The evaluation of human and rabbit diseases in the model transgenic rabbits expressing Venus reporter protein

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1. Introduction

The transgenic (TG) construct, including the Venus coding cDNA driven by the cytomegalovirus enhancer, chicken beta-actin promoter (CAGGS), was successfully integrated into rabbit genome by Sleeping Beauty (SB) transposition and the Venus protein was expressed ubiquitously (Katter *et al.*, 2013). Venus protein is a yellow shifted derivative of the previously identified and widely used green fluorescent protein (GFP) (Shimomura *et al.*, 1962). Venus showed decreased sensitivity to both acidosis and Cl^- compared to GFP (Nagai *et al.*, 2002). Similarly to heterozygote Venus TG rabbits, homozygote Venus TG rabbits are also viable and fertile (Hoffmann *et al.*, 2016). Numerous publications claimed the potential harmful effects of the GFP or its variants on TG animals' health: e.g. dilated cardiomyopathy in mice (Huang *et al.*, 2000), altered cardiac function in zebrafish (Avey *et al.*, 2018; Ho *et al.*, 2007; Huang *et al.*, 2011), growth retardation (Krestel *et al.*, 2004) and increased axon swelling (Bridge *et al.*, 2009) in mice, etc.

In a previous report, Guo and his research group indicated the detrimental role of overexpressed GFP in glomerular physiology in TG mice with ubiquitous reporter protein expression (Guo *et al.*, 2007). More severe proteinuria and higher numbers of sclerotic glomeruli were observed in a GFP TG mouse strain after a single doxorubicin treatment compared with wild-type (WT) mice (Takagi-Akiba *et al.*, 2012). The exact role of GFP in FSGS has not been elucidated yet, but defective

polyubiquitination (Baens *et al.*, 2006) and oxidative stress (Ganini *et al.*, 2017) may be responsible for this condition in GFP TG animals.

Focal segmental glomerulosclerosis (FSGS) is a potential cause of nephrotic syndrome both in humans and pet mammals. FSGS can be primary (the initial cause is not known) or secondary (e.g. obesity- or drug-induced, virus infection-associated, etc. (D'Agati *et al.*, 2011). WT and TG rabbit models for secondary FSGS were established earlier (Matsuo *et al.*, 1987; Packham *et al.*, 1992; Vazquez-Perez *et al.*, 2001), but only a few TG rabbit models were created (Costa *et al.*, 1998; Liu *et al.*, 2007).

The main goals of this project were the following:

i. To examine FSGS in Venus TG and WT rabbits,
ii. To test the hypothesis whether the cholesterol-rich diet (0.5%) causes more severe FSGS in Venus TG compared with WT rabbits,
iii. To examine the potential role of Venus protein-induced oxidative stress in FSGS,
iv. As a continuation of our previous project (NN 108921), the detection and quantification of Venus protein expression in biological fluids and selected organs.

2. Results

2.1. FSGS in Venus TG bucks

Complete blood count and serum biochemistry

Complete blood counts were within the normal reference ranges in all groups (data not shown). Serum parameters (creatinine, albumin, cholesterol and triglycerides) of all rabbits were also within the normal reference values.

Urinalysis

Table 1

Groups	Non-TG control (n = 6)	Venus TG heterozygote (n = 6)	Venus TG homozygote (n = 5)
Microscopic hematuria	not detected	not detected	in 2 bucks
TP (g/L)	0.38 ± 0.51	1.88 ± 1.58	0.45 ± 0.29
TP/Crea ratio	0.29 ± 0.23	1.79 ± 1.61*	0.43 ± 0.34

TP: total protein; Crea: creatinine. The mean value of TP/Crea in Venus TG heterozygote bucks was significantly higher compared to controls. TP (g/L) ratio was also elevated in Venus TG heterozygote bucks, but not significantly compared with the control group (P = 0.052). Data are presented as mean ± SD. Asterisk denotes significant differences: TP/Crea ratio: *F(2.14) = 4.12, P =0.049 compared with control bucks, respectively. Urinalysis, serum biochemistry and complete blood count were done by Department of Clinical Pathology and Oncology, University of Veterinary Medicine, Budapest.

Venus protein expression in histological sections and tissue samples

Fig. 1. Detection of the fluorophore expression in Venus TG rabbits.

