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Increased *LAMP1* Expression Enhances SARS-CoV-1 and SARS-CoV-2 Production in Vero-Derived Transgenic Cell Lines

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Abstract—Coronaviridae is a family of single-stranded RNA (ssRNA) viruses that can cause diseases with high mortality rates. SARS-CoV-1 and MERS-CoV appeared in 2002–2003 and 2012, respectively. A novel coronavirus, SARS-CoV-2, emerged in 2019 in Wuhan (China) and has caused more than 5 million deaths in worldwide. The entry of SARS-CoV-1 into the cell is due to the interaction of the viral spike (S) protein and the cell protein, angiotensin-converting enzyme 2 (ACE2). After infection, virus assembly occurs in Golgi apparatus-derived vesicles during exocytosis. One of the possible participants in this process is LAMP1 protein. We established transgenic Vero cell lines with increased expression of human *LAMP1* gene and evaluated SARS-CoV-1 and SARS-CoV-2 production. An increase in the production of both viruses in *LAMP1*-expressing cells when compared with Vero cells was observed, especially in the presence of trypsin during infection. From these results it can be assumed that LAMP1 promotes SARS-CoV-1 and SARS-CoV-2 production due to enhanced exocytosis.

Keywords: SARS-CoV-1, SARS-CoV-2, LAMP1, COVID-19, Vero, transgenic cells

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Coronaviruses (CoV) belong to the Coronaviridae family of the order Nidovirales. These are viruses containing single-stranded plus-strand RNA (ssRNA), some of which cause serious respiratory and intestinal infections in humans and animals [1]. In the 21st century, members of this family were responsible for three outbreaks of serious human diseases: severe acute respiratory syndrome (SARS) in 2002–2003, Middle East Respiratory Syndrome (MERS) in 2012, and the COVID-19 pandemic which is currently in its third year. The first of these was called SARS-CoV (according to modern terminology SARS-CoV-1), and in 2012, the second was called MERS-CoV. These outbreaks resulted in a total of 1,398 deaths [2]. At the end of December 2019, a new type of coronavirus, SARS-CoV-2, appeared in the city of Wuhan (China) causing the COVID-19 pandemic [3] which quickly spread throughout the world and continues to this day. Over 5 million deaths due to COVID-19 have been recorded to date (https://www.who.int/emergencies/diseases/novel-coronavirus-2019, data as of 08.12.2021).

The coronavirus genome is a (+)ssRNA approximately 30 kb in size with a 5'-cap and 3'-poly(A)-tail. Genomic RNA codes for polyproteins and serves as a template for the synthesis of subgenomic RNA. Analysis of the SARS-CoV-2 genome sequence revealed a high similarity with the genomes of other infectious agents from the *betacoronavirus* genus: bat-SARS-like (SL) ZC45, bat-SL ZXC21, SARS-CoV-1 and MERS-CoV [4]. Bats and palm civets are thought to be the natural reservoir of this pathogen. Entry of the virus into cells is mediated by the binding of the spike glycoprotein (S) located on the surface of the virion to its receptor on the host cell. Angiotensin-converting enzyme-2 (ACE2) has been identified as the main cellular receptor for SARS-CoV-1 and SARS-CoV-2. The receptor-binding domain (RBD) of the S-protein is involved in the interaction with the cellular receptor. SARS-CoV-2 has already been shown to recognize ACE2 in some animal species [1, 5].

In addition, it has been shown that SARS-CoV-2 uses the TMPRSS2 serine protease to prime the S-protein upon cell entry, and the mechanism of this

process is similar to that of SARS-CoV-1 [6, 7]. SARS-CoV-2 RBD has been reported to have stronger affinity for ACE2 than that of SARS-CoV-1 [8]. Proteolytic activation of the S-protein is one of the most important steps in the coronavirus life cycle. In addition to priming by TMPRSS2, the S protein can also be cleaved by host proteases (cathepsin, furin, trypsin) at various sites. In the case of infection of Vero or Vero E6 cells under laboratory conditions, the addition of trypsin led to the same effect as in the presence of cathepsin [9].

The standard cell line for virological research, including the cultivation and study of coronaviruses, is the Vero line. The Vero line is derived from African green monkey kidney cells. An important characteristic of these cells is their inability to produce interferon, an important component of the cellular immune response [10]. The Vero E6 line is often used, which is a clone variant of the Vero culture with contact inhibition i.e., inhibition of cell division upon contact with neighboring cells. SARS-CoV-1 has previously been shown to cause lytic infection when it infects a number of cell cultures, including Vero and Vero E6. Thus, 12 hours after infection of Vero E6 cells, the mean infectious dose ($CCID_{50}$/mL) of the virus was 5.2×10^3 [11, 12].

