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(54) **Titre : VESICULES EXTRACELLULAIRES CIBLEES POUR L'ADMINISTRATION D'AGENTS THERAPEUTIQUES**
(54) **Title: TARGETED EXTRACELLULAR VESICLES FOR DELIVERY OF THERAPEUTICS**

(57) **Abrégé/Abstract:**
The present invention is directed to Saccharomyces-generated extracellular vesicles (EVs) comprising a foreign RNA molecule or protein and at least one foreign membrane surface exposed ligand that specifically binds to a target receptor displayed on a target cell. The present invention also relates to methods of making and using these Saccharomyces-generated EVs for targeted gene silencing. The present invention also relates to fusion proteins comprising a Saccharomyces extracellular vesicle anchor protein and a second peptide designed to bind to cell-specific receptors.

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Abstract:

The present invention is directed to Saccharomyces-generated extracellular vesicles (EVs) comprising a foreign RNA molecule or protein and at least one foreign membrane surface exposed ligand that specifically binds to a target receptor displayed on a target cell. The present invention also relates to methods of making and using these Saccharomyces-generated EVs for targeted gene silencing. The present invention also relates to fusion proteins comprising a Saccharomyces extracellular vesicle anchor protein and a second peptide designed to bind to cell-specific receptors.

TARGETED EXTRACELLULAR VESICLES FOR DELIVERY OF THERAPEUTICS

CROSS-REFERENCE TO RELATED APPLICATIONS

This International PCT application claims the benefit of U.S. Provisional Application Serial No. 63/144,827, filed February 2, 2021, and U.S. Provisional Application Serial No. 63/184,011, filed May 4, 2021, both of which are incorporated by reference in their entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 31,2022, is named "90355.00041-PCT-1" and is 76 Kbytes in size.

TECHNICAL FIELD

The present invention is directed to *Saccharomyces*-generated extracellular vesicles (EVs) comprising a foreign RNA molecule or protein and at least one foreign membrane surface ligand that specifically binds to a target molecule displayed on a target cell. The present invention also relates to methods of making and using these *Saccharomyces*-generated EVs. The present invention also relates to fusion proteins comprising a *Saccharomyces* extracellular vesicle anchor protein and a second peptide.

BACKGROUND

Ever since the discovery that RNA that can be used to silence genes via RNAi, there has been a need for efficient and specific delivery of the RNA to target cells. As naked nucleic acids are difficult to deliver *in vivo* due to rapid clearance, nucleases, lack of organ-specific distribution and low efficacy of cellular uptake, there have been attempts to generate specialized gene delivery vehicles for nucleic acid delivery. Viral vectors and liposomes have been relatively successful in delivering nucleic acids, with a large number of these delivery vehicles in clinical trial. Despite these minor successes, there remain significant limitations that restrict the broad use of nucleic acid delivery to specific target cells, some of which include immune recognition and possible neutralization for most viral vectors, mutagenic integration of viruses such as lentiviruses and inflammation toxicity and rapid clearance of liposomes. Liposomes and viral vectors can sometimes trigger the innate immune system, which leads to acute inflammatory responses, which may, in turn, require the use of immunosuppression strategies to overcome uptake and re-administration issues potentially exposing patients to unwarranted risks of opportunistic

infections. Antibodies generated against the delivery vehicles may also decrease transgene expression on subsequent administration.

Hence, it is imperative to develop technologies that are able to avoid immune recognition and inflammation, while retaining good delivery efficiencies to expand the use of RNAi therapy as a more routine treatment. One of the solutions may lie in the use of extracellular vesicles, e.g., exosomes, for nucleic acid delivery. Extracellular vesicles (EVs) such as exosomes are small membrane-bound vesicles generally of endocytic origin that are released into the extracellular environment. There are several pathways that can result in formation of EVs. The potential of exosome-mediated gene delivery has been shown with delivery of murine mRNAs and miRNAs to human mast cells *in vitro*.

There are questions as to whether "allogeneic exosomes" would trigger an adaptive immune response in the subject receiving the EVs and how quickly and efficient "syngeneic exosomes" could be generated. Thus, there still exists in the art a need to efficiently and quickly produce EVs that are capable of carrying genetic cargo and which the body's immune system will either tolerate or ignore. To date, there has been no successful generation of EVs from yeast that are normally consumed and have been engineered to specifically and directly bind to a target cell to deliver a nucleic acid payload.

SUMMARY OF THE INVENTION

The present invention is directed to extracellular vesicles (EVs) comprising a foreign RNA molecule or protein derived from an Generally Recognized As Safe (GRAS) organism as defined by the U.S. Food and Drug Administration. In this preferred embodiment, the present invention is directed to EVs comprising a foreign RNA molecule or protein derived from *Saccharomyces,* such as *Saccharomyces cerevisiae* (sometimes referred to herein as *Sc*) or *Saccharomyces boulardii* (sometimes referred to herein as *Sb*).

The present invention is directed to EVs comprising a foreign RNA molecule or protein derived from an organism that is probiotic with a subject to be treated. In this preferred embodiment, the present invention is directed to EVs comprising a foreign RNA molecule or protein derived from probiotic *Saccharomyces,* and in particular probiotic *Saccharomyces boulardii.*

In present invention includes systems, methods and compositions for a *Saccharomyces*-generated EVs (sometimes referred to as exosome) platform for the delivery of therapeutic RNA,

particularly small interfering RNA (siRNA) for the treatment of a disease or condition in a subject in need thereof, including viral infection, cancer, or inherited metabolic disorder.

In present invention includes systems, methods and compositions for a *Saccharomyces*-generated EVs (sometimes referred to as exosome) platform for the delivery of therapeutic RNA, particularly small interfering RNA (siRNA) for targeted gene silencing. In a preferred embodiment, the targeted gene silencing may be directed to a pathogen gene, or an endogenous gene expressed by a target cell or host, and preferably a subject.

In present invention includes systems, methods and compositions for a *Saccharomyces*-generated EVs (sometimes referred to as exosome) platform for the delivery of therapeutic RNA, particularly small interfering RNA (siRNA) for the treatment of SARS-CoV-2 virus in a subject in need thereof.

The present invention includes *Saccharomyces*-generated EVs comprising a foreign RNA molecule or protein and at least one foreign membrane surface ligand that specifically binds to a target molecule displayed on a target cell.

The present invention also relates to methods of making and using these *Saccharomyces*-generated EVs. The present invention also relates to fusion proteins comprising a *Saccharomyces* extracellular vesicle anchor protein and a second peptide.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 show NTA analysis of Exosomes (EVs) derived from Sb culture.

FIGS 2A-B. show Pol-II (A) and Pol-I (B) cassettes for expression of shRNA targeting Nsp1 gene of SARS-CoV-2 (siNsp) and eGFP (siGFP).

FIG 3. shows expression of shRNA targeting Nsp1 gene of SARS-CoV-2 by engineered Sb strains.

FIG 4: shows uptake of GFP-labeled EVs by human cells (lungs cancer cell line H1299). Green – GFP-fused Sur7 protein; Blue – Hoechst nuclear staining.

FIGS 5A-E: show EVs produced by *S. boulardii* can deliver functional siRNA and silence targeted gene (GFP). A, B: negative control, H1299-GFP cells treated by mock solution; C, D: H1299-GFP cells treated by application of EVs-siRNA-GFP fraction. A, C: GFP fluorescence, 797 ms exposure time; B,D: phase contrast image of the same field. E. The level of GFP fluorescence signal in EVs-siRNA treated cells (EVs) and control cells (optimem).

FIGS 6A-B: shows the level of SARS-CoV-2 Nsp1 gene expression in H1299 treated with wt EVs isolated from Sb expressing siRNA-nsp1 under control of pol I or pol II promoters or wild type Sb.

FIG 7: shows the level of Nsp1 gene expression in H1299 co-cultivated with wild-type Sb or Sb expressing siRNA-nsp1 under control of pol I or pol II promoters

FIGS 8A-B: Ldh activity assay of Sb-EVs application to (A) Hep-G2 cells and (B) H1299

FIG. 9: depicts a schematic representation of plasmid for expression of anchor proteins with fused GFP reporter protein. Anchor protein and GFP reporter are connected by a flexible peptide linker.

FIG. 10: depicts a schematic representation of plasmid for mammalian expression of partial SARS-CoV-2 sequence fused with GFP reporter protein.

FIG. 11: depicts a schematic representation of plasmid for yeast expression of siRNA.

DETAILED DESCRIPTION OF THE INVENTION

While the invention has been particularly shown and described with reference to a number of embodiments, it would be understood by those skilled in the art that changes in the form and details may be made to the various embodiments disclosed herein without departing from the spirit and scope of the invention and that the various embodiments disclosed herein are not intended to act as limitations on the scope of the claims. All references cited herein are incorporated in their entirety by reference. The terminology used herein is for describing particular embodiments and is not intended to be limiting. As used herein, the singular forms "a," "and" and "the" include plural referents unless the content and context clearly dictate otherwise. Thus, for example, a reference to "a" or "the" marker may include a combination of two or more such markers. Unless defined otherwise, all scientific and technical terms are to be understood as having the same meaning as commonly used in the art to which they pertain. For the purposes of the present invention, the following terms are defined below.

The present invention is directed to *Saccharomyces-generated* extracellular vesicles (EVs) comprising a foreign RNA molecule or protein. In some embodiments, the EVs of the invention may further include at least one foreign membrane surface ligand that specifically binds to a target molecule displayed on a target cell.

In particular, the present invention relates to delivery of RNA to target cells. As used herein, the term RNA is used as it is in the art and is intended to mean at least one ribonucleic acid

molecule. In one embodiment, the RNA is intended to elicit a gene silencing response in the target cell. For example, the RNA delivered to the target cell may be double-stranded RNA (dsRNA) in at least one portion of the entire RNA molecule. In another embodiment, the RNA delivered to the target cell may be single- stranded RNA (ssRNA). In more specific embodiments, the RNA delivered to the target cell may be short interfering RNA (siRNA), short-hairpin RNA (shRNA) or microRNA (miRNA).

For example, dsRNA can be delivered to a target cell and, if necessary, the cell can process the delivered dsRNA into smaller siRNA. As is well-known, siRNA generally is a 21-23 nucleotide duplex with a 2-nucleotide overhang on the 3' region of each strand, *i.e.*, there is a region of single-strandedness of 2 nucleotides on each 3' region of each strand. In one embodiment, the RNA that is delivered to the target cell is a dsRNA that is an RNA duplex longer than 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or even longer than 100 nucleotides in length. As used herein the term RNA duplex means a double-stranded RNA molecule. The duplex may be made from two separate strands that are complementary to one another in specific regions, or the duplex may be formed by one single strand that is internally complementary to itself such that it can fold back on itself to form the RNA duplex. In select embodiments, the dsRNA that is delivered using the methods and compositions of the claimed invention is siRNAs that can be a duplex of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or even 30 nucleotides. In additional select embodiments, the siRNAs delivered to the target cells may or may not have one or two 3' nucleotide overhangs, and, if present, the one or two nucleotide overhangs may separately and independently be zero, one, two, three or four nucleotides in length.

As is well-known, the use of dsRNA to silence gene expression is not limited to specific messenger RNA (mRNA) sequences for each targeted gene. Rather, one strand of the dsRNA should be perfectly complementary or predominantly complementary to a region of the target mRNA that is targeted for silencing. There are now well-established "rules" and guidelines for design of, for example, siRNA molecules that can target mRNAs for cleavage and therefore gene silencing. For example, one of skill may employ the Ui-Tei rule (Ui-Tei, K., *et al., Nucleic Acids Res.*, 32:936-948(2004), incorporated by reference), the Reynolds rule (Reynolds, A., *et al., Nat. Biotechnol.*, 22:326-330 (2004), incorporated by reference) or the Amarzguioui rule (Amarzguioui, M and Prydz, H., *Biochem. Biophys. Res. Commun.*, 316:1050-1058 (2004), incorporated by reference) in designing siRNAs for deliver into the target cell.

Accordingly, the dsRNA delivered to the target cell is an siRNA having a sequence on one strand of the RNA duplex that follows the Ui-Tei rule, the Reynolds rule or the Amarzguioui rule. For example, an siRNA that has one strand following the Ui-Tei rule includes (a) an A or U at position 1, from 5' terminus of siRNA guide strand, (b) a G or Cat position 19, (3) having AU in four or more positions of 1-7 of the guide strand, and (d) no long GC stretches of ten or more nucleotides. Additional characteristics of the siRNA sequence may or may not include other aspects of designer siRNA sequences, such as but not limited to having a UU sequence for at least one of the 3' overhangs, a GC content of between about 30% to about 60%, for example around 50% to 52%. Other characteristics of the siRNA sequence that is delivered to the target cells may or may not include those characteristics noted in Naito, Y. and Ui-Tei, K., *Front. Genet.*, Vol 3, Article 102 (2012).

Generally speaking, the siRNA delivered to the target cell or the RNA delivered to the target cell that is processed into siRNA will contain one strand, *i.e.*, the guide strand, that is perfectly (100%) complementary to a small stretch, about 15-23 bases, to a sequence within the target mRNA. Thus, the siRNA delivered to the target cell or the RNA delivered to the target cell that is processed into siRNA is not necessarily limited to a specific nucleotide sequence, except that the siRNA that is delivered or derived within the cell after delivery may be designed to have one strand that is 100% complementary to between about 15-23 bases of a target mRNA.

