Novel transgenic mice with Cre-dependent co-expression of GFP and human ACE2: a safe tool for study of COVID-19 pathogenesis

Alexandra Bruter (bruter@genebiology.ru)
Core Facility Centre, Institute of Gene Biology

Diana Korshunova
Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Institute of Gene Biology

Marina Kubekina
Institute of Gene Biology of Russian Academy of Sciences

Petr Sergiev
Institute of Functional Genomics, Lomonosov Moscow State University

Anastasiia Kalinina
Federal State Budgetary Institution https://orcid.org/0000-0002-6912-5579

Leonid Ilchuk
Core Facility Centre, Institute of Gene Biology https://orcid.org/0000-0002-0157-2102

Yuliya Silaeva
Core Facility Centre, Institute of Gene Biology

Eugeni Korshunov
Core Facility Centre, Institute of Gene Biology

Vladislav Soldatov
Institute of Gene Biology https://orcid.org/0000-0001-9706-0699

Alexey Deykin
Institute of Gene Biology, Russian Academy of Sciences https://orcid.org/0000-0001-9960-0863

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Abstract

Current coronavirus disease (COVID-19) pandemic still belongs to the most serious problems of public health. A growing body of data shows that infection caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a very complex and multifaceted disease requiring its detailed study to fight with. Nevertheless, there is a row of stumbling blocks in the way of experimental research of COVID-19 determined by the deficiency of appropriate animal models. Herein, we report novel humanized mice with Cre-dependent expression of hACE2, the main entry receptor of SARS-CoV-2. These mice carry hACE2 and GFP transgenes floxed by the STOP-cassette, allowing them to be used as breeders for the creation of animals with the tissue-specific co-expression of hACE2 and GFP. Moreover, inducible expression of hACE2 makes this line biosafe, whereas co-expression with GFP simplifies the detection of transgene-expressing cells. In our study, we tested our line by crossing with Ubi-Cre mice, characterized by tamoxifen-dependent ubiquitous activation of Cre-recombinase. After tamoxifen administration, copy number of the STOP-cassette was decreased and the offspring expressed hACE2 and GFP, confirming the efficacy of our system. We believe that our model can be a useful tool for studying COVID-19 pathogenesis because the selective expression of hACE2 can shed light on the role of different tissues in SARS-CoV-2 associated complications. Obviously, it can also be used for preclinical trials of antiviral drugs and new vaccines.

Introduction

Coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) remains one of the most critical challenges for the medical and scientific communities. Since the first cases of infected patients in Wuhan\textsuperscript{1}, our understanding of the COVID-19 pathogenesis has been repeatedly transformed by results of clinical and molecular research. Most data were obtained from clinical observational studies such as cohort and case-control studies. \textit{In silico}\textsuperscript{2,3} and \textit{in vitro}\textsuperscript{4,5} research also clarified mechanisms of interactions between the virus and human cells. Nevertheless, opportunities to perform experiments for revelation of details of COVID-19 pathogenesis are limited by the absence of available animal test-systems.

Recently, it was shown that SARS-CoV-2 can effectively replicate in several species, including nonhuman primates, ferrets, dogs, and cats\textsuperscript{6}. Macaques infected with the novel virus were used for testing of an inactivated vaccine\textsuperscript{7}. The study in ferrets revealed unexpected details of anti-SARS-CoV-2 immune response\textsuperscript{8}. However, unfortunately, there are no naturally SARS-CoV-2-sensitive species among routine laboratory animals. Obviously, the most convenient, available, and popular laboratory test-system requiring no special housing conditions is mice. As wild type mice support transient coronaviruses infection without clinical signs of the disease\textsuperscript{9}, it was proposed to use genetically modified strains expressing human angiotensin-converting enzyme 2 (hACE2)\textsuperscript{10,11,12,13,14,15} the entry receptor for SARS-CoV-2\textsuperscript{16}. Another proposed option was to deliver an exogenous hACE2 in the alveolar epithelium via intranasal administration of replication-deficient adenovirus Ad5-hACE2 in wild type mice\textsuperscript{17,18}. Moreover,
the SARS-CoV-2 itself was genetically modified to effectively interact with murine ACE2 receptors, making any mice suitable for COVID-19 studies without humanization\textsuperscript{19,20}.

