26188875
3.trimmed_fastq	9685357	7909976
4.trimmed_fastq	39450647	30392649
6.trimmed_fastq	26155377	24124494
7.trimmed_fastq	29094140	27128414
8.trimmed_fastq	24749479	23428313
9.trimmed_fastq	41455039	38928198

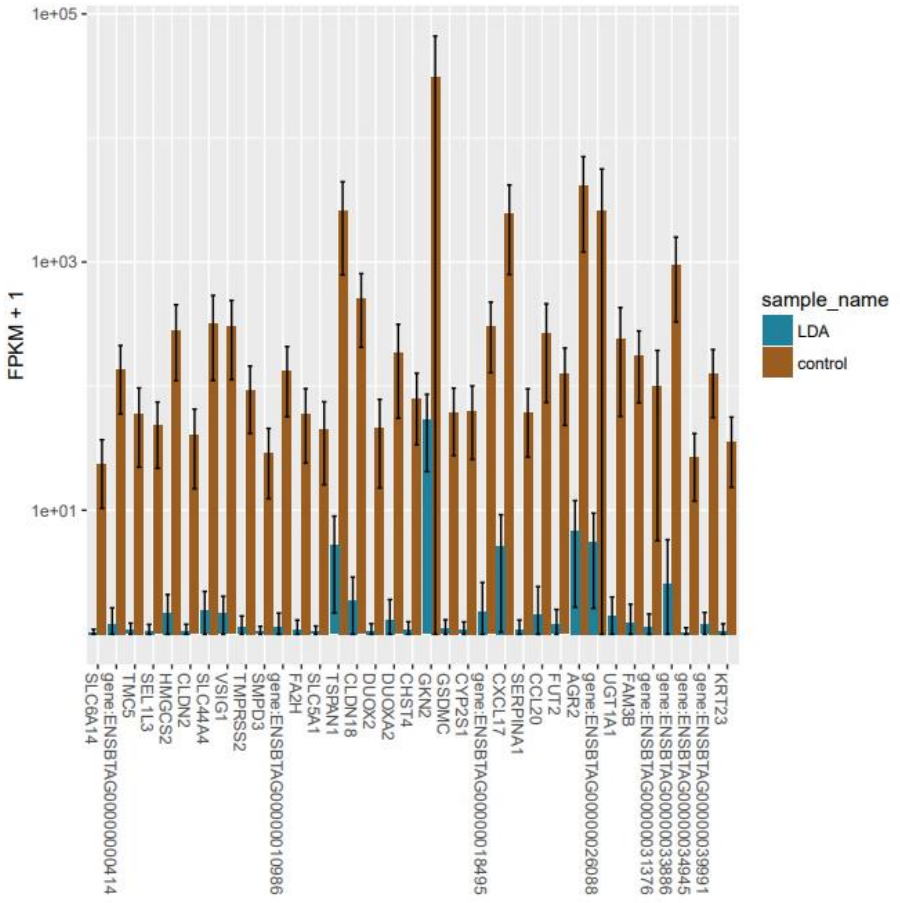
-processing of RNA-seq data

For the evaluation of the fastq files provided by the UD Genomed Ltd we used the Cuffdiff platform at the server of the University of Debrecen. The quality control of the reads was done by utilizing Fastqc, Trimmomatic and sortmerna programs. Shortly, ribosomal reads were removed and reads showing low phred score or adapter sequences were truncated. For genome alignment and differential expression analysis the Tuxedo protocol was used. For gene ontology we assessed protein coding genes showing significant, at least 10x fold change with PANTHER and agriGO. We collected the differentially expressed (DE) genes from the analysis, altogether approximately 450 genes and implemented a GO analysis which showed a downregulation in the group of G-protein coupled receptors. As a result we found, that motilin expression was higher in the control samples compared to healthy ones:



The top 35 up and downregulated genes can be found in these two graphs:





-data verification with qRT-PCR

We selected 15 DE genes for further studies with qRT-PCR method. We could adjust 13 primers and found that in all cases the RNA-seq data were confirmed by the qRT-PCR analysis.