The sequences of various siRNAs directed to SARS-CoV-2 are now well-known. For example, Medeiros, I., et al., *Scientific Reports,* 11: 8849 (2021) (e-published 23 April 2021) (doi.org/10.1038/s41598-021- 88310-8), which is incorporated by reference, discloses siRNA sequences of SARS-CoV-2. In particular, the database located at doi.org/10.17605/OSF.IO/WD9MR, which is incorporated by reference, discloses siRNAs of 18, 19, 20 or 21 nucleotides in length that are directed to SARS-CoV-2. The siRNAs disclosed in these references or databases can be delivered to the target cells using the SGEVs of the present invention.

In one embodiment, the RNA that is delivered to target cells is not chemically modified. In another embodiment, the RNA that is delivered to the target cells is crosslinked RNA, such as but not limited to crosslinked siRNA. Crosslinked siRNA derivatives are as described in U.S. Patent No. 10,087 441, which is incorporated herein by reference in its entirety. Crosslinking can be employed to alter the pharmacokinetics of the composition, for example, to increase the half-

life in the body. Thus, the invention includes delivery of siRNA derivatives that include siRNA having two complementary strands of nucleic acid, such that the two strands are crosslinked. For example, a 3' OH terminus of one of the strands can be modified, or the two strands can be crosslinked and modified at the 3' OH terminus. The siRNA derivative delivered to the target cells can contain a single crosslink, *e.g.,* a psoralen crosslink. In some embodiments, the siRNA derivative has at its 3' terminus a biotin molecule, *e.g.,* a photocleavable biotin, a peptide, a peptidomimetic, a nanoparticle, organic compounds, *e.g.,* a dye such as a fluorescent dye, or a dendrimer. Modifying siRNA derivatives in this way may improve EV uptake or enhance targeting activities of the resulting siRNA derivative as compared to the corresponding siRNA and may be useful for tracing the siRNA derivative in the cell or improving the stability of the siRNA derivative compared to the corresponding siRNA. As such, one skilled in the art can screen crosslinked siRNA derivatives that are modified with various methods to determine whether the crosslinked siRNA derivatives possesses improved properties while maintaining the ability to mediate RNAi as are generally known in the art.

In another embodiment, the RNA delivered to the target cell is ssRNA. The ssRNA delivered to the target cells may act, for example, as a guide strand that would normally be part of an siRNA molecule. In this specific embodiment, the ssRNA that is delivered to the target cells does not need any additional cellular processing, *e.g.,* dicer and/or argonaut, before it can be used to promote gene silencing within the target cell. In another embodiment, the ssRNA that is delivered to the target cell can act as an miRNA.

In yet another embodiment, RNA can be delivered to a target cell and, if necessary, the cell can process the delivered RNA into miRNA. As is well-known in the art, miRNA is a type of RNA that can also suppress gene expression, either by inhibiting translation of mRNA or by promoting degradation of mRNA. Generally speaking, miRNA is ssRNA that can be between about 20-25 nucleotides in length and is often complementary to the untranslated region (UTR) of an mRNA transcript. The RNA that is delivered to the target cell, *e.g.,* dsRNA, may be processed within the target cell to produce the miRNA, or the RNA delivered to the target cell can be miRNA. If the RNA delivered to the cells is to be processed into miRNA, the delivered RNA may be between about 60-100 nucleotides in length and, in one embodiment, is internally complementary to itself such that it can fold back on itself to form short hairpin RNA duplexes. The RNA duplex regions of the short hairpin RNA that include the miRNA need not be 100% perfectly complementary

along the double stranded stretch of the RNA duplex. Thus, the short hairpin RNA containing the miRNA may include "bulges" in which short regions within the duplex structure do not base pair with one another.

Similar to siRNA sequences, the nucleotide sequence of miRNAs delivered to the target cell or the RNA delivered to the target cell that is processed into miRNA is not necessarily limited to a specific nucleotide sequence, except that the miRNA that is delivered or derived within the cell after delivery may be designed to have one strand that is perfectly or predominantly complementary to between about 15-23 bases of a target mRNA. As used herein, a predominantly complementary strand of RNA may have no more than about 4 bases over a 15-23 nucleotide length that are not complementary to the target mRNA.

In specific embodiments, the RNA delivered to the target cells is an miRNA directed against SARS-CoV-2. The sequences of various miRNAs in SARS-CoV-2 are now well-known. For example, Yu, T. *et al., J. Elec. Sci. Tech.* (e-published 6 December 2020) (doi.org/10.1016/j.jnlest.2020.100060), which is incorporated by reference, discloses miRNA sequences of SARS-CoV-2. EI-Nabi, S., *et al., Med Hypotheses,* 143:110203 (2020) (doi: 10.1016/j.mehy.2020.110203), which is incorporated by reference, also discloses miRNAs directed against ORF9, the 3' UTR and the 5'UTR of SARS-CoV-2. Mirzaei, R., *et al., J. Int'l. Immunopharm.*, (e-published 13 November 2020) (doi.org/10.1016/j.intimp.2020.107204), which is incorporated by reference, also discloses miRNAs that can be used for gene silencing of various genes of SARS-CoV-2, and therefore suppress viral replication in the target cell.

In additional embodiments, the RNA that is delivered to the target cells is an miRNA that can suppress innate immunity response in target cells. For example, the RNA delivered to the target cells is human, miR-231, miR-223 or miR-451. Established miRNA databases provide specific sequences of miRNAs in humans and other mammals that are involved in regulation of gene expression. For example, the MiRBase (mirbase.org/) (Kozomara, A. and Griffiths-Jones, S., *Nucleic Acids Res.*, 42:D68-D73 (2014), which are incorporated by reference) discloses miRNAs that may be delivered to the target cells using the SGEVs of the present invention.

In yet another embodiment, the RNA that is delivered to the target cells can be short hairpin RNA (shRNA). As used herein, shRNA is a duplex RNA in which a single RNA strand is internally complementary to itself such that it can fold back on itself to form an RNA duplex. The shRNA may then be further processed in the cell to, for example, miRNA or siRNA.

In some embodiments of the present invention, the delivered RNA is RNA that is directed against at least one of the ORFlab, ORF3a, ORF7a, ORF8, S protein, N protein, the RdRp protein or M protein ORF, or the 5'-nspl region or 5'UTR region of the Severe Acute Respiratory Syndrome-Related Coronavirus 2 (SARS-CoV-2) virus. The polynucleotide sequences of each of these ORFs or region of the SARS-CoV-2 virus are well-known. *See* National Center for Biotechnology and Information (NCBI) Accession Number NC_045512.2 (available on the world wide web at (ncbi.nlm.nih.gov/nuccore/NC_045512.2) and Wu, F., *et al., Nature,* 579 (7798):265-269 (2020) both of which are incorporated by reference.

In some embodiments of the present invention, the RNA delivered to a target cell, may include a shRNA delivered to a target cell by SGEVs. In this preferred embodiment, the SGEV may express a heterologous nucleotide sequence, operably linked to a promoter, according to the nucleotide sequence SEQ ID NO. 1. In another embodiment, of the present invention, the RNA delivered to a target cell, may include a shRNA delivered to a target cell by a SGEV. In this preferred embodiment, the SGEV may express a heterologous nucleotide sequence, operably linked to a promoter, having at least 98% sequence identity to the nucleotide sequence of SEQ ID NO. 1. In a preferred embodiment, the target cell may be infected with, or at risk of infection by SARS-CoV-2.

In some embodiments of the present invention, the RNA delivered to a target cell, may include a shRNA delivered to a target cell by SGEVs. In this preferred embodiment, the SGEV may express a heterologous nucleotide sequence, operably linked to a promoter, according to the nucleotide sequence SEQ ID NO. 18. In another embodiment, of the present invention, the RNA delivered to a target cell, may include a shRNA delivered to a target cell by a SGEV. In this preferred embodiment, the SGEV may express a heterologous nucleotide sequence, operably linked to a promoter, having at least 98% sequence identity to the nucleotide sequence of SEQ ID NO. 18. In a preferred embodiment, the target cell may be infected with, or at risk of infection by SARS-CoV-2.

In some embodiments of the present invention, the RNA delivered to a target cell, may include a shRNA delivered to a target cell by SGEVs transformed by, and expressing by a heterologous expression vector. In this preferred embodiment, the invention may include an expression vector configured to be integrated into the genome of a yeast cell, and preferably a Sb yeast cell, and express an RNA oligonucleotide. The expression vector of the invention may

include a heterologous nucleotide sequence, operably linked to a promoter, having a nucleotide sequence according to SEQ ID NO. 1. In another embodiment, The expression vector of the invention may include a heterologous nucleotide sequence, operably linked to a promoter, having a nucleotide sequence with at least 98% sequence identity to the nucleotide sequence of SEQ ID NO. 1. In a preferred embodiment, the target cell may be infected with, or at risk of infection by SARS-CoV-2.

In some embodiments of the present invention, the RNA delivered to a target cell, may include a shRNA delivered to a target cell by SGEVs transformed by, and expressing by a heterologous expression vector. In this preferred embodiment, the invention may include an expression vector configured to be integrated into the genome of a yeast cell, and preferably a Sb yeast cell, and express an RNA oligonucleotide. The expression vector of the invention may include a heterologous nucleotide sequence, operably linked to a promoter, having a nucleotide sequence according to SEQ ID NO. 18. In another embodiment, The expression vector of the invention may include a heterologous nucleotide sequence, operably linked to a promoter, having a nucleotide sequence with at least 98% sequence identity to the nucleotide sequence of SEQ ID NO. 18. In a preferred embodiment, a target cell is a cell of a subject, and preferably a human subject. The target cell of the invention may be established *in vitro* or *in vivo*, and may be part of an organ or tissue. In a preferred embodiment, the target cell may be infected with, or at risk of infection by SARS-CoV-2.

As noted above, there are well-established "rules" for determining one or more polynucleotide sequences that can be directed against a specific target gene for silencing. As used herein, the phrase "directed against" or "directed to" when used in conjunction with RNA means that the RNA comprises at least one strand that is designed to promote gene silencing for a target gene. In one embodiment, the delivered RNA of the present invention comprises a nucleotide sequence of at least 20 contiguous nucleotides directed against at least one of the ORFlab, ORF3a, ORF7a, ORF8, S protein, N protein, the RdRp protein or M protein ORF, or the 5'-nspl region or 5'UTR region of the SARS-CoV-2 virus. In one embodiment, the invention may include a shRNA directed to the nsp1 gene or region of the SARS-CoV-2 virus. In a preferred embodiment, the invention may include a shRNA according to the nucleotide sequence SEQ ID NO 1 or 18, directed to the nsp1 gene or region of the SARS-CoV-2 virus.

In some embodiments of the present invention, the expression vector of the invention may include one or more nucleotide sequences configured to facilitate integration and expression of a heterologous nucleotide sequence, encoding at least one RNA. In this embodiment, exemplary nucleotide sequence nucleotide sequences configured to facilitate integration and expression of a heterologous expression cassette, encoding at least one RNA may include SEQ ID NO.'s 3-11.

In some embodiments of the present invention, the invention may include a kit for the treatment of a disease or condition in a subject in need thereof. In this preferred embodiment, the kit of the invention may include a quantity of SGEVs containing one or more RNAs directed to inhibit the expression of one or more pathogen genes, or one or more endogenous genes in a subject. The quantity of SGEVs may be provided in a container, or other suitable receptacle or a quantity of SGEVs pre-loaded into a device for administration to a subject, preferably in a standard or customizable dosage. Optionally, the kit of the invention may include instructions for use.

In another preferred embodiment, the kit of the invention may include one or more yeast cells or cultures expressing a heterologous nucleotide sequence, operably linked to a promoter, encoding an RNA oligonucleotide (generally referred to herein as an RNA), and preferably an RNA directed to inhibit the expression of one or more pathogen genes, or one or more endogenous genes in a subject. The quantity of more yeast cells or cultures may be provided in a container, or other suitable receptacle and may be used to seed a culture for growth in a fermenter. Optionally, the kit of the invention may include instructions for use and fermentation.

In some embodiments of the present invention, the invention may include a kit for the treatment of a SARS-CoV-2 virus in a subject in need thereof. In this preferred embodiment, the kit of the invention may include a quantity of SGEVs containing one or more RNAs directed to inhibit the expression of one or more SARS-CoV-2 genes. In a preferred embodiment, the SGEVs of the kit may contain an RNA according to SEQ ID NO. 18, or a sequence having at least 98% sequence identify with SEQ ID NO. 18. The quantity of SGEVs may be provided in a container, or other suitable receptacle or a quantity of SGEVs pre-loaded into a device for administration to a subject, preferably in a standard or customizable dosage. Optionally, the kit of the invention may include instructions for use.

In another preferred embodiment, the kit of the invention may include one or more yeast cells or cultures expressing a heterologous nucleotide sequence, operably linked to a promoter, encoding an RNA, and preferably an RNA directed to inhibit the expression of one or more SARS-

CoV-2 genes. The yeast cells or cultures may express a heterologous nucleotide sequence, operably linked to a promoter, encoding a nucleotide sequence according to SEQ ID NO. 1, or a sequence having at least 98% sequence identify with SEQ ID NO. 1. The quantity of more yeast cells or cultures may be provided in a container, or other suitable receptacle and may be used to seed a culture for growth in a fermenter. Optionally, the kit of the invention may include instructions for use and fermentation.