Using electron microscopy, it was shown that SARS-CoV-1 virus particles attach to the surface of Vero E6 cells and 7 hours after infection viral nucleocapsids enclosed in endosomes appear in the rough endoplasmic reticulum and in the Golgi apparatus. Glycosylation of the viral proteins S, M (membrane protein), and E (envelope protein) occurs in the vesicles of the Golgi apparatus, and is necessary for the correct assembly of viral particles, as well as the assembly itself [13]. The final stage of the infectious cycle is the transfer of lysosomes containing viral particles to the cell membrane and their release through exocytosis. Colocalization of SARS-CoV-1 ORF6 accessory protein with LAMP1 protein in infected Vero E6 cells was found previously [14]. LAMP1 is a lysosome-associated membrane protein that is involved in exocytosis [15]. The glycoprotein (GP) of the Lassa virus, binds with LAMP1 protein after the entry of virions into the cell [16].

We hypothesized that an increase in the amount of LAMP1 in the cell would lead to an increase in exocytosis, causing an increase in the production of SARS-CoV-1 and SARS-CoV-2 viruses by infected cells. In order to test this hypothesis, we created transgenic cell lines based on the Vero culture expressing the human *LAMP1* gene, and evaluated the production of both viruses in the resulting transgenic lines and the original cell culture. As a result of the analysis, differences were found in the production of viruses depending on the expression *LAMP1* and the presence/absence of trypsin used as a serine protease.

EXPERIMENTAL

Preparation and cloning of the open reading frame (ORF) of the *LAMP1* gene. RNA was isolated from HEK293A cells (Cell Storage of the State Research Center of Virology and Biotechnology Vector, Rospotrebnadzor, Russia) using the TRIzol reagent (Thermo Fisher Scientific, USA). The reverse transcription reaction was performed using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) using a specific primer for the *LAMP1* ORF sequence: 5'-GTGCACCAGGCTAGATAGTCTG-3'—according to the following program: decrease in temperature from 75 to 55°C (1.5°C/5 s); 3 min at 55°C and 1 hour at 42°C. The resulting cDNA was used for ORF amplification. The reaction was carried out using the primers: 5'-TAAGCAGAATTCGCCTCG-CGCCATGGCGGC-3' and 5'-TAAGCAGTCGAC-GTGCACCAGGCTAGATAGTCTGGT-3'—according to the following program: 11 cycles (3 min at 94°C, 50 s at 94°C, 1 min at 56°C, 14 min at 68°C), 32 cycles (50 s at 94°C, 10 min 15 s at 61°C) and 10 min at 68°C.

The synthesized fragment was sequenced according to Sanger and cloned under the control of the cytomegalovirus (CMV) promoter into a plasmid obtained from pSBi-GP (#60511, Addgene, USA). The IRES sequence with the puromycin-*N*-acetyltransferase was cloned after the *LAMP1* reading frame. Next, Vero cells (Cell Storage of the State Research Center of Virology and Biotechnology Vector, Rospotrebnadzor, Russia) were transfected with the Lipofectamine 3000 reagent (Thermo Fisher Scientific) with the assembled genetically engineered construct and a plasmid containing the pCMV (CAT) T7-SB100 transposon (#34879, Addgene). Stable transformants were selected on DMEM/F12 medium containing the antibiotic puromycin (10 μg/mL) for one week followed by cultivation in puromycin medium (5 μg/mL) for 6 weeks. *LAMP1* expression in transgenic cell culture was evaluated by real-time PCR using the Bio-Master HS-qPCR SYBR Blue kit (Biolabmix, Russia) and the primers: 5'-AACTCATGAGCTGGACGCTG-3' and 5'-CTCCTGTGGAAAAGAGAACAC-3' to cDNA obtained using the OT-M-MuLV-RH kit (Biolabmix) and degenerate primers. The results were normalized for expression of the housekeeping gene hypoxanthine phosphoribosyltransferase-1 (*HPRT1*).

The experiment to analyze the level of LAMP1 mRNA expression in the Vero cell line was carried out in three technical and four biological replicates. The initial data were processed by the $2^{-\Delta\Delta Ct}$ method [17]. Graphical results are presented as means of biological replicates with standard deviations (SD). Statistical differences between the cell lines Vero.Lu3, Vero.Lu5 and Vero were determined by ANOVA analysis of variance. Significance of differences was assessed using Tukey's HSD significance test. Differences were considered significant at the value $p < 0.05$.

