Chinese Journal of
POLYMER SCIENCE

RESEARCH ARTICLE

https://doi.org/10.1007/s10118-022-2846-6
Chinese J. Polym. Sci.

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

Jun-Xiang Zhou, Xue Yin, and Hao-Jun Liang*

Department of Polymer Science and Engineering, Hefei National Research Center for Physical Sciences at the Microscale, iChEM (Collaborative Innovation Center of Chemistry for Energy Materials), University of Science and Technology of China, Hefei 230026, China

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

Citation: Zhou, J. X.; Yin, X.; Liang, H. J. An effective platform for SARS-CoV-2 prevention by combining neutralization and RNAi technology. *Chinese J. Polym. Sci.* https://doi.org/10.1007/s10118-022-2846-6

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 different treatments[6–9] and vaccines.[10–14] Therefore, it is urgent to develop more effective methods to restrain the spread of COVID-19, meanwhile taking flexibility, bio-stability, and efficiency against multiple variants into consideration.

SARS-CoV-2 is a typical RNA virus, of which the genome is an approximately 30-kb, positive-sense, single-stranded RNA.[15,16] Previous studies have revealed that the invasion process of the SARS-CoV-2 was mainly mediated by the interaction between human ACE2 protein and the receptor-binding domain of the SARS-CoV-2 spike (S) protein.[17] Hence quantities of works were focused on establishing methods to prevent the combination of viral S protein and ACE2 for protecting host cells from infection. A recombinant protein was constructed by fusing the Fc fragment of the human immunoglobulin lgG1 to the extracellular domain of ACE2 protein for the attempt to neutralize the SARS-CoV-2.[18] Another two-step neutralization strategy was also reported to be effective in protecting against COVID-19 by utilizing a decoy nanoparticle targeted both S protein and inflammatory cytokines IL-6.[19] Additionally, many compounds have been identified as efficient in preventing host cells from infection.[8,20,21] Nevertheless, due to the bio-instability of compounds and nanoparticles, these strategies could only reduce the viral infection rate to some extent but lack durability.

The abovementioned researches on neutralizing antibodies played essential roles in the early prevention of SARS-CoV-2. However, it is also crucial to reduce the viral load so as to reduce lung inflammation after infection. For this purpose, RNA interference (RNAi) technology was employed in CDVID-19 treatment.[22–24] It was proved that the siRNA targeting the viral genome could significantly reduce SARS-CoV-2 virus load.[25] It also provided a possibility to overcome the constant emergence of viral variants by targeting deeply conserved regions of the viral genome, whereas, it could only be used as a post-infection treatment. Herein, we developed a new strategy by combining the neutralization and RNAi technology. We demonstrated its effectiveness *in vitro* through a pseudotyped SARS-CoV-2 virus reconstructed from the HIV

* Corresponding author, E-mail: hjliang@ustc.edu.cn

Received June 21, 2022; Accepted July 18, 2022; Published online September 21, 2022

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://rnaidesigner.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% CO_2 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 by lentivirus. ACE2-293T cells were stained with myc-Alexa Flour® 488 (CST #2279) according to the manufacturer's instructions. The cell population with positive signal was selected and collected through flow cytometry for further culture. The selected ACE2-293T cells were fixed and stained with myc-Alexa Flour® 488 and Hoechst 33258 (Beyotime #C1018) before confocal imaging for further demonstration of the correct expression of ACE2 protein. The ACE2-shRNA-293T cells were dealt with puromycin (0.5 μg/ml) for about one week. 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.[26] The schematic diagram of the pseudotyped SARS-CoV-2 virus used in this work is shown in Fig. 1(a). 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 Specially 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.[27] Therefore, the extracellular domain of human ACE2protein 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,

