An Effective Platform for SARS-CoV-2 Prevention by Combining Neutralization and RNAi Technology

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Electronic Supplementary Information

Abstract At present, the coronavirus disease 2019 (COVID-19) pandemic is a global health crisis. Scientists all over the globe are urgently looking forward to an effective solution to prevent the spread of the epidemic and avoid more casualties at an early date. In this study, we establish an effective platform for the prevention of SARS-CoV-2 by combining the neutralization strategy and RNAi technology. To protect normal cells from infection, the customized cells are constructed to stably express viral antigenic receptor ACE2 on the cell membrane. These modified cells are used as bait for inducing the viral entry. The transcription and replication activities of viral genome are intercepted subsequently by the intracellular shRNAs, which are complementary to the viral gene fragments. A pseudotyped virus reconstructed from the HIV lentivirus is utilized as a virus model, by which we validate the feasibility and effectiveness of our strategy in vitro. Our work establishes an initial model and lays the foundation for future prevention and treatment of various RNA viruses.

Keywords SARS-CoV-2; Neutralization; ACE2; RNAi; Pseudotyped virus

INTRODUCTION

A sudden outbreak of pneumonia swept the world at the end of 2019, which was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1,2] According to the data supplied by WHO, more than 520 million people have been diagnosed with COVID-2019, and 6.2 million deaths are included so far.[3] The pandemic severely affected the global economy and caused a great panic among the people all over the world. Alarmingly, due to the constant emergence of viral variants,[4,5] these numbers kept soaring despite global efforts to exploit new strategies by combining the neutralization and RNAi technology. We demonstrated its effectiveness in vitro through a model and lays the foundation for future prevention and treatment of various RNA viruses.

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pseudotyped SARS-CoV-2 virus reconstructed from the HIV lentivirus system. The HEK293T cells were equipped with overexpressed ACE2 protein and shRNAs targeting the viral genome. High concentrations of the ACE2 expressed on cell membranes were verified effectively in trapping the pseudotyped SARS-CoV-2 virus, while the intracellular shRNAs showed validation in reducing viral protein expression. Our study provides a promising, flexible, and effective platform for the prevention and treatment of all RNA viruses including SARS-CoV-2.

EXPERIMENTAL

Plasmids Construction
The sequences of shRNAs targeting eGFP were screened through http://maideigner.lifetechnologies.com. For the ease of fragment insertion, an empty vector equipped with dual BsaI sites and a U6 promoter was synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). Plasmids encoding shRNAs were constructed by Golden Gate Assembly. All the shRNA sequences used in this work were listed in Table S3 (in the electronic supplementary information, ESI). As for plasmids used in cell line constructions, the gene fragments were inserted into a lentivirus vector with puromycin or hygromycin resistance for cell selection. A myc-tag was fused to the N-terminal of the ACE2 protein for fast cell sorting.

Mammalian Cell Culture and Transfection
The HEK293T cell line (ATCC) was maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37 °C in a 5% CO2 environment. Cells were seeded at the density of 160–200 thousand cells per milliliter into 24-well plates for 24 h before transfection. For the shRNA sequences screening test, 150 ng of GFP and 150 ng of shRNA plasmids were co-transfected into HEK293T cells. The transfections were performed according to the manufacturer’s instructions of the transfection reagent (JetPRIME, Polyplus). Cell fluorescence imaging or flow cytometry was performed for 36–48 h after transfection.

Preliminary Test for Pseudotyped Virus Titration
The HEK293T cells were transfected with 500 ng of myc-ACE2-TM in a 24-well plate and seeded in a 96-well plate at 10000 cells per well 24 h after transfection. The pseudotyped SARS-CoV-2 virus (Genomeditech (Shanghai) Co., Ltd.) was diluted to a series of concentrations and added into the corresponding wells. The media was changed 24 h after infection. Flow cytometry analysis was performed 48 h after infection.

Cell Lines
ACE2-293T cells, shRNA-293T cells, and ACE2-shRNA-293T cells were built based on the ACE2-293T cells. For each cell line, 100 selected cells were counted and seeded into 96-well plate for single-cell cloning. The ACE2-shRNA-293T cells were built based on the ACE2-293T cells.

Infection of Pseudotyped SARS-CoV-2 Virus
Cells were seeded at the density of 200 thousand cells per milliliter in a 24-well plate for infection preparation. The pseudotyped SARS-CoV-2 virus was added into samples at the concentration of determined above (1.1 μL per 100 μL media). The media was changed into growth medium 24 h after infection. For the ACE2 cell line identification experiment, the flow cytometry analysis was performed 48 h after infection. While for the final efficiency determination tests, the flow cytometry was performed one week after infection. All the results of flow cytometry were analyzed with Flowjo V10. For each cell sample, 10000 cells were gated and analyzed to calculate the average fluorescence intensity and the positive ratio.