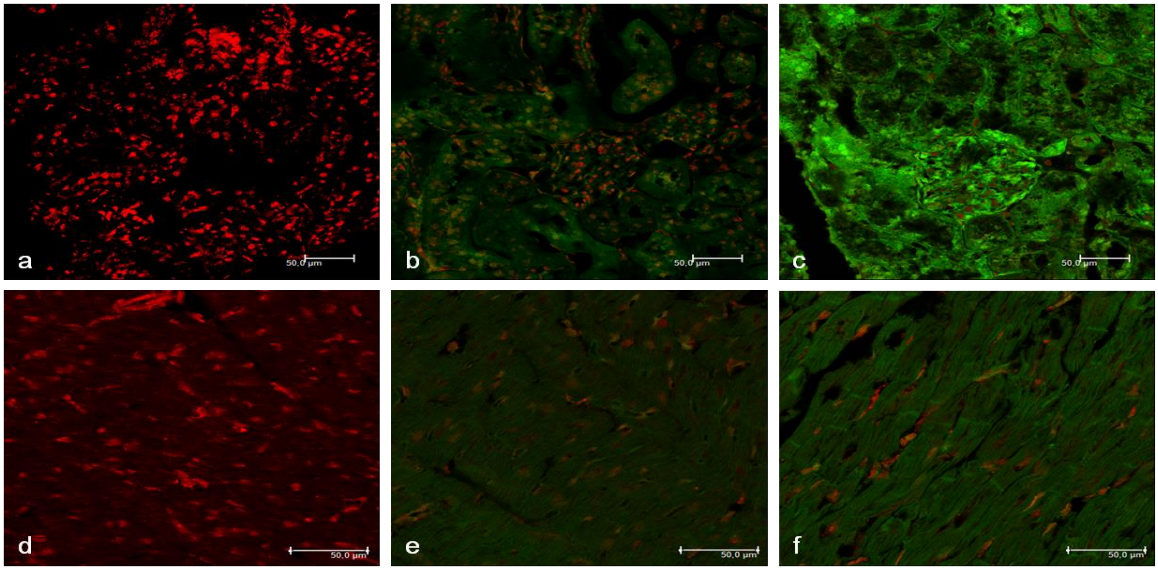

Fig. 1. Propidium iodide staining (×40 objective). *Fig. 1a–c*: renal cortex, *Fig. 1d–f:* myocardium (left ventricular wall). *Fig. 1a, d*: control buck, *Fig 1b, e*: Venus heterozygote TG buck, *Fig. 1c, f*: Venus homozygote TG buck. Scale bars (bottom right) in all images are 50 μm.

Robust fluorophore expression in the renal cortex of Venus heterozygote and homozygote TG rabbits was determined by confocal microscopy (*Fig. 1. a–c*) and microplate reader (*Table 2*).

Table 2

Animals	**Non-TG control (n=2)**		**Venus heterozygote TG (n=2)**		**Venus homozygote TG (n=2)**	
Tissue lysates	Renal cortex	Myocardium	Renal cortex	Myocardium	Renal cortex	Myocardium
Venus protein (μg/mL)	0.52 ± 0.02	0.23 ± 0.02	85.79 ± 2.07	28.56 ± 2.75	137.34 ± 17.74	34.83 ± 2.74

Glomerular histology

Haematoxylin and eosin (HE) and Periodic acid–Schiff (PAS) stainings were performed on paraffin embedded renal cortex sections (Haemalum and eosin Y solution, Carl Roth GmbH, Germany). Fifty glomeruli from the entire renal cortex of each rabbit were examined for glomerulosclerosis. Glomeruli were taken as sclerotic if the mesangial expansion was more than 30% in the glomerular area (Kimura *et al.*, 1991). Tissue sections of each rabbit were analyzed by light microscopy [Olympus BH2 (BH4), Germany] and photographed with a smartphone (Samsung Electronics, South Korea).

Table 3

Groups	**Non-TG control (n=6)**	**Venus TG heterozygote (n=6)**	**Venus TG homozygote (n=5)**
sclerotic glomeruli/ 50 glomeruli ratio (PAS)	2,33 ± 1,51	11,00 ± 3,74*	10,00 ± 7,07*
Glomerulomegaly (H&E)	not detected	in 4 bucks	in 4 bucks

Asterisks denote significant differences. $F_{(2,14)}$= 6,58, p=0.01 Venus TG heterozygote vs. control bucks and p=0,046 Venus homozygote TG compared to control bucks.