Infection of cell cultures with SARS-CoV-1 and SARS-CoV-2 viruses. Work with live SARS-CoV-1 and SARS-CoV-2 viruses was carried out in accordance with the approved Sanitary and Epidemiological Rules for working with pathogens of biosafety group III (https://www.rospotrebnadzor.ru/documents/details.php?ELEMENT_ID=3552). Cells were seeded into wells of a 24-well plate (10^5 cells/well) and incubated until 90% confluence was reached. In experiments involving treatment of a cell monolayer with trypsin, trypsin (up to 4 μg/mL) treated with tosylphenylalanylchloromethyl ketone (TPCK) (Sigma-Aldrich, USA), was added to 1 mL of DMEM/F12 Culture medium (Biolot, Russia) containing antibiotics and incubated for 25 min at room temperature. In experiments without the presence of trypsin, the cells were incubated under the same conditions, but without trypsin. In each well of a 24-well plate, 100 μL of DMEM/F12 medium containing 4.6 log FFU/mL (FFU are focus-forming units) of SARS-CoV-1 virus particles (Urbani strain, Erasmus University Medical Center, Rotterdam; GenBank Acc. no. AY278741.1) or SARS-CoV-2 (SARS-CoV-2/human/AUS/VIC01/2020 strain, GenBank Acc. no. MT007544.1) were added and incubated for 1 h at room temperature. The plate was washed with serum-free Eagle's medium (MEM). 1 mL of a solution of trypsin in DMEM/F12 (2 μg/mL) was added to the plates intended for culturing the cell culture in the presence of trypsin. Only DMEM/F12 medium was added to plates where cultivation took place without trypsin. The plates were incubated for 48 h at 37°C and 5% CO_2. To isolate the viral genetic material, the plates were frozen and thawed three times, followed by lysis using a RIBO-prep kit (AmpliSens, Russia). Each experiment was performed in three biological replicates.

To determine the amount of virus in the cell monolayer and culture medium, the medium was collected separately, centrifuged for 5 min at 1000 *g* and frozen for subsequent lysis as described above. Fresh medium was added to the monolayer, after which it was lysed.

Real-time PCR to determine virus genome equivalents. The number of genome equivalents of SARS-CoV-1 and SARS-CoV-2 was measured using the diagnostic kit for the detection of SARS-CoV-1 and SARS-CoV-2 by RT-PCR with fluorescent probes, Vector-OneStepPCR-CoV-RG (SRC VB "Vector" of Rospotrebnadzor, Russia) according to the manufacturer's recommendations. The results were normalized with respect to the plasmid DNA pJet1.2_SARS, which contained a fragment of the viral genome (sequence 28670–28826 according to strain MN997409.1). The amount of genomic DNA of Vero cells was measured using real-time PCR using the SYBR Green method using BioMaster HS-qPCR SYBR Blue reagents (Biolabmix) with the primers vero_chr11-F (5'-TCCTATGACGGGGGCTGTTA-3') and vero_chr11-R (5'-GGCCCAAGAGGTCGAATTGT-3'). Insofar as the karyotype of the Vero cell line is variable; a fragment of chromosome 11 (chr11:6570784–6570896) was chosen for amplification, for which the stability of the number of copies per cell genome was shown [18]. The number of copies of the genome was determined using a calibration curve by diluting genomic DNA isolated from Vero cells with a known concentration. To calculate the amount of virus per cell, the following formula was used:

$$x = \frac{a}{b},$$

where a is the total number of viral genome equivalents, and b is the total amount of Vero cell genomic DNA in each sample. Three biological and three technical replicates were used to determine the statistical significance of the differences between cell cultures. The normality of distribution was determined by the Shapiro–Wilk test. To determine the statistical significance of the differences between cell cultures, we used the Student's t-test for independent samples with normal distribution. The values were considered reliable at $p < 0.05$.