Here, we report a novel SARS-CoV-2-sensitive transgenic mice strain Tg\textsuperscript{CAGLoxPStopACE2GFP} (hereinafter referred to as hACE2(LoxP-Stop)), carrying hACE2 and GFP (green fluorescent protein) genes under the floxed STOP-cassette. The basic hallmark of this strain is conditional activation of the transgene, allowing to adjust the hACE2 gene expression profile by breeding with different Cre strains. In brief, Cre strains are available transgenic animals expressing Cre-recombinase, an enzyme which catalyses the site-specific recombination between two LoxP sites and, as a result, deletion of the LoxP-limited sequence in the proper orientation. Thus, if Cre-recombinase is expressed, it excises the STOP-cassette, resulting in the expression of hACE2 and GFP. In our study, we crossed hACE2(LoxP-Stop) mice with the Ubi-Cre/ERT2 mice, in which Cre/ERT2-recombinase is expressed ubiquitously but it is able to excise the STOP-cassette only after administration of tamoxifen (Cre/ERT2 needs tamoxifen to be translocated into the nucleus). Their offspring successfully expressed hACE2 and GFP after tamoxifen induction, proving that the system works.

Results

Design of transgene DNA construction

The DNA construction consists of two basic parts: the pKB1 vector and the hACE2 ORF. pKB1, an ampicillin - resistant vector, was constructed for cloning of genes intended for Cre-dependent expression. It contains insulators and terminators to protect the transgene from the position effect\textsuperscript{21,22,23}, the transcriptional unit under the control of the CAG-promoter, and the STOP-cassette\textsuperscript{24}. The Transcriptional unit consists of multiple cloning site for cloning of the transgene, the IRES element of the encephalomyocarditis virus in frame with the GFP gene and the SV40polyA signal. The pKB1 vector was constructed on the basis of the NIF vector\textsuperscript{25}: we inserted a multiple cloning site (AgeI, EcoRV, MluI) between the STOP-cassette and the IRES. The transgene expression in pKB1 is activated only after excision of the STOP-cassette by Cre-recombinase.

The hACE2 ORF was synthesized using mRNA extracted from the human kidney biopsy and cloned in the pKB1 vector via BshTI and MluI restriction sites (Fig. 1). Linearized with Pvul restriction enzyme, the construction was microinjected in zygotes’ pronucleuses; zygotes, survived after 24 hours of cultivation, were transplanted into foster mother mice.

Generation of primary transgenic mice

1511 zygotes were microinjected and transplanted to 138 recipients. 24 recipients gave birth to 48 pups (F0). 9 pups carried the transgene, and 4 of them gave F1 offspring.
Being aware of mosaic patterns of the transgene insertion, we, nevertheless, evaluated the transgene copy number in F0 generation. 7 of 9 transgenes had copy numbers, significantly exceeding 1 – in the range between 4 and 27 copies per genome. This result indicates a high probability of multimers of the construction inserted in the genome\textsuperscript{26,27}. Moreover, it is possible that in some mice, several independent insertions occurred. We expect that independently inserted sequences will be parted by chromosomal crossover in the next generations, whereas multimers will not be.

**Breeding with Cre-mice and tamoxifen-induced activation**

As an activator line, we used B6.Cg-Ndor1Tg(UBC-cre/ERT2)1Ejb/1J from The Jackson Laboratory, also known as Ubi-Cre. We crossed the hACE2(LoxP-STOP) F0 transgenic female #1190, further referred to as the mouse N1, with 8.5 copies of the transgene per genome with the Ubi-Cre transgenic male #1720 and obtained a litter consisting of 7 pups. 2 of them (#2344, further referred to as the mouse N2, and #2346, further referred to as the mouse N3) were hACE2(LoxP-STOP) positive, both with 2 copies of the transgene; 3 of them (the mouse N2, #2347, and #2350) were Ubi-Cre positive, and only the mouse N2 was both hACE2(LoxP-STOP) and Ubi-Cre positive (further referred to as hACE2(LoxP-Cre)) (Fig. 3). Then we activated Cre-recombinase in this mouse N2 with tamoxifen, and just in one week, we observed GFP fluorescence in its skin in the UV-lamp.