In certain embodiments, the invention may include a pharmaceutical composition including a quantity of SGEVs containing one or more RNAs directed to inhibit the expression of one or more pathogen or endogenous host genes, and a pharmaceutically acceptable carrier. In certain embodiments, the invention may include a pharmaceutical composition including a quantity of SGEVs containing one or more RNAs directed to inhibit the expression of one or more SARS-CoV-2 genes, and a pharmaceutically acceptable carrier. In another specific preferred embodiment, the invention may include a pharmaceutical composition including a quantity of SGEVs containing one or more RNAs according to SEQ ID NO. 18, and a pharmaceutically acceptable carrier

In a preferred embodiment, the SGEVs of the kit may contain an RNA according to SEQ ID NO. 18, or a sequence having at least 908% sequence identify with SEQ ID NO. 18. The term "pharmaceutically acceptable" as used herein, refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. The term, "pharmaceutically acceptable carrier" as used herein, includes any and all solvents, or a dispersion medium including, but not limited to, water, ethanol, a polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, stachyose, and the like), suitable mixtures thereof, and vegetable oils, coatings, isotonic and absorption delaying agents, liposomes, commercially available cleansers, and the like. Supplementary bioactive ingredients also can be incorporated into such carriers.

The term "endogenous" gene or protein means that said gene or protein is expressed from a gene naturally found in the genome of a eukaryotic cell. The term "heterologous" gene or protein means that said gene or protein is not expressed from a gene naturally found in the genome of a eukaryotic cell. As used herein, the term "gene" or "polynucleotide" refers to a single nucleotide or a polymer of nucleic acid residues of any length. The polynucleotide may contain deoxyribonucleotides, ribonucleotides, and/or their analogs and may be double-stranded or single

stranded. A polynucleotide can comprise modified nucleic acids (e.g., methylated), nucleic acid analogs or non-naturally occurring nucleic acids and can be interrupted by non-nucleic acid residues. For example, a polynucleotide includes a gene, a gene fragment, cDNA, isolated DNA, mRNA, tRNA, rRNA, isolated RNA of any sequence, recombinant polynucleotides, primers, probes, plasmids, and vectors. Included within the definition are nucleic acid polymers that have been modified, whether naturally or by intervention.

As used herein, the phrase "expression," "gene expression" or "protein expression," such as the level of includes any information pertaining to the amount of gene transcript or protein present in a sample, in a cell, in a patient, secreted in a sample, and secreted from a cell as well as information about the rate at which genes or proteins are produced or are accumulating or being degraded (e.g., reporter gene data, data from nuclear runoff experiments, pulse-chase data etc.). Certain kinds of data might be viewed as relating to both gene and protein expression. For example, protein levels in a cell are reflective of the level of protein as well as the level of transcription, and such data is intended to be included by the phrase "gene or protein expression information." Such information may be given in the form of amounts per cell, amounts relative to a control gene or protein, in unitless measures, etc. The term "expression levels" refers to a quantity reflected in or derivable from the gene or protein expression data, whether the data is directed to gene transcript accumulation or protein accumulation or protein synthesis rates, etc. Polypeptides encoded by a target molecule genes that may be targeted for expression inhibition, for example through an RNAi mediated process herein may reflect a single polypeptide or complex or polypeptides. Accordingly, in another embodiment, the invention provides a polypeptide that is a fragment, precursor, successor or modified version of a protein target molecule described herein. In another embodiment, the invention includes a protein target molecule that comprises a foregoing fragment, precursor, successor or modified polypeptide. As used herein, a "fragment" of a polypeptide refers to a single amino acid or a plurality of amino acid residues comprising an amino acid sequence that has at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 20 contiguous amino acid residues or at least 30 contiguous amino acid residues of a sequence of the polypeptide. As used herein, a "fragment" of poly- or oligonucleotide refers to a single nucleic acid or to a polymer of nucleic acid residues comprising a nucleic acid sequence that has at least 15 contiguous nucleic acid residues, at least 30 contiguous nucleic acid residues, at least 60 contiguous nucleic acid residues, or at least 90% of a sequence of the polynucleotide. In some

embodiment, the fragment is an antigenic fragment, and the size of the fragment will depend upon factors such as whether the epitope recognized by an antibody is a linear epitope or a conformational epitope. Thus, some antigenic fragments will consist of longer segments while others will consist of shorter segments, (e.g., 5, 6, 7, 8, 9, 10, 11 or 12 or more amino acids long, including each integer up to the full length of the polypeptide). Those skilled in the art are well versed in methods for selecting antigenic fragments of proteins.

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," "prevent" and grammatical equivalents (including "lower," "smaller," etc.) when in reference to the expression of any symptom in an untreated subject relative to a treated subject, mean that the quantity and/or magnitude of the symptoms in the treated subject is lower than in the untreated subject by any amount that is recognized as clinically relevant by any medically trained personnel. In one embodiment, the quantity and/or magnitude of the symptoms in the treated subject is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity and/or magnitude of the symptoms in the untreated subject.

The term "introducing," "administered" or "administering", as used herein, refers to any method of providing a composition of SGEVs to a patient such that the composition has its intended effect on the patient. In one embodiment, SGEVs may be introduced to a patient *in vivo*, while in other alternative embodiments, SGEVs may be introduced to subject cells *in vitro* which may then be administered to a patient *in vivo*.

The term "patient," or "subject" as used herein, is a human or animal and need not be hospitalized. For example, out-patients, persons in nursing homes are "patients." A patient may comprise any age of a human or non-human animal and therefore includes both adult and juveniles (i.e., children). It is not intended that the term "patient" connote a need for medical treatment, therefore, a patient may voluntarily or involuntarily be part of experimentation whether clinical or in support of basic science studies.

Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, "expression cassette" refers to a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The peptides of the invention of the present invention may be chimeric.

The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular DNA sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus.

As used herein, a promoter region or promoter element refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one

or both of the limits, ranges excluding either or both of those included limits are also included in the invention. Certain ranges are presented herein with numerical values being preceded by the term "about." The term "about" is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number.

To accomplish delivery of RNA to target cells, the methods and compositions of the present invention comprise *Saccharomyces-generated* extracellular vesicles (SGEVs). The term extracellular vesicles are membranous vesicles released from cells. The extracellular vesicles of the methods and compositions of the invention are composed of lipid bilayers that can envelope and carry cargo in its interior. The lipid bilayer of the EVs may also include proteins embedded therein. In some embodiments, the SGEVs of the compositions and methods of the present invention can be exosomes or ectosomes. As is well-known, exosomes are generally formed upon the endocytosis of multivesicular endosomes (MVEs) to form intraluminal vesicles (ILVs) which are subsequently released into the extracellular environment as exosomes, whereas ectosomes are assembled and released from the plasma membrane. Often, the primary structural feature distinguishing ectosomes and ectosomes is diameter. In some embodiments, the diameter of the SGEVs are between about 30 nm to about 180 nm, between about 50 nm to about 200 nm, between about 75 nm to about 250 nm, between about 100 nm to about 300 nm, between about 125 nm to about 350 nm, between about 150 nm to about 400 nm, between about 175 nm to about 450 nm, between about 200 nm to about 500 nm, between about 250 nm to about 550 nm, between about 300 nm to about 600 nm, between about 350 nm to about between about 650 nm, between about 400 nm to about 700 nm, between about 450 nm to about 750 nm, between about 500 to about 800 nm, between about 550 nm to about 850 nm, between about 600 nm to about 900 nm, between about 650 nm to about 950 nm, between about 700 nm to about 1000 nm, between about 750 nm to about 1050 nm, between about 800 nm to about 1100 nm, between about 850 nm to about 1150 nm or between about 900 nm to about 1200 nm. Thus, exosomes may comprise components on their membrane surface, including but not limited to proteins, glycoproteins, proteoglycans, carbohydrates and lipids, which may be used to direct cargo into to exosome.

For example, the SGEVs of the present invention may comprise one or more protein embedded in the lipid bilayer, such as but not limited to (a) ammonia transport outward protein 2 (SEQ ID NO. 24) (UniProt Database Accession No. P32907, which is hereby incorporated by reference), (b) plasma membrane protein up-regulated during nitrogen stress protein 1 (SEQ ID NO. 25) (UniProt Database Accession No. Q06991, which is hereby incorporated by reference), (c) glucan 1,3-beta-glucosidase 1/11 (SEQ ID NO. 26) (UniProt Database Accession No. P23776, which is hereby incorporated by reference), (d) glucan 1,3-beta-glucosidase (SEQ ID NO. 27) (UniProt Database Accession No. P15703, which is hereby incorporated by reference), (e) 1,3-beta- glucanosyltransferase (SEQ ID NO. 28) (UniProt Database Accession No. P22146, which is hereby incorporated by reference), (f) 1,3-beta-glucanosyltransferase (SEQ ID NO. 29) (UniProt Database Accession No. Q08913, which is hereby incorporated by reference), (g) iron transport multicopper oxidase (SEQ ID NO. 30) (UniProt Database Accession No. P38993, which is hereby incorporated by reference), (h) probable glycosidase protein (SEQ ID NO. 31) (UniProt Database Accession No. P53301, which is hereby incorporated by reference), (i) non-classical export protein 1 (SEQ ID NO. 31) (UniProt Database Accession No. Q02820, which is hereby incorporated by reference) and (j) Sur7 protein (SEQ ID NO. 33) (UniProt Database Accession No. P54003, which is hereby incorporated by reference).

In some embodiments, any one or more of the proteins of (a)-(j) above can serve as an anchor protein within the lipid bilayer membrane of the SGEV. As used herein, an anchor protein is a protein embedded in the membrane of the SGEV such that at least a portion of the protein is exposed to the extravesicular environment. In some embodiments, the anchor protein a protein normally found in SGEVs. In some embodiments, the anchor protein will have another protein domain bound or linked thereto. Thus, the anchor protein can form a complex that can present a protein or protein domain on the membrane surface. In one embodiment, the protein or protein domain linked or bound to the anchor protein is a foreign protein. As used herein, a foreign protein is a protein or portion thereof that the specific species of *Saccharomyces* used to generate the SGEVs does not normally express. For example, the foreign protein may be a full length mammalian proteins or only a portion thereof.

Examples of proteins or portions thereof that may be used as the foreign protein in the foreign protein- anchor protein complex (FPAPC) include human angiotensin I or a portion thereof that binds to angiotensin converting enzyme II (ACE2) (SEQ ID NO. 34), angiotensin II or a

portion thereof that binds to ACE2, Transmembrane protease, serine 2 (TMPRSS2) (SEQ ID NO. 37) or a portion thereof that binds to ACE2, vascular endothelial growth factor A (VEGF-A) (SEQ ID NO. 36) or a portion thereof that binds to neuropilin-1 (NRPl) (SEQ ID NO. 35), or any CendR peptide that binds to NRPl. The term CendR peptide is well understood in the art and, in this instance, means a peptide that binds to NRPl with a (-terminal amino acid sequence of R/KXXR/K. Examples of CendR peptides include but are not limited to the peptide sequence RPARPAR and those disclosed in Teesalu T, *et al., Proc. Nat'/. Acad. Sci. USA,* 106(38):16157-62 (2009) (doi:10.1073/pnas.0908201106), which is incorporated by reference. Other examples of foreign proteins include synthetic proteins or polypeptides. As used herein the terms polypeptide and protein are used interchangeably.

The foreign protein(s) in the FPAPC will bind to a target surface molecule (receptor) displayed on a target cell. As used herein, the term "target surface molecule" is the molecule, *e.g.*, a protein, which is the binding partner to the foreign protein in the FPAPC. In one embodiment, the target surface molecule is a mammalian cell surface protein. Examples of mammalian cell surface proteins include but are not limited to a receptor, an enzyme, an antigen expressed on an immune cell, an antigen expressed on an immune effector cell, a peptide, and an antigen. For example, the target surface molecule may be ACE2 or NRPl. The identity of the target surface molecule will dictate the identity of the foreign protein used in the FPAPC. For example, if the target surface molecule is ACE2, then the foreign protein of the FPAPC may be angiotensin II or a portion or mimetic thereof that binds to ACE2. Similarly, if the target surface molecule is NRPl, then the foreign protein of the FPAPC may be VEGF-A, or a portion or mimetic thereof that binds to NRPl.

Other target surface molecules that may be used to determine the identity of the foreign protein in the FPAPC include but are not limited to TMPRSS2 or Adam17. The term target cell, as used herein, is a cell that expresses the target surface molecule on its cell surface. Thus, a target cell is not necessarily a cell type, but rather a target cell as used herein is determined by the cell surface protein it displays. The target cell may be engineered to express the target surface protein or the target cell may naturally express the target surface protein.

The foreign peptide in the FPAPC may be a peptide mimetic that binds to a membrane protein displayed on a cell surface. A peptide mimetic is used herein as it is in the art. Namely, peptide mimetics are peptides that mimic the binding portion of ligands of hormones, enzymes,

receptors, cytokines or other molecules such that the mimetic can bind to a hormone, enzyme, receptor, cytokine *etc.* The mimetic may be a smaller portion of the full length protein that is responsible for the ligand binding to its binding partner, or the mimetic may be a synthetic molecule whose three dimensional structure can fit into, and possibly bind to, the binding pocket of the naturally occurring ligand's binding partner.