RESULTS

Sequence Integration LAMP1 into the Genome of Vero cells

The *LAMP1* open reading frame was amplified from cDNA derived from HEK293A cells and cloned into a plasmid construct based on the Sleeping Beauty transposon. A standard Vero cell line was transfected with the genetically engineered construct by lipofection, and stable transformants were selected by puromycin selection. Thus, two transgenic cell lines Vero.Lu3 and Vero.Lu5 were obtained. *LAMP1* open reading frame expression was evaluated by real-time PCR. In the cell lines Vero.Lu3 and Vero.Lu5, an 11.01-fold and 12.94-fold increase in the expression of the gene of interest was found, respectively, compared with the original Vero cell line. Differences in the expression level are significant when comparing Vero.Lu3 to Vero.Lu5, and to the original Vero cell line ($p < 0.05$). Thus, both transgenic cell lines were suitable for further experiments (Figs. 1a and 1b).

Viral RNA Replication in Transgenic Cell Cultures

Transgenic cell cultures were infected with SARS-CoV-1 or SARS-CoV-2 viruses. Infected cells were incubated under two conditions: in the presence of trypsin and without it, according to the protocol described earlier [19, 20]. Samples containing viral and cellular genomic material were collected after 48 h [21]. The number of virus genome equivalents was determined relative to a model plasmid containing a fragment of the SARS-CoV-1 viral genome. The results were presented as the number of virus genome

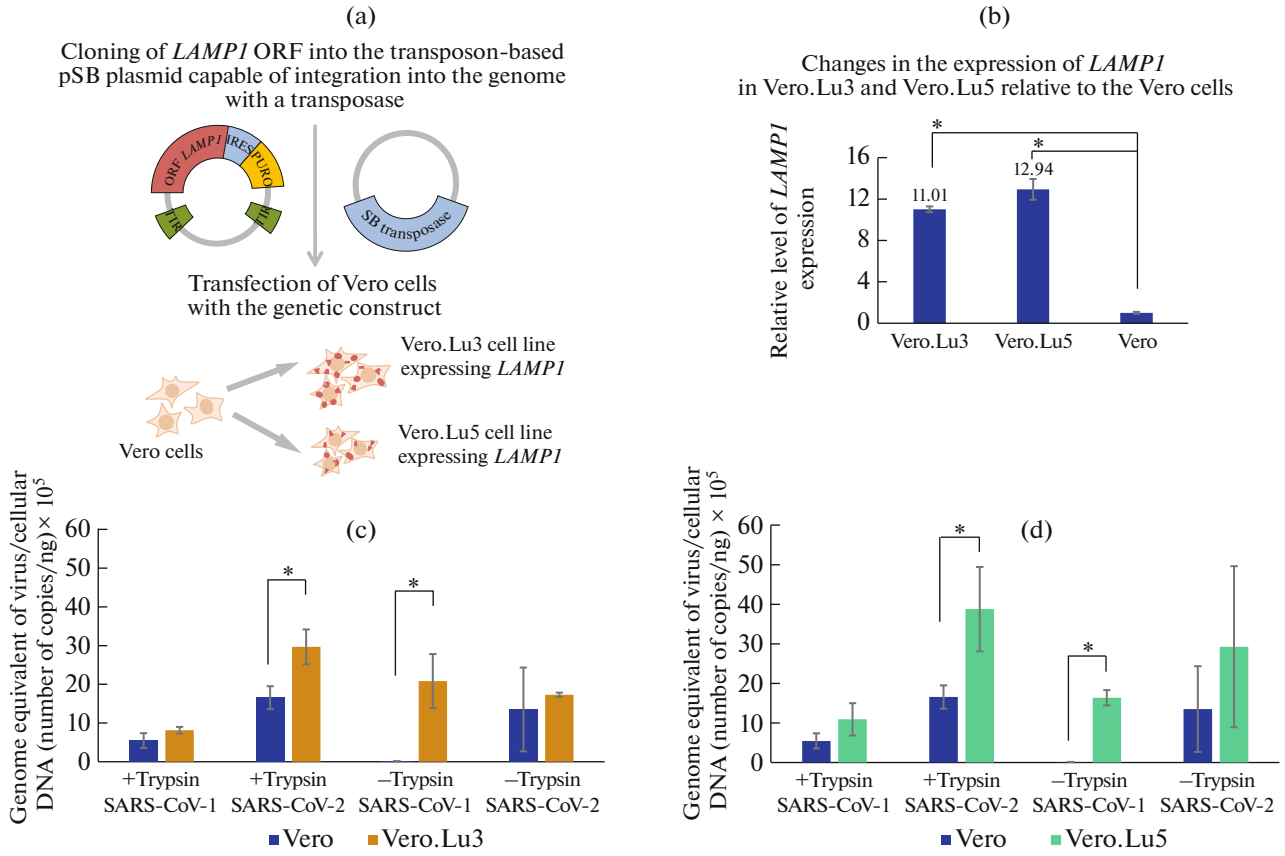

Fig. 1. Scheme for obtaining transgenic cell lines and their infection with coronaviruses. Scheme constructs for obtaining transgenic cell lines Vero.Lu3 and Vero.Lu5 (a) and *LAMP1* gene expression in them (b). Analysis of the number of genome equivalents of SARS-CoV-1 and SARS-CoV-2 viruses per cellular genome of Vero cells and the transgenic lines Vero.Lu3 (c) and Vero.Lu5 (d) 48 hours after infection in the presence (+) and in the absence (–) of trypsin. *$p < 0.05$.