Next, we measured hACE2 (P3 and P4 primers) mRNA level relative to mHprt housekeeping gene (P11 and P12) in PBMCs by real-time PCR. We measured the hACE2 expression level in the mouse N2 hACE2(LoxP-Cre) after tamoxifen activation, in the mouse N3 hACE2(LoxP-STOP) with the transgene copy number, identical to the mouse N2, and in a wild type mouse (Fig. 4A; the hACE2 expression level after activation is taken for 1). In the mouse N2, it appeared to be much higher than in two other mice. hACE2 expression was not detected in wt mice. The transgenic mouse N3 without activation showed an expression level about 3% of the activated mouse N2. This probably indicated a low-range leakage of the STOP-cassette. Transgenic gDNA contamination is not the case because the PCR product was not detectable without reverse transcription (data not shown). In the mouse N2, we measured the copy number of hACE2 and the STOP-cassette before and after activation. For this, we performed real-time PCR with P3 and P4 primers for hACE2 and P13 and P14 primers for the STOP-cassette using genomic DNA extracted before and after activation as the templates. We had doubts that in the case of multimeric insertion, recombination can take place not only between the adjacent LoxP sites but also between the random ones. However, we didn't observe such recombination in our experiment. After tamoxifen activation, the STOP-cassette copy number decreased by 30%, whereas the hACE2 copy number remained exactly the same (Fig. 4B). This experiment shows that tamoxifen induction works properly and effectively, and no transgene excision takes place.

To explore transgenic hACE2 at the protein level, we performed Western blot analysis with polyclonal anti-hACE2 rabbit antibodies (Cloud-Clone Corp., USA-China). We detected a band about 120 kDa corresponding to glycosylated hACE2 form only in the blood of the activated mouse N2. This band was not detected either in wild type mouse or in the non activated transgenic mouse N3. Glycosylation is an important process for membrane proteins such as ACE2. The weight of the detected band affirms proper...
posttranslational modifications because the unglycosylated protein has the molecular weight of about 85–90 kDa\textsuperscript{28}.

Finally, we measured the hACE2 expression using flow cytometry with the same primary antibodies. We observed that 16% of blood mononuclears in the mouse N2 were stained positively for hACE2, whereas no hACE2 + cells were detected in the non-activated mouse N3 or in a wt mouse. As hACE2 was not detected in the mouse N3 by Western blot or flow cytometry, we can conclude that there is no STOP-cassette leakage on the protein level. The difference between mRNA and protein levels can be explained by the SV40polyA sequence in the 3’ part of the STOP-cassette. Even if low rate transcription occurs from the CAG promoter, translation of this transcript terminates at the SV40polyA before the ribosome reaches the \textit{hACE2} ORF. The fact that STOP-cassette leakage is seen only on the mRNA level but not on the protein level supports our claim that our animals satisfy the requirement of tamoxifen-induced expression. As we stained the cells without fixation and permeabilization, we assume that the antibodies reacted only with hACE2 translocated to the cell surface. This is important because only translocated hACE2 can serve as an entry point for the coronavirus and there were doubts if transgenic hACE2 can be properly processed in different types of cells.

\section*{Discussion}

In December 2019, Wuhan, the capital of Hubei Province in China, became the center of the outbreak of pneumonia with an unknown etiology. By January 7, 2020, Chinese scientists identified the causative infectious agent as a novel strain of coronavirus and named it SARS-CoV-2. SARS-CoV-2 causes COVID-19, a very complex disease with a wide spectrum of symptoms. Now it is known for sure that at first SARS-CoV-2 invades the epithelium of the upper and lower airways, but in the case of severe course, it reaches the gas exchange units of the lungs and infects alveolar type II cells\textsuperscript{29}.

Clinical observations quickly established that the main cause of the severe course of COVID-19 is not the lung damage itself, but hyperactive immune response leading to cytokine storm, systemic inflammation, and downstream acute respiratory distress syndrome (ARDS)\textsuperscript{30}. It was shown that the virus has complex interaction with the innate and adaptive immunity and can directly infect the immune cells. Viral particles and SARS-CoV-2 RNA were found in monocytes, CD4 + T cells, CD8 + T-cells, and B-cells\textsuperscript{31,32,33}.