Examples of peptide mimetics that can be used as the foreign protein in the FPAPC include but are not limited to mimetics of angiotensin I, mimetics of angiotensin II, mimetics of VEGF-A and mimetics of the ACE2 binding domain of TMPRSS2. In specific embodiments, the mimetic that is the foreign protein in the FPAPC is at least one of the peptides selected from the amino acid sequences listed in Table II of Huang, L., *et al., J. Biol. Chem.,* 278(18):15532-15540 (2003), which is incorporated by reference. The peptide fragments or mimetics need not have any activity in inhibiting the target surface molecule, provided that the foreign peptide binds with at least some specificity. In specific examples, the mimetic is an angiotensin mimicking peptide with amino acid sequence of GDYSHCSPLRYYPWWKCTYPDPEGGG (SEQ ID NO. 14) or GDDDDCGWIGFANFHLCLHGDPEGGG (SEQ ID NO. 15).

Methods of generating amino acid sequences that, when produced, can serve as peptide mimetics are well-known in the art. In one embodiment, computer modeling can be employed to design and identify peptides, and even small molecules, that can fit into a binding pocket of the target surface molecule. For example, Farhadi, T. and Hashemian, S., *Drug Des. Devel. Ther.,* 12:1239-1254 (2018), which is incorporated by reference, provides a review of current computer modeling methods that can be employed to generate putative peptide mimetics that can be used as the foreign protein in the FPAPC. Other methods of designing peptide mimetics include determining the region of binding between a ligand and its binding partner and shortening the full length ligand and/or mutating, *e.g.,* alanine scanning, to determine the identities of amino acid identity that contribute to binding.

In one embodiment, the FPAPC is a fusion protein. The term fusion protein as used herein is used as it is in the art. Namely, the fusion proteins used in the methods and compositions of the present invention involve two separate proteins or protein domains that are linked by a covalent bond. In one embodiment, the covalent bond linking the two domains is an amine bond. In more specific embodiments, the anchor protein and the foreign protein is a fusion protein comprising a single-chain polypeptide. In even more specific embodiments, the single-chain polypeptide

comprising the anchor protein and the foreign protein further comprises a linker peptide sequence. Any linker sequence can be used to covalently link the anchor protein and the foreign protein.

As used herein, the term "peptide linker(s)," "linker(s)," or "linker moiety" refers to a peptide or polypeptide sequence, *e.g.*, a synthetic peptide or polypeptide sequence, which connects two domains in a linear amino acid sequence of a polypeptide chain. In one embodiment, the polypeptides of invention are encoded by nucleic acid molecules that encode peptide linkers which either directly or indirectly connect the anchor protein and foreign protein which make up the construct. These linkers may be interposed between the anchor protein and foreign protein. If the linker connects two protein moieties contiguously in the linear polypeptide sequence, it is referred to as a "direct" linkage. In contrast, the linkers may link the first protein moiety, *i.e.*, anchor protein or foreign protein, to a binding moiety which is, in turn, linked to the second protein moiety, *i.e.*, anchor protein or foreign protein, thereby forming an indirect linkage. Linkers are typically located at the N or C terminus of the protein moieties.

In one embodiment, the linker linking the anchor protein and the foreign protein is a peptide comprised of glycine (Gly)n, wherein n is an integer that is the same or higher than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10. In another embodiment, the linker linking the anchor protein and the foreign protein is a gly-ser linker. As used herein, the term "gly-ser peptide linker" (GS) refers to a peptide comprising or consisting of glycine (G or Gly) and serine (Sor Ser) residues. Exemplary gly-ser peptide linkers comprise the amino acid sequence (Gly4 Ser)n or (Gly3 Ser)n. Another exemplary gly-ser peptide linker comprises the amino acid sequence S(Gly4 Ser)n wherein n is an integer that is the same or higher than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10. In another embodiment, the linker linking the anchor protein and the foreign protein is a peptide comprising the sequence of GSAGSAAGSGEF (SEQ ID NO. 16).

The extracellular vesicles are generated from *Saccharomyces*. *Saccharomyces* is a single-celled organism, but the term "extracellular vesicle," as it relates to the SGEVs, refers to vesicles that are secreted from *Saccharomyces* into the local environment, such as, but not limited to cell culture medium and organisms that may have ingested or consumed or been administered the *Saccharomyces* secreting the vesicles containing the foreign RNA. In one embodiment, the SGEVs are secreted from *Saccharomyces cerevisiae or Saccharomyces boullardii.*

The *Saccharomyces* are engineered to produce EVs that contain FPAPC such that the foreign protein in the FPAPC is on the exterior side of the vesicle. The foreign protein is to be

complexed with an anchor protein. The anchor proteins, as disclosed herein, will derive from the species of *Saccharomyces* used. Thus, the term *"Saccharomyces-derived"* as used herein means that at least one of the anchor proteins within the EVs used in the compositions and methods of the present invention are normally expressed in *Saccharomyces*. In one embodiment, at least one of the anchor proteins of the SGEVs used in the methods and compositions of the present invention are normally expressed in *Saccharomyces cerevisiae or Saccharomyces boullardii*. One of skill in the art can determine the source of the anchor protein, generally speaking, by its amino acid sequence. For example, if a specific anchor protein has an amino acid sequence of a *Saccharomyces boullardii* anchor protein, then the SGEV would be "derived" *Saccharomyces boullardii*. In some instances, the amino acid sequence of the anchor proteins are from the same species. In other instances, the amino acid sequence of the anchor proteins are from the different species, but the anchor proteins should all be from within the *Saccharomyces* genera. To be clear, if the EVs used in the methods and compositions of the present invention contain proteins that are not from any *Saccharomyces* species, the EV could still be considered an SGEV if those *non-Saccharomyces* proteins are not used as anchor proteins to form a complex with a foreign protein in the FPAPC.

The SGEVs used in the methods and compositions of the present invention may or may not also comprise a detectable signal. The signal can be complexed with a protein within the SGEVs, or the signal can be within the interior of the SGEV. In one embodiment, the detectable signal is a green fluorescent protein (GFP).

The SGEVs are to be employed in methods of silencing target genes. In select embodiments, the invention relates to methods of gene silencing comprising administering the SGEVs of the present invention, comprising foreign RNA, to a cell or population of cells that express a target gene. The SGEVs can deliver their foreign RNA cargo, comprising a nucleotide sequence that targets a target gene for silencing, to the target cells, thereby silencing the target gene.

As used herein, a target gene is a gene whose expression is to be selectively inhibited or "silenced." This silencing is achieved by promoting the degradation of the mRNA of the target gene that is induced by the binding between the delivered RNA, *e.g.*, a shRNA, miRNA, siRNA, and the mRNA of the target gene. One portion or segment of these molecules is an anti-sense strand that is substantially complementary to a portion, *e.g.*, about 16 to about 40 or more

nucleotides of the mRNA of the target gene. Any gene previously identified by genetics or by sequencing may represent a target. Target genes may include, viral structural genes, such as but not limited to, capsid proteins, envelope proteins and membrane fusion proteins, viral non-structural genes such as but not limited to, virus replicon genes and virus immunomodulatory genes, viral regulatory and/or accessory genes. Other target genes include nuclear-encoded developmental genes and regulatory genes as well as metabolic or structural genes or genes encoding enzymes. In one embodiment of the present invention, the gene to which the delivered RNA is targeting for silencing is a viral gene that is necessary for virus replication.

As used herein, the gene silencing need not be a complete silencing. In one embodiment, the silencing is a "complete" silencing in that the gene expression is completely suppressed such that there is no detectable expression of the target gene. In other embodiments, the silencing is not a complete silencing and, instead, the silencing is partial. A partial gene silencing means a reduction in expression of the target gene such that expression may still be detectable. A reduction of gene expression can be assessed by determining gene expression levels before and after treatment or administration of the SGEVs. Gene expression levels can be measured using well-known methods, including but not limited to, measuring protein expression levels of the target gene and measuring mRNA levels of the target gene. Measuring protein expression levels can be accomplished directly, *e.g.*, Western Blot, ELISA, *etc.* or indirectly, *e.g.*, protein activity, metabolite levels, etc. In one embodiment, gene expression levels are measured with "RNA-seq," which is a well-known methodology for RNA profiling. See Wang, Z., *et al., Nat Rev Genet.*, 10(1): 57-63 (2009), which is incorporated by reference. The levels of gene expression of a target gene in a cell or group of cells can be measured prior to administration of the SGEVs by culturing the cells and measuring gene expression levels from the cells in culture. Then the SGEVs can be administered to the cells in culture and target gene expression levels can be reassessed to determine changes in gene expression levels.

The term "administering" as used herein means that the SGEVs are brought into contact or the same environment as the target cells. For example, if the SGEVs are administered to a subject having or suspected of having a viral infection, the SGEVs may be administered to the subject by a routine route of administration, such as but not limited to, oral, intravenous, topical, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal route. If the SGEVs are

administered to cells in culture, for example to assess differential gene expression levels, the SGEVs can be added to the culture medium.

In more specific embodiments, the target gene against which the delivered RNA is directed is selected from the group consisting of the ORFlab, ORF3a, ORF7a, ORF8, S protein, N protein, the RdRp protein or M protein ORF, or the 5'-nspl region or S'UTR region of SARS-CoV-2. In more specific embodiments, the delivered RNA comprises a nucleotide sequence of between about 20 to about 100 contiguous nucleotides of the ORFlab, ORF3a, ORF7a, ORF8, S protein, N protein, the RdRp protein or M protein ORF, or the 5'-nspl region or S'UTR region of SARS-CoV-2.

The present invention also relates to polynucleotides encoding the fusion proteins of the present invention. As is known in the art, for any DNA sequence determined by an automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

The polynucleotides of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the peptides may be identical to the coding sequence shown in the sequence listing, or that of any of the deposited clones, or may be a different coding sequence which, as a result of the redundancy or degeneracy of the genetic code, encodes the same fusion proteins as shown in the sequence listing.

The term "polynucleotide encoding a peptide" encompasses a polynucleotide which includes only coding sequences for the polypeptide, *e.g.*, fusion protein, as well as a polynucleotide which includes additional coding and/or non-coding sequences. Thus, for example, the

polynucleotides of the present invention may encode for a peptide, *e.g.,* a fusion protein, or for a peptide having a prosequence or for a protein having both a prosequence and presequence.

The polynucleotides of the present invention may also have the coding sequence fused in frame to, for example, a marker sequence which allows for identification of the polypeptide of the present invention. The marker sequence may be a GFP protein, a hexa-histidine tag to provide for purification of the fusion protein is used.

The invention also relates to vectors, including but not limited to, expression vectors comprising the polynucleotides encoding the fusion proteins of the present invention. Types of vectors for expression for proteins and fusion proteins are well known in the art. In one embodiment, the vector is an expression vector for protein expression in *Saccharomyces.* Yeast expression vectors are commercially available from manufacturers.

The present invention also relates to methods of making and using these *Saccharomyces*-generated EVs. In one embodiment, the methods of making the SGEVs of the present invention comprise introducing into the *Saccharomyces* the expression vector encoding a fusion protein of the present invention to generate a host *Saccharomyces* cell. The host cell is then cultured under conditions to permit protein production from the vector encoding the fusion protein. In one embodiment, the host cells of the present invention *Saccharomyces cerevisiae or Saccharomyces boullardii.*

Culture conditions for culturing yeast host cells are well-known in the art. The continued culture of the host cell will permit production and secretion of the SGEVs into the cell culture environment, where they can be isolated from culture.

Methods of isolating extracellular vesicles, such as exosomes, from cell culture media are well- known in the art and are reviewed in Li, P. *et al., Theranostics,* 7(3):789-804 (2017), which is incorporated by reference herein. Generally speaking, methods of isolating the SGEVs from culture include but are not limited to ultracentrifugation methods, size-based exclusion methods, immunoaffinity capture-based methods, precipitation methods, microfluidics-based methods or some combination thereof.

The foreign RNA may or may not be present in the SGEVs immediately isolated from culture. For example, the foreign RNA can be introduced into the SGEVs by a number of different techniques. In select embodiments, the SGEVs are loaded with the foreign RNA by electroporation or the use of a transfection reagent. Extrapolation of the voltages used for electroporation of cells

to take into account the size of the exosomes would suggest that excessively high voltages would be required for electroporation of exosomes. Surprisingly however, it is possible to use electroporation to load exosomes with RNA using voltages in the range of between about 20V/cm to 1000V/cm, for example 20V/cm to 100V/cm, with capacitance between about 25 μF and about 250 μF, for example between 25 μF and 125 μF.

In an alternative aspect of the present invention, it is possible to load the SGEVs with the foreign RNA using transfection agents. Despite the small size of the exosomes, conventional transfection agents can be used for transfection of exosomes with genetic material. In some embodiments, transfection reagents for use in accordance with the present invention include cationic liposomes.

In still other embodiments, the *Saccharomyces* host cell harboring an expression vector encoding the fusion protein of the present invention will include at least a second expression vector. The second expression vector would comprise a coding sequence for the foreign RNA to be loaded into the SGEVs as cargo. In these embodiments, the host cell would comprise at least two vectors, one of which comprises a polynucleotide encoding at least the foreign protein portion of the FPAPC and a second vector comprising expressing the foreign RNA that will become the cargo within the SGEV. United States Patent No. 10,174,338, which is incorporated by reference, discloses various expression vectors that can be used to carry and express the foreign RNA in yeast cells.