Gene	up/down regulated	primer F	primer R
SLC6A14	lowest35	CGATTACCACGACAATTCAAGA	TCCAGCACAGAAGTGGTCAA
SMPD3	lowest35	AGCTGGAGCAGCAGCACT	GTCCTGGTCCAGCAGAGTTC
FA2H	lowest35	GTACCTCATCCATCGCTTCC	GACAGGATGAGCCGTAGCAC
DUOX2	lowest35	GGCCAGGACTCACTCTCACT	TCCTCGAGAGCCTGATATACG
DUOXA2	lowest35	TCAGCGCAGAATGGTCAGT	AGTCGATGGTCTCGTTCAGC
CHST4	lowest35	TGGCCAGAGGAAGAAGACAG	CTTCATTCTTTTGGGCGGTA
GKN2	lowest35	AGATGGACCACAAAGCCATC	CCAAACAGGATCCAATCCAC
GSDMC	lowest35	GGGACATTCGAGATGCTCTT	AATGACGCAGGGTCTTGACT
GAL	highest35	ACAGCGCTGGGTACCTTCT	TTCGTCTTCAGGCTCGAGTT
CCDC136	highest35	TTCCTGAACGAGGAGTACCG	GGAAATCCTCTTCTGAGGTCTG
MYL9	highest35	AGAAGCTGAACGGCACAGAC	GCCTCTCGGTACATCTCGTC
ITGA7	highest35	GCAGAAGGAGAGTAAGGAGAACC	GTCCTGGCTGAGAACAAAGC
ITGA5	highest35	CTCACTGGCCAGGATGAGTT	AACCTGGGAAGGCTTAGACG
ACTA1	highest35	CCGAGAGAAGTCTCGCTTCC	GCTTTCACCAGGCCAGAG
CARTPT	highest35	CGAGCCCTGGACATCTACTC	GGGGACTTGGCCATACTTCT
MLN		CCCACTCAGACCGCAAGT	AGATGGGAACAAAGGCTTCC

-HRM analysis

In the interest of easy and quick identification of the SNP in the bovine DNA samples we designed an HRM method. The HRM was implemented with a following primers: Bt_MLN_HRM_1 5'-cagccggaccccagaaga- 3' and Bt_MLN_HRM_2 5'-ggggtgcagctctctcca-3'.

Three types of tissues were compared to pre-analyzed samples. Genotyping was successful in all cases.

ID	origin of sample	result	method
T3319	tissue	T/C	sequencing, HRM
T6865	tissue	T	sequencing, HRM
T6905	tissue	C	sequencing, HRM
B1	blood	T/C	HRM
B2	blood	C	HRM
B4	blood	T/C	HRM
B5	blood	T/C	HRM
H1	hair	T	HRM
H2	hair	T/C	HRM
H3	hair	T/C	HRM
H4	hair	T	HRM
H5	hair	T/C	HRM

-publication

These data will be published in 2019, preparation of the manuscript is in progress.

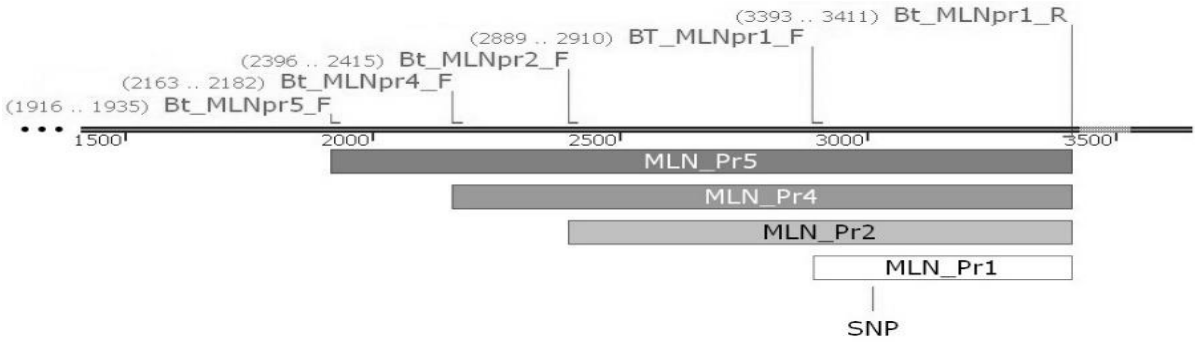
During the period of the grant some bioinformatic knowledge was necessary to learn, thus the PI spent two short term internships at University di Bologna, this fruitful collaboration resulted a publication in DNA Research.