Many reports show that besides the lungs and the immune system, an important target of SARS-CoV-2 is the vascular endothelium\textsuperscript{34}. After the beginning of the disease outbreak, quickly accumulated autopsy data revealed thromboembolic complications and microthrombosis in COVID-19 patients\textsuperscript{35,36}. Recently, Monteil et al. reported successful SARS-CoV-2 inoculation in blood vessel organoids\textsuperscript{37}. Furthermore, some researchers directly observed the presence of viral particles in endothelial cells of patients\textsuperscript{38,39}. In sum, these data show that the vasculature involvement may be the key link in the pathogenesis of COVID-19 because endothelium is a master regulator of hemodynamics, hemostasis, and immune cells homing.

Another tissue classically involved in the coronavirus infection is the nervous system. Clinical observations of COVID-19 patients have shown neurological complications such as headache, loss of
smell, encephalopathy, cognitive impairment, and ischemic stroke\textsuperscript{40}. Most likely, these symptoms are caused by the systemic inflammatory syndrome or direct SARS-CoV-2 replication in the nervous system. Using human brain organoids, mice overexpressing human ACE2, and brain autopsy, Song et al. have shown neuroinvasive activity and direct infection of neurons by SARS-CoV-2 through the ACE2-dependent pathway\textsuperscript{41}.

Unfortunately, the list of potential targets for COVID-19 is growing. There are reports of the involvement of other organs with the high expression of ACE2, such as the intestines, kidneys, and heart\textsuperscript{42}. As ACE2 is highly expressed in tissues of the male reproductive system, there are concerns that SARS-CoV-2 can cause infertility in men\textsuperscript{43,44}. These concerns are partially confirmed by cases of orchitis following the outbreak of SARS-CoV in 2002\textsuperscript{45} and sex hormones imbalance in male patients with COVID-19\textsuperscript{46}.

Thus, COVID-19 has very complex and multifaceted pathogenesis. The revelation of its pathways is an essential way in the search for effective approaches to therapy. Herein, we describe novel transgenic mice with the Cre-dependent co-expression of hACE2 and GFP, designed to study the role of different tissues in SARS-CoV-2 infection. For instance, specific activation of hACE2 in endothelial, immune, or alveolar cells, can help to reveal the primary causative tissue, which involvement leads to the acute respiratory distress syndrome in COVID-19. Another option, for example, is to study the fertility of SARS-CoV-2-infected male mice with testicular-specific activation of hACE2. In this way the fertility will depend only on testicular but not on systemic and behavioral changes caused by COVID-19.

In our study, we examined F1 generation, obtained after crossing the hACE2(LoxP-Stop) F0 mouse with the Ubi-Cre mouse. The human ubiquitin C (UBC) promoter that drives Cre expression in this strain provides a relatively consistent expression level across different cell types. One of the offspring carried both $hACE2GFP$ and $Cre$ alleles. In this mouse, the expression of hACE2 and GFP was then successfully triggered by tamoxifen administration at least in blood mononuclears and skin.

After activation, we detected the STOP-cassette excision, hACE2 mRNA presence, and hACE2 protein assembling. Westernblot analysis found hACE2 with the molecular weight about 120 kDa, indicating proper protein glycosylation. Using FACS, we confirmed that hACE2 is located on the cell surface, which means that transgenic protein successfully underwent the surface translocation and can interact with SARS-CoV-2. Interestingly, our dosage scheme of tamoxifen administration led to relatively modest activation of the transgene, but it can be increased in further experiments if required\textsuperscript{47}. Thus, tamoxifen-dependent activation is an additional option to adjust the transgene expression and, therefore, the SARS-CoV-2 sensitivity.

We think that use of Ubi-Cre/ERT2 strain is an important point in our research. We chose this approach because tamoxifen-dependent activation is a two-step defense against SARS-CoV-2 infection, simplifying work with animals. Before activation, the mice are epidemiologically safe for laboratory staff, and workers are safe for the mice. Thus, it minimizes all the risks related to undesired infection of the hACE2-expressing mice by SARS-CoV-2 before placing them in specific conditions of a virological laboratory.
Developing our strategy for the generation of mice with Cre-dependent activation of hACE2, we were guided by several points. Firstly, we decided to co-express hACE2 with GFP because we think it simplifies visualization procedures. GFP fluorescence confirms the transgene activation allowing to visualize hACE2-expressing tissues without the use of complicated and expensive immunostaining protocols. In our study we demonstrated that activation of hACE2/GFP expression can be confirmed just by the fluorescence in the transgenic mouse skin. Noteworthy, some authors notice that floxed transgenes can be expressed at a small level even before Cre-dependent activation\textsuperscript{48}, so co-expression with GFP also allows immediately detecting this leak.