The present invention also relates to fusion proteins comprising a *Saccharomyces* extracellular vesicle anchor protein and a second peptide. The second peptide may be any one of the foreign proteins listed herein as part of the FPAPC. In some embodiments, the fusion protein comprises a linker peptide. In more specific embodiments, the linker in the fusion proteins are any of the linker peptides disclosed herein.

For example, the fusion proteins of the present invention may comprise one or more protein embedded in the lipid bilayer, such as but not limited to such as but not limited to(a) ammonia transport outward protein 2 (SEQ ID NO. 24) (UniProt Database Accession No. P32907, which is hereby incorporated by reference), (b) plasma membrane protein up-regulated during nitrogen stress protein 1 (SEQ ID NO. 25) (UniProt Database Accession No. Q06991, which is hereby incorpo-rated by reference), (c) glucan 1,3-beta-glucosidase 1/11 (SEQ ID NO. 26) (UniProt Database Accession No. P23776, which is hereby incorporated by reference), (d) glucan 1,3-beta-

glucosidase (SEQ ID NO. 27) (UniProt Database Accession No. P15703, which is hereby incorporated by reference), (e) 1,3-beta- glucanosyltransferase (SEQ ID NO. 28) (UniProt Database Accession No. P22146, which is hereby incorporated by reference), (f) 1,3-beta-glucanosyltransferase (SEQ ID NO. 29) (UniProt Data-base Accession No. Q08913, which is hereby incorporated by reference), (g) iron transport multicopper oxidase (SEQ ID NO. 30) (UniProt Database Accession No. P38993, which is hereby incorporated by reference), (h) probable glycosidase protein (SEQ ID NO. 31) (UniProt Database Accession No. P53301, which is hereby incorporated by reference), (i) non-classical export protein 1 (SEQ ID NO. 31) (UniProt Database Accession No. Q02820, which is hereby incorporated by reference) and (j) Sur7 pro-tein (SEQ ID NO. 33) (UniProt Database Accession No. P54003, which is hereby incorporated by reference).

In more specific embodiments, the second protein or protein domain linked or bound to the first protein is a foreign protein, as the term is used herein. For example, the foreign protein may be a full length mammalian protein or only a portion thereof. Examples of proteins or portions thereof that may be used as the second protein or protein domain of the fusion proteins include but are not limited to human angiotensin I, angiotensin II, vascular endothelial growth factor A (VEGF-A), mimetics of the ACE2 binding domain of TMPRSS2. Other examples of the second protein or proteins of the fusion proteins of the present invention include synthetic proteins or polypeptides.

Other examples of the second protein or proteins of the fusion proteins of the present invention include a mammalian cell surface protein ligand. Examples of mammalian cell surface proteins include but are not limited to a receptor, an enzyme, an antigen expressed on an immune cell, an antigen expressed on an immune effector cell, a peptide, and an antigen. For example, the mammalian cell surface protein may be angiotensin converting enzyme II (ACE2), neuropilin-1 (NRPl), TMPRSS2 or Adam17. The identity of the mammalian cell surface protein will dictate the identity of the second protein or protein domain used in the fusions proteins of the present invention. For example, the second protein or protein domain of the fusion proteins of the present invention may be angiotensin II or a fragment or mimetic thereof. Similarly, the second protein or protein domain of the fusion proteins of the present invention may be VEGF-A, or a fragment or mimetic thereof.

The present invention also relates to methods of administering the SGEVs to a subject. In one specific embodiment, the present invention relates to methods of treating subject having or suspected of having a virus infection by administering the SGEVs to the subject. The treatment methods comprise administering the SGEVs of the invention to a subject infected with or suspected of being infected with the virus, where the foreign RNA within the administered SGEVs comprises at least one nucleotide sequence that targets at least one of the viral genes for gene silencing. In one specific embodiment, the subject has or is suspected of having a SARS-CoV-2 virus infection.

As used herein, the methods include administering the SGEVs to the subject prior to a medical diagnosis of having a virus infection. The methods therefore include administering the SGEVs to the subject if the subject displays at least one symptom of a virus infection such as, but not limited to, fever, headache, body ache, dizziness, loss of sense of smell, loss of sense of taste, fatigue, chills, nausea, vomiting, diarrhea, loss of appetite, disorientation, rash, cough, sore throat, congestion, difficulty breathing and low blood oxygen levels, to name a few. Accordingly, the methods include administering the SGEVs of the present invention to a subject exhibiting one or more of these symptoms.

The present invention also provides methods of prophylaxis of a virus infection. These methods include administering the subject the SGEVs of the present invention before any symptoms of virus infection appear. Thus, the invention includes methods of preventing or reducing the likelihood of acquiring a virus infection by administering the SGEVs of the present invention to a subject prior to detecting any symptoms of the virus infection or being diagnosed with the virus infection.

When administration is for the purpose of treatment, the SGEVs are provided at, or after the onset of, a symptom or condition in need of treatment. The therapeutic administration of the SGEVs serves to attenuate any symptom or prevent additional symptoms from arising. When administration is for the purposes of preventing a condition from arising ("prophylactic administration"), the SGEVs are provided in advance of any visible or detectable symptom. The prophylactic administration of the SGEVs serves to attenuate subsequently arising symptoms or prevent or reduce the likelihood of symptoms from arising altogether. The route of administration of the SGEVs includes, but is not limited to, topical, transdermal, intranasal, rectal, oral, subcutaneous, intravenous, intraarterial, intramuscular, intraosseous, intraperitoneal, epidural and

intrathecal as disclosed herein. In one example SGEV's may be derived or isolated from a GRAS and/or probiotic yeast cell, such as *Saccharomyces cerevisiae*, and preferably *Saccharomyces boullardii.* For example, *Saccharomyces boullardii* probiotics, releasing wild type exosomes, have been shown to diminish disease severity by reducing the expression of inflammatory cytokines and stimulating the expression of anti-inflammatory cytokines in multiple organs including the lungs and cardiovascular system. *Saccharomyces boullardii* cells also have low immunogenicity and positively modulate host immune response in the presence of additional antigens. *Sb* is well established for genetic manipulation which allows the present inventors to engineer the *Sb* strain for expression and loading of specific siRNAs in exosomes. Cultivation of *Sb* is fast, low-cost, and easy to scale up using established procedures. Finally, the lipids present in EVs are natural and thus not likely to be cytotoxic when used therapeutically unlike artificial lipids frequently used to package mRNA for vaccines

In specific embodiments, the oral administration of the SGEVs include administering engineered yeast, producing the SGEVs, as a probiotic. As used herein, a probiotic is a microorganism, such as a bacteria or yeast, generally recognized as safe for human or animal consumption. The probiotics of the present invention may or may not have additional health benefits to the consumer. In specific embodiments of the present invention, the probiotics is a *Saccharomyces cerevisiae* or a *Saccharomyces boullardii.* For example, *Saccharomyces boullardii* probiotics, releasing wild type exosomes, have been shown to diminish disease severity by reducing the expression of inflammatory cytokines and stimulating the expression of anti-inflammatory cytokines in multiple organs including the lungs and cardiovascular system. *Saccharomyces boullardii* cells also have low immunogenicity and positively modulate host immune response in the presence of additional antigens. *Sb* is well established for genetic manipulation which allows the present inventors to engineer the *Sb* strain for expression and loading of specific siRNAs in exosomes. Finally, Cultivation of *Sb* is fast, low-cost, and easy to scale up using established procedures The probiotic used in the methods of administering will be engineered to produce the SGEVs of the present invention.

As used herein, the term "RNAi molecules" "interfering RNA molecules" or "interfering RNA" or RNA molecules configured to mediate RNA interference generally refers to an RNA which is capable of inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g., the full

translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNAi molecules include dsRNAs such as siRNAs, miRNAs and shRNAs, sgRNA, CRISPR RNA (crRNs). In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression. As used herein, an RNA molecule or even RNAi molecule may further encompass lincRNA molecules as well as lncRNA molecules.

In some embodiments of the invention, the nucleic acid agent is a double stranded RNA (dsRNA). As used herein the term "dsRNA" relates to two strands of anti-parallel polyribonucleic acids held together by base pairing. The two strands can be of identical length or of different lengths, provided there is enough sequence homology between the two strands that a double stranded structure is formed with at least 60%, 70% 80%, 90%, 95% or 100% complementary over the entire length. According to an embodiment of the invention, there are no overhangs for the dsRNA molecule. According to another embodiment of the invention, the dsRNA molecule comprises overhangs. According to other embodiments, the strands are aligned such that there are at least 1, 2, or 3 bases at the end of the strands which do not align (i.e., for which no complementary bases occur in the opposing strand) such that an overhang of 1, 2 or 3 residues occurs at one or both ends of the duplex when strands are annealed. It will be noted that the dsRNA can be defined in terms of the nucleic acid sequence of the DNA encoding the target gene transcript, and it is understood that a dsRNA sequence corresponding to the coding sequence of a gene comprises an RNA complement of the gene's coding sequence, or other sequence of the gene which is transcribed into RNA.

The inhibitory RNA sequence can be greater than 90% identical or even 100% identical, to the portion of the target gene transcript. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript under stringent conditions (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 60 degrees C hybridization for 12-hours; followed by washing). The length of the double-stranded nucleotide sequences complementary to the target gene transcript may be at least about 18, 19, 21, 25, 50, 100, 200, 300, 400, 491, 500, 550, 600, 650, 700, 750, 800, 900, 1000 or more

bases. In some embodiments of the invention, the length of the double-stranded nucleotide sequence is approximately from about 18 to about 530, or longer, nucleotides in length.

The present teachings relate to various lengths of dsRNA, whereby the shorter version i.e., x is shorter or equals 50 bp (e.g., 17-50), is referred to as siRNA or miRNA. Longer dsRNA molecules of 51-600 are referred to herein as dsRNA, which can be further processed for siRNA molecules. According to some embodiments, the nucleic acid sequence of the dsRNA is greater than 15 base pairs in length. According to yet other embodiments, the nucleic acid sequence of the dsRNA is 19-25 base pairs in length, 30-100 base pairs in length, 100-250 base pairs in length or 100-500 base pairs in length. According to still other embodiments, the dsRNA is 500-800 base pairs in length, 700-800 base pairs in length, 300-600 base pairs in length, 350-500 base pairs in length or 400-450 base pairs in length. In some embodiments, the dsRNA is 400 base pairs in length. In some embodiments, the dsRNA is 750 base pairs in length.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 17-30 base pairs, but also longer e.g., 31-50 bp) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC. It has been found that position of the 3'-overhang influences potency of a siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand. This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

In certain embodiments, dsRNA can come from 2 sources; one derived from gene transcripts generated from opposing gene promoters on opposite strands of the DNA and 2) from fold back hairpin structures produced from a single gene promoter but having internal complimentary. For example, strands of a double-stranded interfering RNA (e.g., a siRNA) may be connected to form a hairpin or stem-loop structure (e.g., a shRNA). Thus, as mentioned, the RNA silencing agent may also be a short hairpin RNA (shRNA). The term "shRNA", as used

herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

As used herein, the phrase "microRNA (also referred to herein interchangeably as "miRNA") or a precursor thereof" refers to a microRNA (miRNA) molecule acting as a post-transcriptional regulator. Typically, the miRNA molecules are RNA molecules of about 20 to 22 nucleotides in length which can be loaded into a RISC complex and which direct the cleavage of another RNA molecule, wherein the other RNA molecule comprises a nucleotide sequence, essentially complementary to the nucleotide sequence of the miRNA molecule. Typically, a miRNA molecule is processed from a "pre-miRNA," or as used herein, a precursor of a pre-miRNA molecule by proteins, such as DCL proteins, and loaded onto a RISC complex where it can guide the cleavage of the target RNA molecules. Pre-microRNA molecules are typically processed from pri-microRNA molecules (primary transcripts). The single stranded RNA segments flanking the pre-microRNA are important for processing of the pri-miRNA into the pre-miRNA. The cleavage site appears to be determined by the distance from the stem-ssRNA junction (Han et al. 2006, Cell 125, 887-901, 887-901).

As used herein, a "pre-miRNA" molecule is an RNA molecule of about 100 to about 200 nucleotides, preferably about 100 to about 130 nucleotides, which can adopt a secondary structure comprising an imperfect double stranded RNA stem and a single stranded RNA loop (also referred to as "hairpin"), and further comprising the nucleotide sequence of the miRNA (and its complement sequence) in the double stranded RNA stem. According to a specific embodiment, the miRNA and its complement are located about 10 to about 20 nucleotides from the free ends of the miRNA double stranded RNA stem. The length and sequence of the single stranded loop region are not critical and may vary considerably, e.g., between 30 and 50 nucleotides in length. The

complementarity between the miRNA and its complement need not be perfect, and about 1 to 3 bulges of unpaired nucleotides can be tolerated. The secondary structure adopted by an RNA molecule can be predicted by computer algorithms conventional in the art such as mFOLD. The particular strand of the double stranded RNA stem from the pre- miRNA which is released by DCL activity and loaded onto the RISC complex is determined by the degree of complementarity at the 5' end, whereby the strand, which at its 5' end, is the least involved in hydrogen bonding between the nucleotides of the different strands of the cleaved dsRNA stem, is loaded onto the RISC complex and will determine the sequence specificity of the target RNA molecule degradation. However, if empirically the miRNA molecule from a particular synthetic pre-miRNA molecule is not functional (because the "wrong" strand is loaded on the RISC complex), it will be immediately evident that this problem can be solved by exchanging the position of the miRNA molecule and its complement on the respective strands of the dsRNA stem of the pre-miRNA molecule. As is known in the art, binding between A and U involving two hydrogen bonds, or G and U involving two hydrogen bonds is less strong that between G and C involving three hydrogen bonds.