3. In vitro examination of the mutation of motilin promoter:

Another goal of this project was to perform functional promoter analysis with bovine motilin/ mutated motilin gene which is associated with LDA. A regulatory SNP within the bovine motilin gene was reported as a possible causative mutation (Mömke, 2012) but some necessary and important functional experiments are missing from the Mömke paper. To examine the influence of the SNP on gene expression, reporter plasmid vectors were constructed for transient transfection assay.

-cloning of mutant and wild type motilin promoter to reporter constructs

Cattle motilin promoter fragments have been cloned to EGFP reporter plasmids, the results were confirmed by both enzymatic digestions and Sanger sequencing. The clones are prepared for long term storage.



-transient transformation of in vitro cell cultures with reporter constructs containing wild type and mutant promoters

We managed to construct four different lengths motilin promoter fragments amplified from a wild type bovine genomic DNA. To determine the minimal promoter region needed for the motilin expression we will transfect a construct, which contains an EGFP reporter protein driven by these four different sizes of the bovine motilin promoter to mouse fibroblast cells. From the four fragments of the promoter two was long enough to redeem the reporter gene expression, MLN_Pr5, MLN_Pr4.

-publication

The results of the cloning of the bovine motilin promoter published in international conferences.

4. In vivo examination of the mutation of motilin promoter:

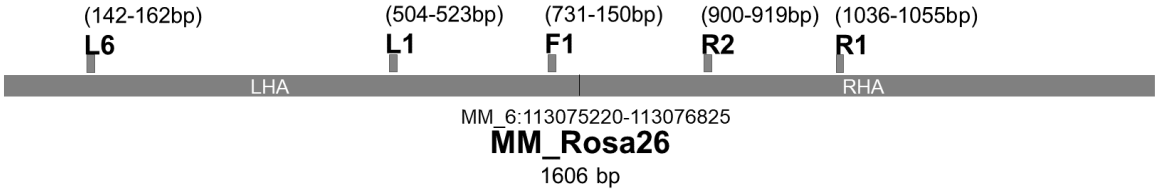
The goal was to create transgenic mouse founders, which express reporter gene driven by the bovine motilin promoter (T or C allele). to measure the protein and mRNA expression level of the reporter gene and to compare the strength of the promoter variants.

To get rid of the position effect of the transgene integration a CRIPR/Cas9 based knock in method is a suitable technique. The CRISPR/Cas9 system is an RNA-guided gene-editing platform, which can make a double strand break in the DNA at the specific location. If a donor template which carrying the transgene flanked by the specific homology arms also present a homologous recombination can be occur.

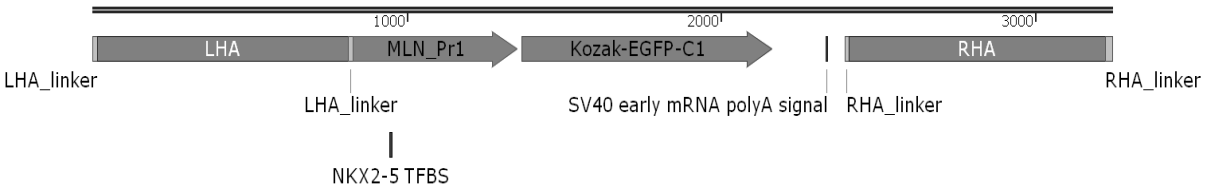
-molecular cloning of the constructs

For the CRISPR-based transgenesis process the target sequences were designed (in collaboration with Adrienn Borsy) and they were cloned into vectors containing both the gRNAs and the Cas9 nuclease.