equivalents in the sample and the number of virus genome equivalents per unit mass of Vero cell genomic DNA. An increase in the production of SARS-CoV-1 and SARS-CoV-2 was found in Vero.Lu3 and Vero.Lu5 cells compared to Vero cells (Figs. 1c and 1d, respectively). A significant difference in virus production between Vero cells and transgenic cells was more evident and significant in the presence of trypsin in the culture medium upon infection ($p < 0.05$). So, in the cell lines Vero.Lu3 and Vero.Lu5 there was a 5- and 6-fold increase in SARS-CoV-1 production, respectively, compared with the original cells (Figs. 1c and 1d). For SARS-CoV-2, the differences were even more pronounced: virus production increased by 1,236 and 362 times, respectively. Recalculation of the number of genome equivalents of the virus, adjusted for the genomic equivalents of Vero cells, led to only minor changes. In the absence of trypsin, the production of SARS-CoV-1 in transgenic cells increased by only 1.3–3.0 times, and SARS-CoV-2 production was almost identical to that in the original line. Transformation into cellular genomes resulted in similar profiles. However, all differences in the absence of trypsin were not significant.

It is also shown that 48 hours after infection without trypsin, the amount of virus in the cell monolayer is 1.6 times higher than in the culture medium in Vero.Lu3 cells, 1.4 times higher in Vero.Lu5 cells, and 1.3 times higher in the initial Vero lines. When infected with trypsin, these values are 1.5, 1.5, and 1.4, respectively.

DISCUSSION

In this work, the production of SARS-CoV-1 and SARS-CoV-2 viruses in transgenic Vero cells expressing the human *LAMP1* gene was investigated. The number of virus genome equivalents was analyzed, as well as the number of virus genome equivalents normalized to the genomic equivalent of Vero cells. The established differences in viral production profiles between the transgenic lines and the original cells were statistically significant. An equal number of cells and an equal number of viral particles were used in the infection experiments. However, coronaviruses exhibit a cytopathic effect, so a decrease in virus production may be observed due to the death of a monolayer of cells. Differences were found in the produc-

tion of viral particles during infection of cells in the presence of trypsin and without it. A significant difference was found between the original Vero cell culture and transgenic cell lines expressing *LAMP1*, in the presence of trypsin. Previously, it was shown that trypsin promotes the processing and activation of the S protein by hydrolysis with the formation of two subunits, S1 and S2. The activated protein interacts with ACE2 and the virus enters the cell [22]. It is known that mature coronavirus virions leave the cell in 12 hours after infection [11, 12], which can lead to secondary infection of the cell culture and an increase in virus production.

We have shown that increased expression *LAMP1* leads to an increase in the production of the studied coronaviruses in both lines of transgenic Vero cells. It should be noted that in the original Vero cell line, the production of SARS-CoV-2 was higher than that of SARS-CoV-1, which may be due to its increased virulence. As for the LAMP1 protein, its most important function is the regulation of lysosome exocytosis [15]. Previously, LAMP1 was reported to be involved in the life cycle of viruses that use endosomes to enter the cell. Thus, this protein increases the overall efficiency of cell infection with the Lassa virus [23]. LAMP1 allows viral particles to exit the endocytic pathway before they encounter the more acidic proteolytic environment, resulting in more viable virions.

In our study, we have shown an increase in the production of SARS-CoV-1 and SARS-CoV-2 viruses in Vero cells with *LAMP1* gene overexpression. Also, 48 hours after infection, an increased amount of virus in the cells was found relative to its content in the culture medium in transgenic lines compared to the initial line. Based on these data, it can be assumed that the LAMP1 protein is involved in life cycle of the SARS-CoV-1 and SARS-CoV-2 viruses, and it can be considered as a potential target for the treatment of coronavirus infection.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare they have no conflicts of interest.

This study does not contain any research involving humans or animals as research objects.

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