Naturally occurring miRNA molecules may be comprised within their naturally occurring pre-miRNA molecules, but they can also be introduced into existing pre- miRNA molecule scaffolds by exchanging the nucleotide sequence of the miRNA molecule normally processed from such existing pre-miRNA molecule for the nucleotide sequence of another miRNA of interest. The scaffold of the pre-miRNA can also be completely synthetic. Likewise, synthetic miRNA molecules may be comprised within, and processed from, existing pre-miRNA molecule scaffolds or synthetic pre- miRNA scaffolds. Some pre-miRNA scaffolds may be preferred over others for their efficiency to be correctly processed into the designed microRNAs, particularly when expressed as a chimeric gene wherein other DNA regions, such as untranslated leader sequences or transcription termination and polyadenylation regions are incorporated in the primary transcript in addition to the pre-microRNA.

According to the present teachings, the dsRNA molecules may be naturally occurring or synthetic. The dsRNA can be a mixture of long and short dsRNA molecules such as, dsRNA, siRNA, siRNA+dsRNA, siRNA+miRNA, or a combination of same.

In a preferred embodiment, one or more nucleic acid agents are designed for specifically targeting a target gene of interest. It will be appreciated that the nucleic acid agent can be used to downregulate one or more target genes (e.g., as described in detail above). If a number of target

genes are targeted, a heterogenic composition which comprises a plurality of nucleic acid agents for targeting a number of target genes is used. Alternatively, the plurality of nucleic acid agents is separately formulated. According to a specific embodiment, a number of distinct nucleic acid agent molecules for a single target are used, which may be used separately or simultaneously (i.e., co-formulation) applied.

For example, in order to silence the expression of an mRNA of interest, synthesis of the dsRNA suitable for use with some embodiments of the invention can be selected as follows. First, the mRNA sequence is scanned including the 3' UTR and the 5' UTR. Second, the mRNA sequence is compared to an appropriate genomic database using any sequence alignment software, such as the BLAST software available from the NCBI server (wwwdotncbidotnlmdotnihdotgov/BLAST/). Putative regions in the mRNA sequence which exhibit significant homology to other coding sequences are filtered out. Qualifying target sequences are selected as templates for dsRNA synthesis. Preferred sequences are those that have as little homology to other genes in the genome to reduce an "off-target" effect. It will be appreciated that the RNA silencing agent of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

The terms "comprises", "comprising", are intended to have the broad meaning ascribed to them in U.S. Patent Law and can mean "includes", "including" and the like.

The invention now being generally described will be more readily understood by reference to the following examples, which are included merely for the purposes of illustration of certain aspects of the embodiments of the present invention. The examples are not intended to limit the invention, as one of skill in the art would recognize from the above teachings and the following examples that other techniques and methods can satisfy the claims and can be employed without departing from the scope of the claimed invention.

The invention now being generally described will be more readily understood by reference to the following examples, which are included merely for the purposes of illustration of certain aspects of the embodiments of the present invention. The examples are not intended to limit the invention, as one of skill in the art would recognize from the above teachings and the following examples that other techniques and methods can satisfy the claims and can be employed without departing from the scope of the claimed invention. Indeed, while this invention has been particularly shown and described with references to preferred embodiments thereof, it will be

understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

EXAMPLES

Example 1: Rationale for SGEVs as anti-viral therapeutic platform.

Mammals, and in particular humans are constantly exposed to viruses that can lead to substantial disease burden, morbidity, and potentially mortality. The mutations of known viruses, such as the Delta and Omicron variants of the SARS-CoV-2 coronavirus, and arising of new viruses from zoonotic sources present a constant threat of epidemics and pandemics and a never-ending challenge to medicine. Unlike bacterial diseases, which generally can be treated with antibiotics, so far there is no common approach for fighting viral diseases. As SARS-CoV-2 coronavirus pandemic has demonstrated, the development of efficient treatment for new viruses could take months or years of medical struggle while the virus takes its toll on human lives and the world economy. As noted above, RNA interference is a mechanism of regulation of gene expression by blocking and disruption mRNA molecules mediated by small (19-27 bp) siRNA specifically complimentary to targeted mRNA sequence. Antiviral action of exogenous siRNA was demonstrated for broad range of both RNA and DNA viruses (for review see Levanova et al, 2018 (incorporated herein by reference)). As such, siRNA technology has potential to become a universal approach for anti-viral therapy.

The bottleneck of siRNA technology is the challenge of delivery of siRNA to targeted cells. Generally, siRNA molecules are not very stable itself and could be easily digested by extracellular nucleases. Also, despite of small size of siRNA molecules, electrostatic repulsion prevents their passive diffusion through cell membranes which brings in the necessity to encapsulate siRNA for protection of its integrity and improving membrane penetration. Two main types of vesicles currently used for siRNA encapsulation are chemically synthesized nanoparticles (proteins based or liposomes) or naturally occurring extracellular vesicles aka exosomes derived from mammalian cells. Both methods are expensive, there are concerns regarding administration of frequent and large doses of artificial lipids, and scaling up mammalian exosomes production is problematic. The cost of drug production could be potential roadblock for creating therapeutics for treatment of viral infection since such drugs need to be produced in high quantity to meet the needs of the market.

In one embodiment, the present invention addresses these technical hurdles by engineering a yeast, and preferably a Sb strain producing exosomes carrying anti-viral siRNA. Such yeast strain(s) can be grown in fermenters allowing easy and inexpensive commercial production of exosomes for pharmaceutical use, that could further be used as broad platform for siRNA delivery for silencing replication of viruses as well host genes expression in the range of pathological conditions such as inflammation, cancer or auto-immune diseases.

While considerable efforts to develop a drug delivery system employing EVs is currently being pursued, most of the work is on vesicles derived from human cells, especially EVs from macrophages or mesenchymal stem cells. Cultivation of mammalian cells for EVs requires the use of expensive growth media and serums and sometimes special, sophisticated techniques such as 3D-cell culturing. On the other hand, growing yeast is low-cost, simple, and fast: the average time for division of mammalian cells is more than 24 hours, while yeast double their population approximately every 90 minutes. Furthermore, human cells have the potential to introduce human pathogens which cannot grow in yeast.

Extracellular vesicles must be safe for injection into humans, which means their administration should not induce any adverse reactions such as inflammation or a strong immune response. In certain embodiment, the use of Sb as a source of EVs source organism as there is evidence of it being safe for humans in the prior art. Sb has also been used as a probiotic for decades, and it is currently approved by the FDA as a Generally Recognized As Safe (GRAS) microorganism. Oral administration of Sb has demonstrated clinical effectiveness in treatment of many gastrointestinal diseases both inflammatory, such as IBD or Crohn's disease, or infectious, such as *Clostridium difficile* or *E. coli* infections (review Kelesidis T, Pothoulakis C, 2012). Moreover, the positive effect of Sb is not limited by local responses on intestinal surfaces but has a systemic beneficial effect on the organism level. Sb administration prevents tissue damage and decreases inflammation in hosts by down-regulating the production of pro-inflammatory while up-regulating anti-inflammatory cytokines (Fidan et al, 2009; Duman et al, 2013; Durmaz et al, 2020).

Specifically, the protective effect of Sb pretreatment was demonstrated in an experimental model of lung injury in rats (Karen et al, 2010) and for a cardiovascular system in a diabetic mice model (Brandalo et al, 2018). Additionally, Sb also has unique immunomodulatory properties. While Sb itself has low immunogenicity and doesn't induce significant production of antibodies recognizing Sb antigens (Hudson et al, 2016; Joossens, 2005), the presence of Sb stimulates an

immune response to pathogens. For example, in experiments an oral administration of Sb significantly increased specific antibody production in mice infected by *Clostridium difficile* (Bagherpour et al, 2016), and increased antibody production in response to a vaccination against leptospirosis (Silveira et al, 2014). Sb cells routinely shed EVs into the environment, so subjects exposed to Sb are simultaneously exposed to Sb-derived EVs as well. As such, administration of Sb-derived EVs can be safe and have as low immunogenicity as the administration of Sb itself. Moreover, EV-based treatments and therapeutics are not susceptible to antibiotic resistance due to their unique mode of action. This allows wide-spread deployment of the invention without the negative potential of developing strains of antibiotic resistant pathogens.

Unlike EVs derived from mammalian cells, engineering of yeast EVs has yet to be systematically explored and a few important gaps need to be filled to use yeast EVs as vesicles for therapeutic delivery. To close these gaps, present inventors established a siRNA expression system in Sb cells for the synthesis of therapeutic siRNA and packing them into EVs.

Example 2: Characterization of Sb-derived EVs.

EVs were isolated *via* ultracentrifugation from 1L of Sb culture grown in flask for 24 h and resuspended in 1 ml of phosphate-buffered saline (PBS). The size distribution and number of EVs were determined using nanoparticle tracking analysis (NTA) on a ZetaView instrument. Typical yields of EVs obtained from single extraction were approximately $3x10^{11}$ with median size of particles 125nm, StDev 70nm (Fig. 1) and zeta potential about -15 mV.

Example 3: Establishment of siRNA expressing system in Sb.

Sb doesn't have its own RNAi machinery including proteins of the RISC complex such as dicer or argounate. Though some miRNA-like structures were found in Sb by RNA sequencing, the general mechanism of small RNA processing in Sb is unclear. To express siRNA of pre-defined size in Sb cells the present inventors designed integration cassettes where shRNA were expressed either under RNA polymerase II type TDH3 promoter (Fig. 2A), or under control of RNA polymerase I rRNA promoter (Fig. 2B). Both expression cassettes contained two siRNAs flanked with tRNAs or tRNA and ribozyme HDV. Post-transcriptional processing of tRNA by yeast RNases P and Z or auto-cleavage of HDV ribozyme leads to removal of flanking sequences and separation of single small RNAs (Zhang et al, 2019). This approach also allowed the present inventors to engineer Sb expressing pool of siRNAs for simultaneous targeting more than one locus in viral genomes. Expression cassettes used in this work contained two short hairpin RNA

(shRNA), one targeting Nsp1 gene from SARS-CoV-2 genome (SEQ ID NO. 1, and 18), and second targeting eGFP gene (SEQ ID NO. 1, and 18). ShRNA expression cassettes were genome integrated in YPRCt3 locus on XVI chromosome. It was previously shown that integration at this locus doesn't affect the cell growth and gene expression in *S. boulardii* (Durmusoglu et al, 2020)

To demonstrate that engineered Sb cells indeed express shRNA, the present inventors extracted total RNA from wild-type and engineered Sb cells, and performed northern dot blot analysis using an RNA probe sequence-specific to the yeast expressed shRNA-Nsp1 (Fig. 3). ShRNA-Nsp1 was detected in both SB strains using RNA pol I and RNA pol II expression cassettes as well as in EVs fraction extracted from engineered Sb, but not in wild type Sb cells.

Example 3: Verification of cellular uptake of Sb-derived EVs.

Cellular uptake of different yeast-derived EVs has been previously demonstrated (for review Rizzo, Rodrigues and Janbon, 2020). To verify that Sb-derived EVs also could be utilized by human cells, the present inventors extracted EVs from engineered Sb strain expressing Sur7 protein localized in EVs and fused with GFP reporter gene, and applied these EVs to H1299 cells. The presence of GFP labeled protein allowed us to visualize the localization of EVs. Following the application of the EVs, we observed the presence of GFP signals in H1299 cells and the spread of fluorescence in cytoplasm and endosomes-like structures, which demonstrates that EVs are indeed being absorbed and degraded by human cancer lung cells (Fig. 4).

Example 4: Silencing of targeted genes in human cells by siRNA delivered by Sb-derived EVs. through functional delivery of siRNA externally loaded to Sb-derived EVs to human cells.

To evaluate the possibility for delivery of functional siRNA for gene silencing, Sur7 externally loaded EVs derived from wild-type Sb strain with siRNA targeting GFP using the lipofectamine 3000 agent, and then removed leftover lipofectamine by rinsing EVs mixture using centrifugation with 100 kDa cutoff filter. EVs loaded with siRNA-GFP were applied to H1299 cells expressing GFP reporter protein. The level of GFP fluorescence was measured for an evaluation of GFP expression.

To make sure that any possible silencing effect was not an artifact of lipofectamine-formed liposomes carrying siRNA, as a negative control we performed mock lipofectamine loading of siRNA to OptiMem media (Gibco, 11058021) without EVs and rinsed the mixture on a 100 kDA filter before application to the cells. The present inventors observed an average 35% decrease in GFP signal in human H1299 cells 72 hours after application of EVs loaded with siRNA compared

to cells treated with mock transfection solution in two independent experiments (Fig. 5). These results demonstrate that Sb-derived EVs can deliver functional siRNA and induce silencing of targeted genes in human cells.

Example 5: silencing of targeted viral gene by EVs derived from engineered Sb strains expressing specific shRNA.