Five gRNAs were designed to the mouse Rosa 26 locus.

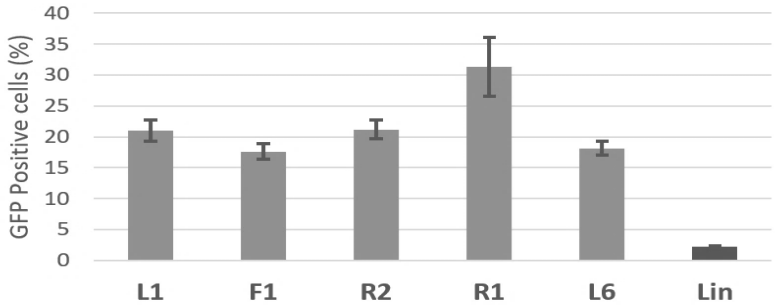


The donor vector which required to the homologous recombination was constructed including an EGFP reporter gene between the LHA and RHA sequences of the mouse Rosa 26 loci. The reporter gene is driven by the bovine motilin promoter.



-testing the efficiency in vitro

Erwin Welker's group as a collaborator transfected the five target sequence to mouse brain cells. The efficiency of the transfection measured with FACS flow cytometry. Based on the results the effectiveness of R1 was the best of the five target (31,3%).



-transgenesis

Mouse zygotes were collected at 20 h after injection of human chorionic gonadotropin (hCG) from superovulated FVB/N females mated with FVB/N males. Pronuclei were injected using a manual injector with continuous flow. Following visualization of pronuclear swelling, the needle was pulled out through the cytoplasm, injecting a small amount of additional RNA delivery to the cytoplasm. The microinjection mix contained a sgRNA in a final concentration of 150 ng/µL. Injected zygotes were transferred to pseudopregnant CD-1 females. All animals born from embryo transfer were genotyped by polymerase chain reaction (PCR) and T7 assay. PCR primers were the following: MM_Rosa26_RHA_ 5'-AACACCGCGGCAGTTTATAAAT-3', MM_Rosa26_RHA_L 5'-TTATTGCGGCCGCAGATGGGC-3'.

Interestingly, although the efficiency of knockout was good (one from five born animals), we could not receive any homologous recombination events within the experiments of knock in constructs during the grant period.

experiment day	injection buffer	number of injected embryos	number of transferred embrios	number of borned pups
2017.10.19	R1_gRNA (KO)	10	10	0
2017.11.10	R1_gRNA (KO)	20	18	5 (1 TG, 20%)
2017.12.19	MLN_Pr4_R1	15	12	0
2017.12.21	MLN_Pr4_R1	25	20	13
2017.12.22	MLN_Pr4_R1	11	9	2
2018.02.05	MLN_Pr4_R1	15	15	8
2018.04.11	MLN_Pr4_R1	16	8	1
2018.04.13	MLN_Pr4_R1	23	20	8
2018.06.26	MLN_Pr4_R1	20	16	5
2018.06.27	MLN_Pr4_R1	21	18	11
2018.07.02	MLN_Pr4_R1	29	26	13
2018.07.10	MLN_Pr4_R1	23	23	0

-publication

The gRNA efficiency analysis was published at DNA Research.

-3R rules

Animals were housed in groups of 2–5 with free access to food and water. Animals were kept under a standard light–dark cycle (06.00–18.00 h) at 22 °C. This study was carried out in strict accordance with the recommendations and rules in the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes. The protocol was approved by the Animal Care and Ethics Committee of the Agricultural Biotechnology Institute, NAIK and the Pest County's governmental office (permission number: PEI/001/3463-6/2014). The method used for euthanasia was cervical dislocation. All efforts were made to minimize suffering.