Conclusions

SARS-CoV-2-sensitive murine models are an important part of studies of COVID-19. In our laboratory, a novel mouse strain was obtained with the conditional co-expression of hACE2 and GFP. We believe that our strain hACE2(LoxP-Stop) can serve as a useful tool for studying the role of different tissues in COVID-19 pathogenesis. Moreover, Cre-dependent expression of hACE2 makes hACE2(LoxP-Stop) mice convenient for housing because before activation, they can not serve as a reservoir for SARS-CoV-2 and do not require specific biosafety conditions. Finally, co-expression with GFP simplifies visualization of hACE2-expressing tissues. We are ready to transfer hACE2(LoxP-STOP) mice to any interested research group. We believe that our hACE2(LoxP-STOP) strain has a number of unique properties that can be useful in preclinical trials and especially in studying COVID-19-pathogenesis.

Materials And Methods

Bioethics and legality of the work

The human biomaterial was obtained in the process of medical intervention under the supervision of the Local Ethics Committee of the Sechenov (Medical) University. All the animal experiments were carried out in accordance with the rules of humane treatment, with the permission and control of the IGB RAS Bioethics Commission (the conclusion of the Bioethical Commission #06 – 01/02 – 1 dated 02/03/2020). The animal experiments did not imply any biological hazard and did not require a biosafety facility.

Development of transgene DNA construction

Total RNA was extracted from the human kidney biopsy material. The tissue was homogenized with Precellys 24 homogenizer, and RNA was extracted with the QIAGEN RNeasy Mini Kit. cDNA was synthesized with reverse transcriptase Superscript II (Invitrogen) and d(T)\textsubscript{16} oligo. hACE2 gene (2400 bp) was amplified with Q5 polymerase (NEB), using primers P15 and P16 (all primer sequences are presented in the Appendix). The amplified fragment was digested with restriction enzymes BshTI and MluI (ThermoFisher Scientific) and inserted in the pKB1 vector digested with the same enzymes. The circular plasmid DNA was then digested with PvuI and gel purified; a 13.5 kbp linear fragment without the
bacterial propagation elements was extracted with the QIAquick Gel Extraction Kit (Qiagen) and dissolved in the TE-buffer at concentration 1 ng/µl.

**Animals, pronuclear microinjections, and embryo transfer**

To generate primary transgenic animals, we used (CBAxC57BL/6)F1 zygotes obtained from 3 wk of age CBA and C57BL/6 breeders (Stolbovaya breeding station, Russian Federation). The transgene DNA construction was introduced by pronuclear microinjections as described earlier. Surviving zygotes were cultivated in M16 medium, 37°C, 6% CO₂ for 24 h and then transplanted to oviducts of foster mothers at the 2-cells stage as previously described.

B6.Cg-Ndor1Tg(UBC-cre/ERT2)1Ejb/1J, also known as Ubi-Cre, mouse strain was purchased from The Jackson Laboratory. In this strain, chimeric Cre-recombinase is expressed under the control of the UBC promoter. Chimeric Cre-recombinase consists of a Cre-recombinase domain and a domain representing a mutant form of the estrogen receptor, which lacks the ability to bind natural 17β-estradiol and binds only synthetic tamoxifen. Without tamoxifen, the protein is restricted to the cytoplasm, and only in the presence of tamoxifen translocates to the nucleus and performs recombination. Ubi-Cre mice can be maintained only in hemizygodites.

Animals were kept in the IGB RAN vivarium with artificial lighting (12 h/12 h mode) at the temperature of 21–23°C, the humidity of 38–50%; mice had free access to food and water.

**Tamoxifen treatment**

Tamoxifen (Hexal) was administered by a gavage at a dose 1 mg/100 g once a day for 20 days (2% suspension formulations).

**Genotyping**

DNA samples were extracted from tail tissues samples. The first step of F0 genotyping was performed using the primer pair P1 and P2, specific to the 3'-region of the STOP-cassette and the 5'-region of IRES, respectively (the 3000 bp PCR product band, Fig. 2). Advanced genotyping of F0 combined detection of the STOP-cassette (P13, P14), the hACE2 ORF (P15, P16), eGFP (P21, P22), and the terminator (P23, P24).