The present inventors next sought to determine if EVs isolated from engineered Sb cells expressing siRNA are able to induce the silencing of a siRNA-targeted gene in human cells. For this purpose, we applied EVs isolated from both Sb-pol-I-siNsp1-siGFP and Sb-pol-II-siNsp1-siGFP as well as from wild type Sb to H1299-nsp1 human cells. 48 h after the application we extracted RNA from H1299 cells and used qRT-PCR to compare the level of nsp1 expression in cells treated with EVs from siRNA-expressing strains and cells treated with EVs derived from wt Sb. As shown in Figure 6, treating H1299-nsp1 cells with EVs from Sb with siRNA-Nsp1 expression driven by RNA pol II caused 37% decrease in the level of nsp1 expression compared to cells treated with wild-type EVs (Fig. 6a) , while the treatment with EVs isolated from Sb strain having RNA pol I driven siRNA expression induced only 15% of silencing of nsp1 gene (Fig. 6b)

Example 6: EVs-mediated silencing of targeted viral gene by co-cultivation shRNA-expressing Sb with human cells.

Growing yeast constantly release EVs into their environment. Therefore, co-cultivation of targeted cells with engineered Sb strains producing EVs loaded by siRNA might be another way of siRNA delivery. In order to evaluate this possibility, we assembled a growth chamber with two sections divided by 0.4 um cutoff membrane. H1299 cells expressing the nsp1-GFP gene fusion construct were placed on the bottom section of the chamber, and wild type Sb or Sb strains expressing siRNA-nsp1 and siRNA-gfp were grown in the top section. This configuration allows the exchange of liquid and passage of EVs but not yeast themselves between two sections of the chamber. After 4 days of co-cultivation, the present inventors extracted RNA from H1299 cells and performed qPCR with primers to nsp1 gene to measure the nsp1 expression level. As shown in Figure 7, the H1299 cells grown in the presence of both Sb strains expressing shRNA targeting nsp1 gene under control of pol I or pol II promoters the level of nsp1 expression was significantly lower (30% reduction for pol I-driven, and 52% reduction for pol II-driven siRNA expression) compared to the cells co-cultivated with wt Sb, though this effect was more prominent in cells co-cultivated with Sb-pol-II-siNsp1-siGFP strain.

Example 7: The level of Nsp1 gene expression in H1299 co-cultivated with wt Sb or Sb expressing siRNA-nsp1 under control of pol I or pol II promoters, further demonstrating Sb-derived EVs don't have cytotoxic effect on human cells.

No previous research was done to evaluate the safety of Sb-derived EVs in human cell lines. As such, the present inventors conducted *in vitro* experiments to evaluate potential cytotoxicity of Sb-derived EVs for human lung cell line H1299 and human liver cancer cell line Hep-2 (often used as in vitro hepatotoxicity model). The present inventors performed lactate dehydrogenase activity assay of H1299 and Hep G2 cells following application of Sb-derived EVs in concentration 10^9 EVs/ml. LDH is cytosolic enzyme which is released in the medium from dead cells due to damage of cell membranes, therefore higher amount of LDH in the medium indicate the cytotoxic effect. No significant toxicity was detected in both cell lines at a period 6-20h after EVs application (Fig. 8).

Example 8: Materials and Methods.

Sb strains design and construction: To create Sb strains expressing shRNA targeting nsp1 and eGFP genes, wild type Sb strain was transformed by dsDNA segments including siRNA expressing cassette and geneticin-resistance gene flanked on 5' and 3' ends by integration sequences homological to sequences from YPRCt3 locus on the *Sb* XVI chromosome. Transformation was performed by electroporation method following protocol described by Benatuil et al (Benatuil et al, 2010)

Description of integration constructs: All constructs were synthesized by Genscript.

Polymerase I – based expression cassette: To utilize the RNA polymerase I gene promoter responsible for synthesis of rRNA which is major component of cells RNA pool, we designed expression cassette based on rRNA operon of Sb. Since upstream of rDNA has multiple promoter elements positioned on different distance from the start of transcription, in our design we included 5' Non-transcribed Spacer (NTS) from rRNA operon with 191 bp of the Major promoter element following by minimal promoter element and first 25 bp of External translated spacer (Gallagher at al, 2019). The shRNA expression cassette contained tRNA-Ala, shRNA-nsp1, tRNA-Gly, shRNA-GFP and tRNA-Gly. 3' end of the expression cassette contained the terminator part of rRNA operon consisted from 210 bp of External Translated Spacer and 183 bp of non-translated spacer (described in Gallagher et al, 2019)

RNA Polymerase II – based expression cassette: Consisted of the TDH3 promoter followed by tRNA-Ala, shRNA-nsp1, tRNA-Gly, shRNA-GFP, Hepatitis Delta Virus ribozyme and CYC1 terminator

Sb EVs labeling by fluorescent reporter gene: We cloned Sur7-GFP fused genes construct into psf-TEF1-G418 yeast selection vector (Sigma-Aldrich, OGS542) under control of TEF1 promoter and CYC1 terminator. The Sur7 protein (a membrane protein present in membrane compartments occupied by Can1) was found to be present in EVs produced by *Saccharomyces cerevisiae strains (*Dawson et al, 2020). For the visualization under the microscope, Sur7 was fused with monomeric mUkG1-GFP protein from soft coral was codon-optimized for expression in Saccharomyces (Kaishima et al, 2016). The Sur7-mUKG fusion gene was synthesized by Genscript and cloned into psf-TEF1 vector using inFusion kit (Takara).

Engineering of H1299 cells expressing partial sequence of SARS-CoV-2 genome and eGFP reporter gene for gene silencing experiments: 5' UTR and first 396 bp of nsp1 gene from genome of Sars-CoV-2 virus were sub-cloned into mammalian expression vector pcDNA3.1 (genscript.com/gsfiles/vector-map/mammalian/pcDNA3.1-reduce.pdf?2084916751) in frame with the self-cleaving 2A peptide and eGFP. Gene synthesis was performed by Genscript. H1299 cells (ATCC CRL-5803™) were transformed with plasmid pcDNA3.1-nsp1-eGFP using the lipofectamine 3000 reagent (Thermofisher L3000001) and grown on RPMI-1640 complete media (10% FBS, 1% penicillin-streptomycine) with addition of Geneticin® for selection of transformants. The procedure yielded the H1299-nsp1-GFP cell line expressing both 5'UTR-nsp1 gene and eGFP. This line was used in our experiments as a reporter for siRNA efficiency in targeted gene silencing.

Evaluation of siRNA efficiency: The siRNA-GFP targeting eGFP gene sequence was previously described by Reshke et al (Reshke et al, 2020). For the design of siRNAs targeting nsp1 gene, we used Genscript target finder tool (genscript.com/tools/sirna-target-finder). All nsp1 siRNA sequences are listed in Table 2. For analysis of the efficiency of nsp1 siRNAs, we transfected H1299-nsp1 cells with each nsp1 siRNA construct using Lipofectamine 3000 reagent according to manufacturer protocol (thermofisher.com/us/en/home/brands/product-brand/lipofectamine/lipofectamine-3000.html). All nsp1 siRNA constructs were used at a 50nM final concentration. 24 h after transfection the level of nsp1 gene expression was quantidfied by qPCR.

QRT-PCR. Relative nsp1 or GFP gene expression was measured by quantitative real-time PCR (qRT-PCR). Total RNA was isolated using RNA plus Nucleospin kit (TakaraBio). Real-time PCR amplification was performed using a Mx3000P QPCR system (Agilent technologies). A Luna® Universal One-Step RT-qPCR Kit (NEB) was used to perform one step RT-PCR. Oligonucleotides concentration and cycling conditions were according to manufacturer recommendations. Gene specific primers are listed in Table 2. Approximately 25 ng of total RNA was used in each reaction. Relative expression levels of the specific transcripts were calculated using the Geneticin resistance gene mRNA as the internal reference for normalization.

Cell culture. H1299 cells (ATCC CRL-5803™) and Hep G2 cells (Sigma, 85011430) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA; #26140) and 100 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA; #15140) and grown at 37 °C and 5% CO_2

Cells visualization and GFP fluorescence measurements: GFP fluorescence images of H1299-nsp1 cells were taken on an inverted fluorescent microscope (VWR 89404-464) using a GFP filter set and x20 objective. Images were processed using ImageJ (1.47v) software. For live cell imaging of EVs uptake, 100 ul of Sur7-GFP labeled EVs were added to H1299 cells plated on glass-bottomed dishes and images were taken on Zeiss confocal microscope using a 63x oil immersion objective.

EVs extraction. Overnight cultures of *Saccharomyces boulardii* were diluted in 100 times with YPD medium. Cultures were then incubated for 16-24 h at 30 °C with shaking (200 rpm). For EV isolation, cells and debris were removed first by centrifugation at 3500 × g for 35 min and then 15,000 × g for 35 min. Supernatants were collected and ultracentrifuged at 100,000 × g for 70 min at 4 °C (rotor SW32Ti, Optima EX-100 Ultracentrifuge, Beckman) to isolate EVs. Pellets were collected and washed once with 1 × phosphate-buffered saline (PBS). The resulting EVs pellets were resuspended in 1 ml of PBS filtered through a 0.22 μm filter and stored at −80 °C

Application of EVs to H1299-nsp1 cells for gene silencing: EVs isolated from siRNA-Nsp1 expressing or from wild-type *Sb* strains were added to a sub-confluent monolayer of H1299 cells in 3 subsequent applications at 12 hour intervals. 10^{11} EVs/mL were used for each application. 48 h after first application of the EVs RNA was extracted from EV- treated cells and the level of Nsp1 expression was measured by qRT-PCR as described above.

Co-cultivation of H1299-nsp1 cells with Sb strains: H1299-nsp1 cells were plated in 6-well plates in complete RPMI-1640 medium. Permeable cell culture inserts with membrane pore size of 0.4 µm (Falcon™ 353090, 08-771) were placed on the top of each well to allow liquid exchange. The inserts were permeable for medium and EVs but not for yeast cells. 100 ul overnight culture of wild-type Sb or siRNA-nsp1 expressing Sb strains were added to the top section of the inserts. Half of the medium was carefully removed and replaced with fresh medium every 2 days during the experiment. The level of nsp1 expression was measured in H1299 cells co-cultivated with wild-type siRNA expressing strains 4 days after co-cultivation.

Cytotoxicity assay: 10^9 EVs/ml were added to H1299 or Hep G2 cells and the release of lactate dehydrogenase (LDH) was measured in cell medium 3, 6 and 20 h after EVs application using the CyQUANT LDH Cytotoxicity Assay kit (invitrogen, C20300) following the manufacturer protocol.

miRNA dot blots. The purified miRNA samples were diluted in nuclease-free water to final concentration 0.2, 1.0 and 1.5 µg/µl, and 10-15 µl aliquots were then spotted to a positively charged nylon transfer membraned (Whatman Nytran SuPerCharge, GE Helthcare Life Sciences, Germany), resulting in total amount of 2.0, 3.0, 10.0, and 15.0 µg miRNA per dot, respectively. Chemically synthesized RNA oligonucleotides siGFP (5′--3′), and siNSP1-2 (5′--3′) were obtained from Integrated DNA Technologies, Inc. (USA, San Diego). RNA probes were labeled to high specific activity using a DIG Oligonucleotide 3'-End labeling kit, 2 Generation (Roshe Diagnostics GmbH, Germany). After UV cross-linking to UVP HL-2000 using a HybriLinker Hybridization Oven UV Crosslinker (USA, Upland), the membranes were prehybridized at 42°C for 30 min in ULTRAhybTM-Oligo Hybridization Buffer (Thermo Fisher Scientific Baltics UAB, Lithuania). After prehybridization, the purified labeled probe was added to the prehybridization buffer, and the membrane was hybridized at 42°C for 14 – 18 h. After hybridization, the membranes were washed with twice 2x SSC-0.2% SDS (20 min at 42°C), 2x SSC-0.2% SDS (20 min at 55°C, twice), 1x SSC-0.1% SDS (20 min at 55°C, twice). The membranes were then blocked and washed with the DIG Wash and Block Buffer Set (Roshe Diagnostics GmbH, Germany). After washing the membranes chemiluminescence detection was performed using Anti-Digoxigenin-AP Fab fragments (Roshe Diagnostics GmbH, Germany), CDP-Star, ready-to-use (Roshe Diagnostics GmbH, Germany) and the ChemiDoc XRS+ Imaging System (Bio-Rad). The signal intensities of

the dots were quantified by densitometry using the Volume Tools of the Image Lab software, version 6.0.1 build 34 (Bio-Rad).

Applicant incorporates by reference Examples 1-9 of priority reference U.S. Provisional Application Serial No. 63/184,011, filed May 4, 2021.

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TABLES

Table 1. Exemplary strains and cells lines.

Strains	Genotype	Origin
Saccharomyces boulardii (Sb)	WT	ATCC type strain MYA-796
Sb-pol-I-siNsp1-siGFP	pol-I-siNsp1-siGFP	This invention
Sb-pol-II-siNsp1-siGFP	pol-II-siNsp1-siGFP	This invention
Sb-Su7	Sur7-GFP in psf-TEF1 vector	This invention
Cell lines	**Genotype**	**Origin**
H1299	Derived from Non-small cell lung cancer	ATCC CRL-5803™
H1299-Nsp1	H1299 Expressing 5'UTR and partial Nsp1 gene of SARS-CoV-2 virus in fusion with eGFP under CMV promoter	This invention
Hep G2	Derived from human liver	Sigma, 85011430

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Table 2: Exemplary Oligonucleotides sequences.