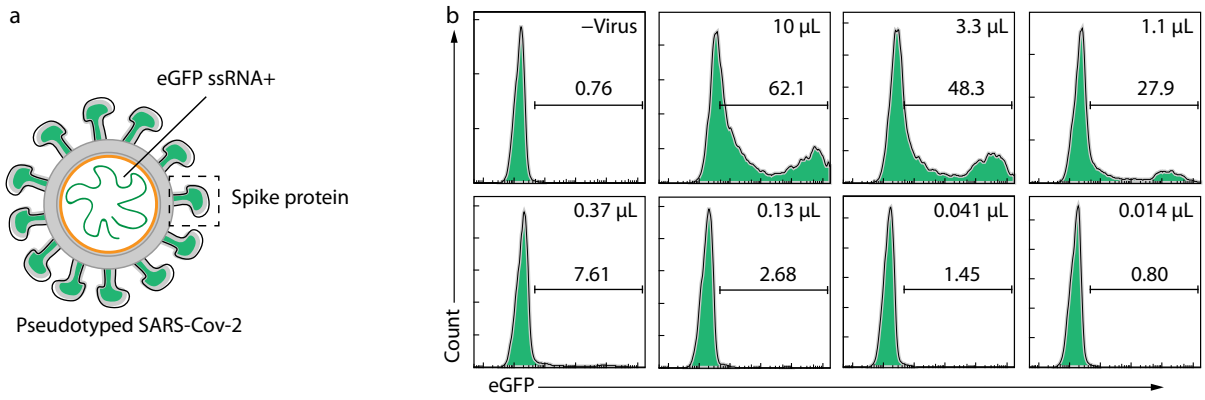

Fig. 1 The schematic representation of the pseudotyped SARS-CoV-2 virus and the results of the preliminary test for the pseudotyped virus titration. (a) The envelope protein VSVG of the HIV lentivirus system was replaced with the full-length SARS-CoV-2S protein to construct the pseudotyped virus. Moreover, 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.