RESULTS AND DISCUSSION

Preliminary Validation of Effectiveness of Pseudotyped SARS-CoV-2 Virus
In order to avoid the physical security risks in traditional virus experiments, numerous kinds of pseudotyped viruses have been developed as experimental alternatives to wild-type SARS-CoV-2. The schematic diagram of the pseudotyped SARS-CoV-2 virus used in this work is shown in Fig. 1a. As the most important surface membrane protein of coronavirus, the spike (S) protein is critical to the initial stage of viral invasion. The original envelope protein VSVG in the HIV lentiviral vectors was replaced by S protein for constructing pseudotyped SARS-CoV-2 virus. An additional RNA encoding eGFP was conducted into the pseudotyped virus for facilitating the determination of efficiency. To determine the appropriate virus titer for subsequent experiments, HEK293T cells transfected with ACE2 protein were dealt with a series of diluted pseudotyped viruses (details were displayed in Fig. S1, ESI). The results of cell flow cytometry 48 h after infection showed there was a positive correlation between the infection efficiency and the viral tilter (Fig. 1b). It was shown that the samples with virus volume less than 0.37 μL were uninfected, while those samples treated with viruses in higher titers displayed positive fluorescence signals. According to the result, a ratio of 1.1 μL virus per 100 μL media was adopted in subsequent infection experiments.

Testing the Ability of ACE2-293T Cells to Specialy Capture Pseudotyped Virus
It has been reported that ACE2 protein played a crucial role in identifying and combining with the spike protein of SARS-CoV-2. Therefore, the extracellular domain of human ACE2 protein was fused to the transmembrane (TM) domain of the platelet-derived growth factor receptor (PDGFR). A myc-tag was added to the N-terminal of the recombinant ACE2 for cell sorting (amino sequence was listed in Table S1 in ESI). The lentivirus infection efficiency was measured by flow cytometry of stained cells (Fig. 2a). The subsequent immunofluorescence also demonstrated that the ACE2 protein was located on the cellular membrane surface (Fig. S4 in ESI). For further verifying the specificity of ACE2-293T cells, the HEK293T and ACE2-293T cells were dealt with or without the pseudotyped SARS-CoV-2 virus.
Fig. 1 The schematic representation of the pseudotyped SARS-CoV-2 virus and the results of the preliminary test for the pseudotyped virus titration. (a) The envelope protein VSVG of the HIV lentivirus system was replaced with the full-length SARS-CoV-2S protein to construct the pseudotyped virus. Moreover, the eGFP was co-transfected with the modified lentiviral plasmids to simulate the viral RNA genome. Therefore, the pseudotyped SARS-CoV-2 viral infection efficiency could be characterized by the eGFP signals. (b) The ACE2 protein was transfected into HEK293T cells for pseudotyped SARS-CoV-2 virus titration. A series of diluted pseudotyped SARS-CoV-2 viruses were added to the ACE2 overexpressed cells to determine appropriate virus titers.

Fig. 2 Construction and identification of stable ACE2-293T cell line. (a) Lentivirus infected 293T cells to construct an ACE2 overexpressed stable cell line. The myc-tag was fused to the N-terminal of the ACE2 protein for cell sorting (left). Flow cytometry images of the infected cells stained with myc-488 (right). (b) Verification for the ability of the stable ACE2-293T cell line to trick the pseudotyped SARS-CoV-2 virus. The capture efficiencies were noted at the top of the cell gate. (c) Determination of the viral capture efficiency of the ACE2-293T cells in the condition of co-exiting with mCherry-293T cells.

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respectively. Compared with the wild-type HEK293T cells, the ACE2-293T cells displayed a remarkable ability to trap the pseudotyped SARS-CoV-2 virus (Fig 2b). To further validate the specificity and efficiency of ACE2-293T cells in capturing the pseudotyped SARS-CoV-2 virus, the ACE2-293T cells were mixed with the mCherry-293T cells in a ratio of 1:1 before infection (Fig. 2c and Fig. S5 in ESI). As expected, the mCherry-labeled normal 293T cells were protected from infecting against the pseudotyped SARS-CoV-2 virus by the ACE2-293T cells. It preliminarily confirmed that our strategy that using ACE2 overexpressed cells to trap the SARS-CoV-2 virus to protect normal human cells from infection seemed practicable.