F1 genotyping was performed by detection of hACE2 and eGFP ORFs presence as described above and Ubi-Cre presence according to the protocol, recommended by Jackson Laboratory using P17, P18, P19, P20 primers. All Ubi-Cre probes had a 324 bp band as the internal positive control, and transgenic Ubi-Cre probes had a 475 bp band.

**Copy number measurement**

The transgene copy number was determined using logarithmic interpolation based on Cₜ for the transgene and reference genes with known copy numbers. We have chosen murine Hprt (1 copy per genome in males, 2 in females, primers P5 and P6), Hba1 (4 copies per genome, primers P7 and P8), and H3c7 (18 copies per genome, primers P9 and P10) genes as the reference for comparison. For qPCR, we...
used the SYBR Green mix with HS Taq polymerase (Evrogen). We used primers P3 and P4 for hACE2 copy number measurement. In experiments assessing the Cre-recombinase effectivity, we also measured the STOP-cassette copy number with primers P13 and P14.

**qPCR and gene expression measurement**

Blood for evaluation of hACE2 expression was collected from the retro-orbital venous sinus. 100 ul of blood samples were mixed with 1 ml of ammonium-chloride-potassium RBC lysis buffer, incubated for 5 minutes at 25°C and centrifuged for 5' at 300 g at room temperature. The observed cell pellet was washed with PBS twice and used for downstream applications.

gDNA-free total RNA was extracted from blood cells with the RNeasy Plus Mini Kit (Qiagen). cDNA was synthesized with the iScript™ Reverse Transcription Supermix (Bio-Rad), and real-time PCR was performed using the SYBR GREEN mix (Evrogen) and primers P3, P4, P11, and P12. Real-time PCR with extracted RNA instead of cDNA was performed to prove the absence of genomic DNA contamination. The results were analyzed with the CFX Manager Software (Bio-Rad).

**Western blot**

Peripheral blood mononuclear cells (PBMCs) of F1 mice hACE2(LoxP-Cre) were extracted as previously described. After RBC lysis, PBMCs were lysed in 200 µl of the RIPA buffer (1 ml RIPA: 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris, pH 8.0; 2 µl PIC, 20 µl PMSF). The Protein concentration was measured by the Bradford assay, the calibration curve was built using BSA serial dilutions. The samples (80 mg of protein each) were loaded into 8% PAAG. The electrophoresis was performed in the SDS buffer at 120V for 1.5 h, the proteins were then transferred to the nitrocellulose membrane (0.2 µm) at 250 mA for 1 hour in the transfer buffer with 20% MeOH. After the transfer, the membrane was incubated in 5% non-fat dried milk dissolved in TBS. The Primary anti-hACE2 antibodies PAB886Hu01 (Cloud-Clone Corp., USA-China) were dissolved 1:1500 in 1% BSA in the TBS-buffer, and the membrane was stained for 18 hours at 4°C. The membrane was then washed in the TBS-buffer and incubated with the secondary anti-rabbit antibodies (Anti-rabbit IgG, HRP-linked Antibody #7074, Cell Signaling) diluted 1:1000 in 5% non-fat dried milk dissolved in TBS for 1 hour. After washing in the TBS-buffer, chemiluminescent labels were activated with ECL and visualized in iBright.

**FACS analysis**

Extracted PBMCs were pre-incubated with Fc block (clone 2.4G2, BD Pharmingen, Franklin Lakes, NJ) (10 min, 4°C), incubated with the polyclonal anti-hACE2 antibodies (Cloud-clone Corp., Product No.PAB886Hu01) (40 min, 4°C), and then stained with the secondary fluorescent -labeled antibodies (Invitrogen, Catalog # A-11010) (40 min, 4°C). The analysis was performed on the flow cytometer BD FACSCanto II (BD Bioscience) using the FACS DIVA 6.0 software (BD Bioscience). Dead cells were excluded from the analysis based on the parameters of the forward and side scatter and staining with propidium iodide (BD Bioscience). At least $10^4$ events per sample were collected to characterize the
Peripheral leukocytes populations. Data were processed using the FlowJo 7.6 software (TreeStar Inc., Ashland, OR).