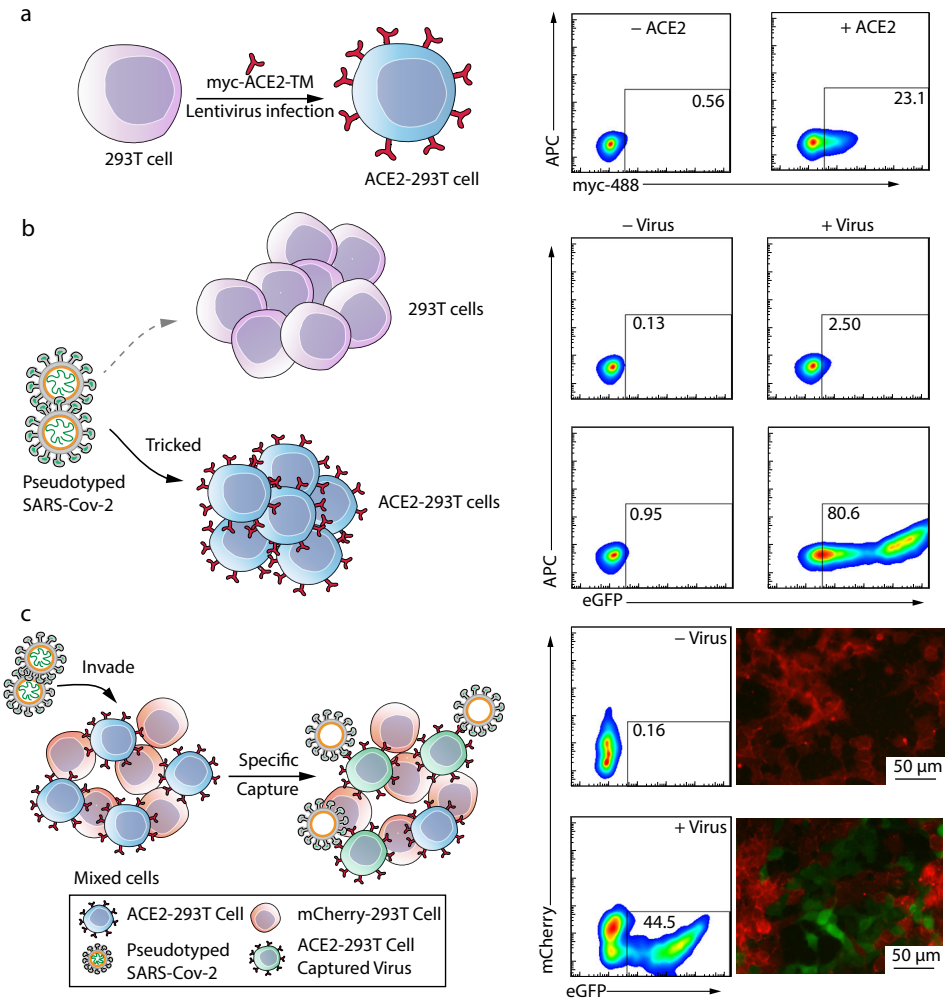

Fig. 2 Construction and identification of stable ACE2-293T cell line. (a) Lentivirus infected 293T cells to construct an ACE2 overexpressed stable cell line. The myc-tag was fused to the N-terminal of the ACE2 protein for cell sorting (left). Flow cytometry images of the infected cells stained with myc-488 (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.

https://doi.org/10.1007/s10118-022-2846-6

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-labled 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.

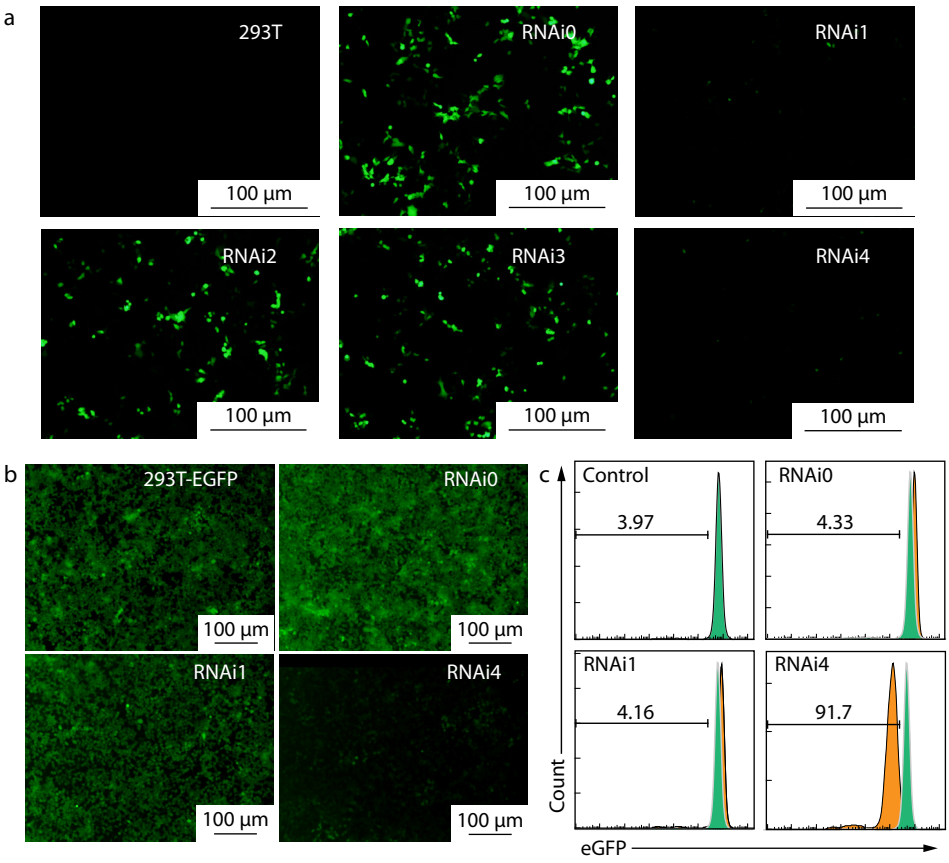

Fig. 3 Screening of shRNA sequences targeting eGFP and the corresponding capacities to silence transiently transfected and stably expressed eGFP. (a) Fluorescent images of shRNA sequence screening experiments. Four shRNA target sequences and the control samples were tested for silencing transient transfected eGFP protein. Scale bars, 100 μm. (b) Fluorescent images of the experiments testing the two selected shRNA target sequences for silencing stable-expressed eGFP protein. Scale bars, 100 μm. (c) Flow cytometry diagram of the corresponding samples in (b). The 293T-EGFP cells were used as control group and displayed in green peak. The experiment groups were displayed in orange peaks. The numbers donated the percentage of the cells whose eGFP were knocked down.