Figure 2: **Glomerulosclerosis and glomerulomegaly in Venus TG rabbits.**

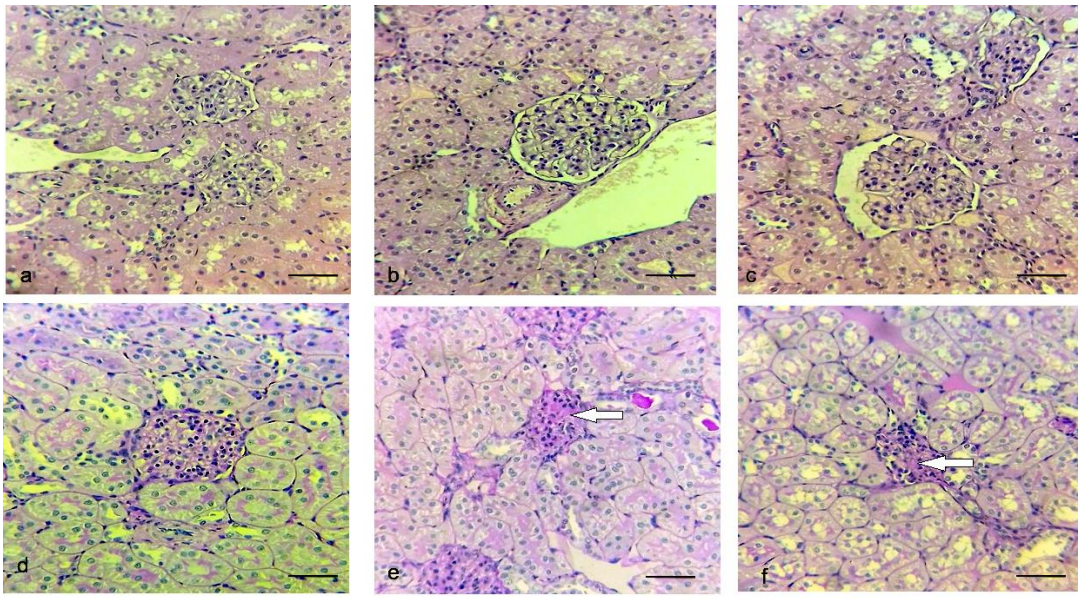

Fig 2a-c: H&E staining, **Fig 2d-f:** PAS staining (20X objective). The glomeruli of control bucks showed normal morphology (**Fig. 2a, d**). Sclerosis and hyalinosis were detected in the sections of Venus heterozygote (**Fig. 2e**) and homozygote TG bucks (**Fig. 2f**). Glomerulomegaly was also observed in Venus heterozygote (**Fig. 2b**) and homozygote TG bucks (**Fig. 2c**).

Scale bars (bottom right) in all images are 50 μm. Arrows denote the sclerotic lesions. Histological processing and light microscopy imaging were done by Balka, Gyula DMV, PhD and Dékay, Valéria DMV, respectively.

Overall, Venus TG bucks were diagnosed with FSGS; thus, this type of glomerulopathy could be a common disease in TG animals overexpressing GFP (Liptak *et al.*, 2018).

2.2. Hypercholesterinemia and oxidative stress in Venus TG rabbits

Two months old male WT and Venus heterozygote TG rabbits (thereafter: Venus TG) fed either standard rabbit chow (Purina Uni) or cholesterol (0.5%, ssniff Spezialdiäten GmbH) enriched diet for 8 weeks. Serum cholesterol level was elevated in cholesterol-fed Venus TG and WT rabbits after 4 (WT: 41 ± 4.9, Venus TG: 38.6 ± 12.1 mmol/L) and 8 weeks (WT: 54.3 ± 8, Venus TG: 39.8 ± 14.3 mmol/L), while hypercholesterinemia was not detected in standard chow-fed rabbits. Catalase, superoxide dismutase 1-3, glutathione peroxidase 1 (GPX1), peroxiredoxin 1 and NADPH oxidase 4 (NOX4) mRNA expressions were measured in renal cortex, myocardium (left ventricular wall) and liver samples. GPX1 mRNA expression was significantly higher in renal cortex of WT than Venus TG rabbits. NOX4 mRNA expression was markedly elevated in renal cortex of Venus TG rabbits compared to WT rabbits. Histology and urinalysis did not reveal differences in normal chow-fed and cholesterol-fed Venus TG rabbits.

Overall, NOX4 may have a role in FSGS in Venus TG rabbits, but further experiments are needed to clarify the role of NOX4 in disease progression. Hypercholesterinemia did not cause more severe FSGS in Venus TG rabbits.

2.3. Venus protein expression in biological fluids and selected organs

Venus mRNA expression and protein fluorescence were measured by rt-qPCR and microplate reader, respectively. Cholesterol and normal-chow fed Venus TG and WT rabbits were the same as were described in section 2.2. Significant differences in Venus mRNA expression or Venus fluorescence were not observed in any organs (renal cortex, myocardium, liver). In conclusion, hypercholesterinemia did not influence the expression of Venus mRNA or protein in Venus TG rabbits.

Lactating Venus heterozygote and homozygote TG does were injected with oxytocin (Kela Laboratoria) and milked in the second week of lactation**.** Mammary gland tissue samples of Venus TG does were collected during lactation and Venus fluorescence were analyzed by confocal imaging. Tear and oral saliva samples were collected from sexually mature (5-6 months) bucks with medical swab sticks. Western blot and macroscopic excitation analysis revealed fat and milk cell fractions from both Venus homozygote and heterozygote TG does were Venus positive. The strongest Venus fluorescence was found in the whey fraction of the Venus homozygote TG doe. This expression pattern was also verified with Western blot analysis in tear, saliva and seminal fluids.