**Cutting Off the Viral Gene Delivery Process by RNAi Technology**

Viral invasion is usually divided into several processes: adsorption, entrance, uncoating, biosynthesis, assembly and release. As a typical RNA virus, the SARS-CoV-2 releases its RNA positive-sense chain to the cytoplasm after entry and uncoating. Therefore, it is hopeful to cut off viral gene delivery through the RNAi technique. For ease of observation, an RNA encoding eGFP was parceled into the pseudotyped virus to simulate the viral genome. Four sequences (named RNAi1, RNAi2, RNAi3 and RNAi4, respectively) targeting GFP and an off-target sequence (RNAi0) were screened and designed as experimental groups and control groups, respectively (all target sequences are listed in Table S2 in ESI). The transient co-transfections were performed to preliminarily assess the knock-off efficiencies of these shRNA sequences (Fig. 3a and Fig. S2 in ESI). RNAi1 and RNAi4 displayed good performance in silencing transiently transfected eGFP while RNAi2 and RNAi3 exhibited slight difference compared with the control group RNAi0. Moreover, it is also essential to evaluate the interference ability of these shRNA sequences to knock down stably expressed proteins. In this way, it showed a possibility that not only gene delivery could be blocked in the early stage of virus invasion, but also made it capable to interfere with the expression of the corresponding proteins later. Due to the fact that RNAi1 and RNAi4 exhibited a higher capacity to silence the transfected eGFP, the two target sequences were applied for the test of silencing stably expressed eGFP (Figs. 3b and 3c and Fig. S3 in ESI). According to the results, the RNAi4 showed a significant efficiency in knocking down stably expressed GFP although the RNAi1 seemed inefficient. Taking the above results into account, the RNAi1 and the RNAi4 were predicted to be potent in subsequent experiments.

![Fig. 3](https://doi.org/10.1007/s10118-022-2846-6)
Trapping the Pseudotyped SARS-CoV-2 Virus and Blocking the Viral Gene Delivery by the Customized ACE2-shRNA-293T Cells

It has been successfully achieved to specially capture the pseudotyped SARS-CoV-2 virus by ACE2-293T cells in the abovementioned experiments. Additionally, several shRNA target sequences were screened for effectively silencing transient transfected and stably expressed GFP. Therefore, it should be feasible to establish a platform for the prevention and treatment of SARS-CoV-2, where the cells could trap the virus by the overexpressed ACE2 protein on the cell surface and block the viral gene delivery process by the intracellular shRNA (Fig. 4a). To verify the effectiveness of this strategy, the cell lines stably expressing ACE2 protein and the selected shRNA sequences were constructed based on the ACE2-293T cells. To further determine the silencing efficiency and stability of shRNA in these cells, the constructed cell lines were infected with pseudotyped SARS-CoV-2 virus, and the GFP positive proportions of these cells were measured one week after infection. (Fig. 4b). As a control, 61% of ACE2-RNAi0-293T cells was infected. In test groups, the infection rates of the ACE2-RNAi1-293T cells and ACE2-RNAi4-293T were reduced to 52.6% and 29.5%, respectively. Furthermore, the relative expression of viral genes (here referred to GFP) in these cell lines was determined by measuring the average GFP fluorescence intensity (Fig. 4c). Although the ACE2-RNAi1-293T cells displayed a slight effect in reducing the viral infection rate, their GFP expressions (orange column) were obviously reduced compared with the control group (ACE2-RNAi0-293T cells, green column). On the other hand, it was found that ACE2-RNAi4-293T cells also showed an outstanding performance in inhibiting protein expression, which indicated that these modified cells had powerful potency in viral prevention and treatment.

CONCLUSIONS

Unknown viruses attacked human time after time and seriously endangered public health. People have to seek effective solutions to survive every crisis. The Spanish flu broke out in 1918 caused more than 260,000 deaths in Spain. With a large family and numerous hosts, the coronavirus may bring about the potential danger of the outburst of zoonotic diseases. In addition, the Ebola virus caused the 2013-2015 West African epidemic, and the HIV led to a vast number of deaths. It kept reminding us of the urgency of finding a universal strategy against these horrible viruses. Taking SARS-CoV-2 as an example, we constructed decoy cells according to the mechanism of virus infection to trap and “kill” the virus. In this work, to facilitate the detection of virus infection in the experiments, we utilized a pseudotyped SARS-CoV-2 virus as a research model, which was reconstructed from the HIV lentivirus system. The virus filter was firstly determined by...
measuring the infection efficiency of HEK293T cells transfected with ACE2 protein, which were dealt with a series of diluted pseudotyped viruses. The ACE2-293T cell line that stably overexpressed ACE2 protein on the cell membrane was then proved to be capable to specially trap the pseudotyped SARS-CoV-2. To cut off the delivery process of the viral genome after trapping, two shRNA sequences were selected, and the corresponding cell lines were constructed based on the ACE2-293T cells. Both customized cell lines have shown excellent performance in reducing the viral infection efficiency and silencing the expression of viral genes. Our strategy could provide a novel fundamental platform for establishment of general solution to tricky RNA viruses in the future.

NOTES
The authors declare no competing financial interest.

Electronic Supplementary Information
Electronic supplementary information (ESI) is available free of charge in the online version of this article at http://doi.org/

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