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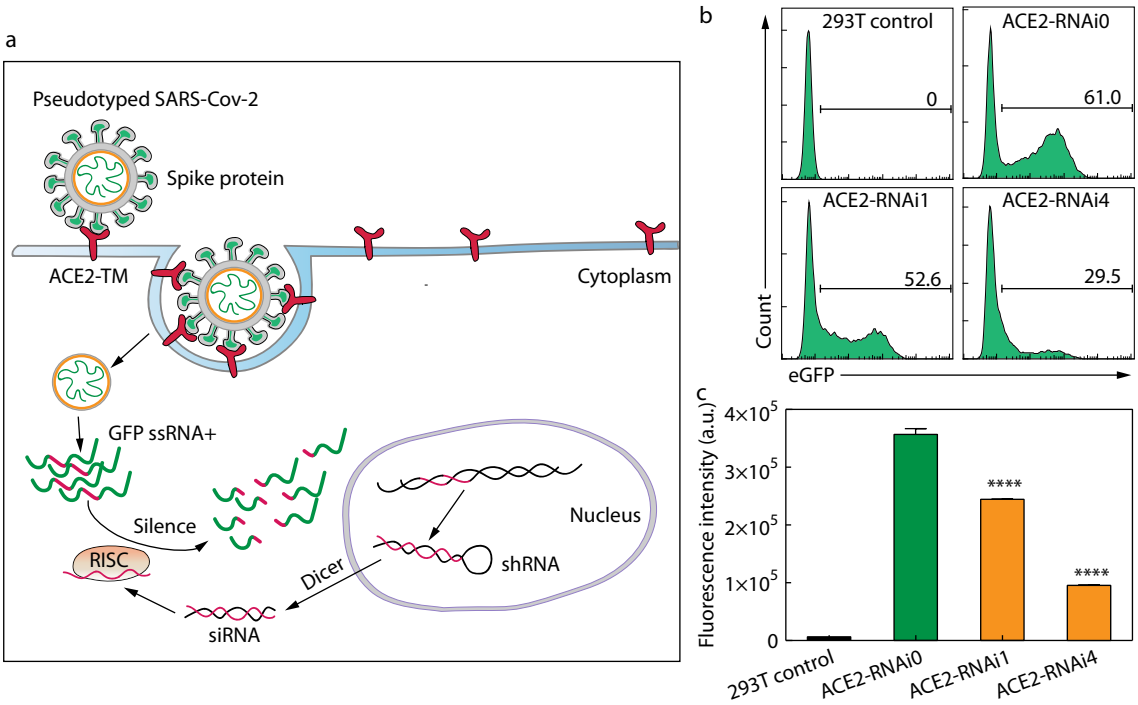

Fig. 4 The mechanism and results diagrams of the ACE2-shRNA-293Tcell line trapping and gene silencing pseudotyped SARS-CoV-2. (a) Mechanism diagram of the custom therapy. HEK293T cells were equipped with overexpressed ACE2 protein and shRNA targeting viral RNA (here refers to eGFP). The pseudotyped SARS-CoV-2 was first trapped by the high concentrate ACE2 protein on the cell membrane. The single viral positive-sense RNA chain was delivered into the cytoplasm and cut by the RISC subsequently. (b) The effectiveness of the strategy was characterized by measuring the positive proportion of GFP in each sample. The HEK293T cell was shown as a negative control while the ACE2-RNAi0-293T cell was displayed as a positive control. (c) The average intensity of GFP in each cell line for testing the silencing efficiency of the viral RNA (GFP). The green columns for control groups, and the orange columns for experimental groups.

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 SRAS-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.[28] With a large family and numerous hosts, the coronavirus may bring about the potential danger of the outburst of zoonotic diseases.[29] In addition, the Ebola virus caused the 2013-2015 West African epidemic,[30] and the HIV led to a vast number of deaths.[31] 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 tilter was firstly determined by

https://doi.org/10.1007/s10118-022-2846-6

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/10.1007/s10118-022-2846-6.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (Nos. 21991132, 52033010 and 52021002), the National Key R&D Program of China (Nos. 2020YFA0710700 and 2020YFA0710703), the fundamental Research funds for the central universities (No. WK9110000005), and the Open Research Fund of State Key Laboratory of Polymer Physics and Chemistry (Changchun Institute of Applied Chemistry, Chinese Academy of Sciences).

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