In the vast majority of studies, mammary gland-specific promoters and regulatory elements were used to express recombinant proteins in the milk of TG animals, e.g. the whey acid protein gene promoter in mice (Pittius *et al.,* 1988)and in rabbits (Hiripi *et al.,* 2003), the β-casein gene promoter in cattle (Yang *et al.,* 2011); etc.

Our results demonstrate the secretion of recombinant protein into milk despite the lack of gland specific promoters and signal peptide cDNA for the secretory pathway, without posttranslational modifications (PTMs). This phenomena was previously discovered by our collaborator research group, using Venus TG pigs (Mukherjee *et al.*, 2016). This is a novel approach for the production of recombinant proteins into the milk of TG rabbits (Kerekes *et al.*, 2017). If the secreted recombinant protein does not require PTMs, our method could be useful for large-scale production in the future.

3. Main finding and conclusions

The main findings and achievements of this project were the following:

i. Venus TG bucks had mild proteinuria and/or microscopic hematuria and FSGS with normal complete blood count. Our data indicated that glomerulosclerosis in TG animals expressing the reporter GFP or its variants (EGFP, Venus) was not limited to mice.
ii. Similarly to human FSGS patients, male predominance was also observed in the severity of glomerulosclerosis in Venus TG rabbits.
iii. Venus protein was detected in the milk of lactating Venus TG rabbits. A new method for secretion of recombinant proteins into the milk of TG rabbits was published.
iv. Hypercholesterinemia had no effect on FSGS in Venus TG rabbits.
v. Studies about GFP-induced pathology in TG animals were collected and summarized in our recent review.

In conclusion, our data suggested that glomerulosclerosis in TG animals expressing GFP or its variants was not restricted to mice. Our findings should be considered when *in vivo* studies using GFP TG animals are designed.

4. Publications in which NKFIH PD16 120870 grant was acknowledged:

Andrea Kerekes, Orsolya Ivett Hoffmann, Gergely Iski, ***Nándor Lipták***, Elen Gócza, Wilfried A. Kues, Zsuzsanna Bősze and László Hiripi. Secretion of a recombinant protein without a signal peptide by the exocrine glands of transgenic rabbits. *PLoS One*, 2017, 12(10): e0187214. Doi: 10.1371/journal.pone.0187214, Impact factor (2017): 2.766. Medicine (miscellaneous) SJR Quartile Score (2017): Q1

Nándor Lipták*, Orsolya Ivett Hoffmann, Gabriella Skoda, Elen Gócza, Andrea Kerekes, Zsuzsanna Bősze and László Hiripi. Glomerulosclerosis in transgenic rabbits with ubiquitous venus protein expression. *Acta Veterinaria Hungarica*, 2018, 66 (2), pp. 281–293. Doi: 10.1556/004.2018.026. Impact factor (2018): 1.059. Veterinary (miscellaneous) SJR Quartile Score (2018): Q2

Nándor Lipták*, Zsuzsanna Bősze and László Hiripi. GFP transgenic animals in biomedical research: a review of potential disadvantages. *Physiological Research*, 2019, 68: 525-530. Doi: 10.33549/physiolres.934227, Impact factor (2018): 1.701[#]. Medicine (miscellaneous) SJR Quartile Score (2018): Q2

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[#] 2019 impact factor will be announced in 2020

Cumulative impact factor: 5.526

PI was corresponding and first author in two papers. Two papers are open access, published by *Physiological Research* and *PloS One*, while one paper, published by *Acta Veterinaria Hungarica* is available for free on REAL MTAK (http://real.mtak.hu/81536/) and Researchgate websites. https://www.researchgate.net/publication/326083470_Glomerulosclerosis_in_transgenic_rabbits_with_ubiquitous_Venus_protein_expression

5. Conference abstracts and posters in which NKFIH PD16 120870 grant was acknowledged

Nándor Lipták: Glomerulosclerosis Venus transzgénikus nyulakban. Genetikai Műhelyek Magyarországon XVIII. Minikonferencia, Szeged, 2017.

Nándor Lipták, Orsolya Ivett Hoffmann, Gabriella Skoda, Elen Gócza, Andrea Kerekes, Zsuzsanna Bősze and László Hiripi. Mild focal segmental glomerulosclerosis in Venus transgenic rabbits. SALAAM Final Meeting, Halle (Saale), 2017.

Nándor Lipták, Orsolya Ivett Hoffmann, Gergely Iski, Dávid Ernszt, Krisztián Kvell, Andrea Kerekes, Zsuzsanna Bősze and László Hiripi. The assessment of Venus transgenic cell migration during pregnancy in non-transgenic rabbits, Hungarian Molecular Life Sciences, Eger, 2017.

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