**Statistical analysis**

Statistical processing was not applied due to the limited number of animals (only one in each group).

**Appendix**

Sequences of primers:
| Code | Sequence 5’->3’ |
|------|---------------|
| P1   | AAAATTGTGTACCTTTAGCTTTTTTA |
| P2   | CCTAGGAATGCTCGTCAAGA |
| P3   | CTTATGTGCACAAAGGTGAC |
| P4   | TGTGGCTGCAGAAAGTGAG |
| P5   | GACCGTGGGCTTACCTCAC |
| P6   | GACTGCGGGGTCGGCATGA |
| P7   | CCACCTCGATGACCTGCCCCG |
| P8   | CTGATGCCTCTGCTCCCCCTTC |
| P9   | GACCTCAAGACCGACCTGCG |
| P10  | TTGGGCATGATGGTGACACG |
| P11  | GCAGTACAGCCCCAAAAATGG |
| P12  | CTTTTCCACCAGCAAGCT |
| P13  | GTTAGATCTGCTGCCACCGT |
| P14  | AGGTGGCAAGTGGTATTCG |
| P15  | ATTAACCGGTATGTCAGCTCTTCTGC |
| P16  | ATTAACCGGTCTAAAGGGAGGTCTGAAACATCAT |
| P17  | GACGTACCCCGTTCTGGT |
| P18  | AGGCAAATTTTGTGTACGG |
| P19  | CTAGGCCACAGAATTTGAAAGATCT |
| P20  | GTAGGTGAAATTCTACATCATCC |
| P21  | CGACGTAAACCGGCCAAGCT |
| P22  | GCGGACTTGAAGAAGTCTG |
| P23  | GCGAGTCCATGTCACTCAG |
| P24  | GTGTGCCCTTTGGAGCTTG |

**Declarations**

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**Conflict of Interests**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

**Author contributions**

**AD** – main idea, design of the experiments, embryo transfer, obtaining the results, writing the article

**DK** – animal surgery, animal care, tamoxifen administration, pronuclear microinjections, tissue sampling

**AB** – molecular biology, DNA transgenic construction development, writing the article

**EK** – animal surgery, animal care, animal breeding, animal handling, obstetrics procedures, veterinary, blood collection

**MK** – molecular biology, DNA transgenic construction development

**AK** – FASC, writing the article

**YuS** – FASC

**PS** – cDNA cloning

**LI** – bioinformatics, mathematical proceeding

**SV** – main idea, design of the experiments, graphical design, writing the article
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Figures

Figure 1

pKB1-hACE2 linear construction intended for transgenesis
Figure 2

A) primer positioning for primary genotyping; B) F0 primary genotyping. Mouse N1 involved in further experiments is indicated; C) Advanced genotyping of mouse N1. Presence of hACE2, GFP, the STOP-cassette, and the terminator is confirmed.
Figure 3

Scheme of transgenic mice breeding and F1 genotyping. 7 pups characterized by hACE2 (~500 bp band) and GFP (~200 bp band) presence on the upper gel and by Cre-Ubi presence (2 bands in the transgenic mice, 1 band in wt mice) on the lower gel.
Figure 4

Effect of tamoxifen administration on transgene activation. A) The hACE2 expression was found only in hACE2(LoxP-Cre) mouse (N2) after tamoxifen activation; B) The Stop-cassette copy number in hACE2(LoxP-Cre) mouse (N2) after tamoxifen activation decreased by 30%, whereas the hACE2 copy number did not change;
Figure 5

Detection of hACE2 and GFP. A) hACE2 presence on the cell surface of PBMC. A) Activated transgenic mouse N2 hACE2(LoxP-Cre) (right lower panel) compared to non-activated transgenic mouse N3 hACE2(LoxP-Stop) (left lower panel). Unstained controls are represented at the upper panel. B) - The N2 mouse’s skin fluorescence in UV-lamp was observed after tamoxifen-induction due to eGFP expression. C) - Western blot analysis of hACE2 expression in PBMC. Glycosylated 120 kDa hACE2 at the upper panel, 37 kDa GAPDH at the lower panel. WT mouse to the left, activated transgenic mouse in the middle, non-activated transgenic mouse to the right.

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