Oligo	Sequence	Comments
shRNA-GFP	AUGAACUUCAGGGUCAGCUUGCGUUGACCCUGAAGUUCAUUC (SEQ ID NO. 17)	SiRNA targeting GFP
shRNA-Nsp1	GGCAUUCAGUACGGUCGUAUAUUGGAGAUACGACCGUACUGAAUGCCUU (SEQ ID NO. 18)	SiRNA targeting nsp1 SARS-CoV-2 gene
Nsp1 North	AGGCATTCAGTACGGTCGTA (SEQ ID NO. 19)	Probe for detection in Northern blot analysis
qNsp1-5'	CGTACGTGGCTTTGGAGACT (SEQ ID NO. 20)	Primer used for qPCR
qNsp1-3'	ACCATGAGGTGCAGTTCGAG (SEQ ID NO. 21)	Primer used for qPCR
qGen-5'	TGCTCGACGTTGTCACTGAA (SEQ ID NO. 22)	Primer used for qPCR

qGen-3’	GATGTTTCGCTTGGTGGTCG(SEQ ID NO. 23)	Primer used for qPCR

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CLAIMS

What is claimed is:

1. A pharmaceutical composition for the treatment of SARS-COV-2, comprising a quantity of *Saccharomyces*-generated extracellular vesicle (EVs) containing one or more heterologous RNAs directed to inhibit the expression of one or more SARS-CoV-2 genes, and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein the EV is generated from *Saccharomyces cerevisiae*, or *Saccharomyces boullardii*.

3. The pharmaceutical composition of any of claims 1-2, wherein the heterologous RNA is dsRNA, miRNA, shRNA or siRNA.

4. The pharmaceutical composition of any of claims 1-3, wherein the heterologous RNA comprises an RNA according to SEQ ID NO. 18.

5. The pharmaceutical composition of any of claims 1-3, wherein the heterologous RNA comprises an RNA having at least 98% homology with SEQ ID NO. 18.

6. The pharmaceutical composition any of claims 1-5, further comprising at least one heterologous membrane surface ligand that specifically binds to a target surface molecule displayed on a target cell.

7. The pharmaceutical composition of claim 6, wherein the at least one heterologous membrane surface ligand is linked to an anchor protein embedded in the lipid bilayer of the *Saccharomyces*-generated EV.

8. The pharmaceutical composition of claim 7, wherein the anchor protein is selected from the group consisting of ammonia transport outward protein 2, plasma membrane protein up- regulated during nitrogen stress protein 1, glucan 1,3-beta-glucosidase 1/11, glucan 1,3-beta- glucosidase, 1,3-beta-glucanosyltransferase, 1,3-beta-glucanosyltransferase, iron transport multicopper

oxidase, probable glycosidase protein and non-classical export protein 1, Sur7 protein, or a combination of the same.

9. The pharmaceutical composition of claim 7-8, wherein the linked is a peptide linker

10. The pharmaceutical composition of claim 6, wherein the target surface molecule is a mammalian cell surface protein receptor.

11. The pharmaceutical composition of claim 10, wherein the target surface molecule is a mammalian cell surface protein selected from the group consisting of an enzyme, an antigen expressed on an immune cell, an antigen expressed on an immune effector cell, a peptide, and an antigen.

12. The pharmaceutical composition of claim 10, wherein the target surface molecule is human angiotensin converting enzyme 2 (ACE2), neuropilin 1 (NRPl) or transmembrane protease, serine 2 TMPRSS2.

13. The pharmaceutical composition of any of claims 6-7, wherein the at least one heterologous membrane surface ligand comprises angiotensin II, vascular endothelial growth factor A (VEGF-A) or a fragment thereof.

14. The pharmaceutical composition of any of claims 6-13, further comprising a detectable label or a purification label.

15. A method treating a subject having or suspected of having a SARS-CoV-2 virus infection, the method comprising administering the pharmaceutical composition of any of claims 1-14 to a subject in need thereof.

16. The kit containing the pharmaceutical composition of any of claims 1-15, a container, and optionally instructions for use.

17. The kit of claim 16, wherein the a container contains a metered dose of the pharmaceutical composition.

18. A method of manufacturing a pharmaceutical composition for the treatment of SARS-COV-2, comprising

- transforming a *Saccharomyces* host cell to express a heterologous nucleotide, operably linked to a promoter, encoding one or more heterologous RNAs directed to inhibit the expression of one or more SARS-CoV-2 genes; and
- culturing the host cell under conditions that promote extracellular vesicle generation; and
- isolating the *Saccharomyces*-generated extracellular vesicle (EVs) from culture, wherein said isolated EVs contain the one or more heterologous RNAs;
- combining the EVs with a pharmaceutically acceptable carrier.

19. The method of claim 18, wherein the EV is generated from *Saccharomyces cerevisiae*, or *Saccharomyces boullardii*.

20. The method any of claims 18-19, wherein the heterologous RNA is dsRNA, miRNA, shRNA or siRNA.

21. The method any of claims 18-20, wherein the heterologous RNA comprises an RNA according to SEQ ID NO. 18.

22. The method any of claims 18-20, wherein the heterologous RNA comprises an RNA having at least 98% homology with SEQ ID NO. 18.

23. The method any of claims 18-22, further comprising presenting on the surface of said EVs at least one heterologous membrane surface ligand that specifically binds to a target surface molecule displayed on a target cell.

24. The method of claim 23, wherein the at least one heterologous membrane surface ligand is linked to an anchor protein embedded in the lipid bilayer of the *Saccharomyces*- generated EV.

25. The method of claim 24, wherein the anchor protein is selected from the group consisting of ammonia transport outward protein 2, plasma membrane protein up- regulated during nitrogen stress protein 1, glucan 1,3-beta-glucosidase 1/11, glucan 1,3-beta- glucosidase, 1,3-beta-glucanosyltransferase, 1,3-beta-glucanosyltransferase, iron transport multicopper oxidase, probable glycosidase protein and non-classical export protein 1, Sur7 protein or a combination of the same.

26. The method of claim 14, wherein the linked is a peptide linker

27. The method of claim 23, wherein the target surface molecule is a mammalian cell surface protein.

28. The method of claim 27, wherein the target surface molecule is a mammalian cell surface protein selected from the group consisting of an enzyme, an antigen expressed on an immune cell, an antigen expressed on an immune effector cell, a peptide, and an antigen.

29. The pharmaceutical composition of claim 27, wherein the target surface molecule is human angiotensin converting enzyme 2 (ACE2), neuropilin 1 (NRPl) or transmembrane protease, serine 2 TMPRSS2.

30. The method of any of claims 23-24, wherein the at least one heterologous membrane surface ligand comprises angiotensin II, vascular endothelial growth factor A (VEGF-A) or a fragment thereof.

31. A method treating a subject having or suspected of having a SARS-CoV-2 virus infection, the method comprising administering a pharmaceutical composition manufactured by the method of any of claims 18-30 to a subject in need thereof.

32. A method of producing *Saccharomyces*-generated extracellular vesicles (SGEVs), the method comprising:

- transforming a *Saccharomyces* host cell to express a heterologous nucleotide, operably linked to a promoter, encoding one or more heterologous RNAs directed to inhibit the expression of one or more SARS-CoV-2 genes; and
- culturing the host cell under conditions that promote extracellular vesicle generation and isolating the EVs from culture; wherein said isolated EVs contain the one or more heterologous RNAs.

33. The method of claim 32, wherein the EV is generated from *Saccharomyces cerevisiae*, or *Saccharomyces boullardii*.

34. The pharmaceutical composition of any of claims 32-33, wherein the heterologous RNA is dsRNA, miRNA, shRNA or siRNA.

35. The method any of claims 32-34, wherein the heterologous RNA comprises an RNA according to SEQ ID NO. 18.

36. The method any of claims 32-34, wherein the heterologous RNA comprises an RNA having at least 98% homology with SEQ ID NO. 18.

37. A method treating a subject having or suspected of having a virus infection, the method comprising administering the SGEV of any of claims 32-36 to a subject infected with or suspected of being infected with the virus, wherein the heterologous RNA within the SGEVs comprise a nucleotide sequence that targets at least one of the viral genes for gene silencing.

38. A pharmaceutical composition comprising: a quantity of *Saccharomyces*-generated extracellular vesicle (EVs) containing one or more heterologous RNAs directed to inhibit the expression of one or more pathogen genes, or one or more endogenous genes or a target cell and a pharmaceutically acceptable carrier.

39. The pharmaceutical composition of claim 38, wherein the EV is generated from *Saccharomyces cerevisiae*, or *Saccharomyces boullardii*.

40. The pharmaceutical composition of claim 38, wherein the pathogen is a viral pathogen.

41. The pharmaceutical composition of claim 40, wherein viral pathogen is SARS-CoV-2 virus.

42. The pharmaceutical composition of any of claims 38-41, wherein the heterologous RNA is dsRNA, miRNA, shRNA or siRNA.

43. The pharmaceutical composition of claim 41, wherein the heterologous RNA comprises an RNA according to SEQ ID NO. 18.

44. The pharmaceutical composition of claim 41, wherein the heterologous RNA comprises an RNA having at least 98% homology with SEQ ID NO. 18.

45. The kit containing the pharmaceutical composition of any of claims 38-44, a container, and optionally instructions for use.

46. The kit of claim 45, wherein the a container contains a metered dose of the pharmaceutical composition.

47. A *Saccharomyces*-generated extracellular vesicle (EV) comprising a heterologous RNA molecule and optionally at least one heterologous membrane surface ligand that specifically binds to a target surface molecule displayed on a target cell.

48. The extracellular vesicle of claim 47, wherein the EV is generated from *Saccharomyces cerevisiae*, or *Saccharomyces boullardii*.

49. The extracellular vesicle of any of claims 47-48, wherein the heterologous RNA is dsRNA, miRNA, shRNA or siRNA.

50. The extracellular vesicle of any of claims 47-49, wherein the at least one heterologous membrane surface ligand is linked to an anchor protein embedded in the lipid bilayer of the *Saccharomyces*-generated EV.

51. The extracellular vesicle of any of claims 47-50, wherein the anchor protein is selected from the group consisting of ammonia transport outward protein 2, plasma membrane protein up-regulated during nitrogen stress protein 1, glucan 1,3-beta-glucosidase 1/11, glucan 1,3-beta-glucosidase, 1,3-beta-glucanosyltransferase, 1,3-beta-glucanosyltransferase, iron transport multicopper oxidase, probable glycosidase protein and non-classical export protein 1, Sur7 protein or a combination of the same.

52. The extracellular vesicle of claims 50 or 51, wherein the linked is a peptide linker.

53. The extracellular vesicle of any of claims 47-6, wherein the target surface molecule is a mammalian cell surface protein.

54. The extracellular vesicle of any of claims 47-53, wherein the target surface molecule is a mammalian cell surface protein selected from the group consisting of an enzyme, an antigen expressed on an immune cell, an antigen expressed on an immune effector cell, a peptide, and an antigen.

55. The extracellular vesicle of any of claims 47-54, wherein the target surface molecule is human angiotensin converting enzyme 2 (ACE2), neuropilin 1 (NRPl) or transmembrane protease, serine 2 TMPRSS2.

56. The extracellular vesicle of claim 55, wherein the at least one heterologous membrane surface ligand comprises angiotensin II, vascular endothelial growth factor A (VEGF-A) or a fragment thereof.

57. The extracellular vesicle of any of claims 47-56, further comprising a detectable label or a purification label.

58. A fusion peptide comprising a *Saccharomyces* extracellular vesicle anchor protein and a second peptide.

59. The fusion protein of claim 58, wherein the anchor protein and the second peptide are linked *via* a peptide linker.

60. The fusion protein of claims 58 or 59, wherein the second peptide is a peptide that binds to a mammalian cell surface protein.

61. A polynucleotide encoding the fusion protein of any of claims 58-60.

62. An expression vector comprising the polynucleotide of claim 61.

63. A Saccharomyces host cell comprising the expression vector of claim 62.

64. A method of producing *Saccharomyces*-generated extracellular vesicles (SGEVs) comprising a fusion protein, the method comprising culturing the host cell of claim 63 under conditions that promote extracellular vesicle generation and isolating the EVs from culture.

65. A method treating a subject having or suspected of having a virus infection, the method comprising administering the SGEV of any of claims 47-57 to a subject infected with or suspected of being infected with the virus, wherein the heterologous RNA within the SGEVs comprise a nucleotide sequence that targets at least one of the viral genes for gene silencing.

66. The method of claim 65, wherein the virus is SARS-CoV-2.

67. A method of reducing the expression of a target gene in a target cell, the method comprising administering the SGEVs of any of claims 47-57 to the target cell, wherein the heterologous RNA within the SGEVs comprise a nucleotide sequence that targets the target gene for gene silencing.

68. The extracellular vesicle of any of claims 47-57, wherein heterologous RNA comprises a heterologous RNA according to SEQ ID NO. 18.

69. The extracellular vesicle of any of claims 47-57, wherein the heterologous RNA comprises an RNA having at least 98% homology with SEQ ID NO. 18.

70. A *Saccharomyces*-generated extracellular vesicle (EVs) generated from *Saccharomyces cerevisiae*, or *Saccharomyces boullardii* containing a heterologous RNA according to SEQ ID NO. 18, and a heterologous Sur7 protein

71. The extracellular vesicle of claim 70, wherein the Sur7 protein is fused with a Green-Fluorescent Peptide (GFP).

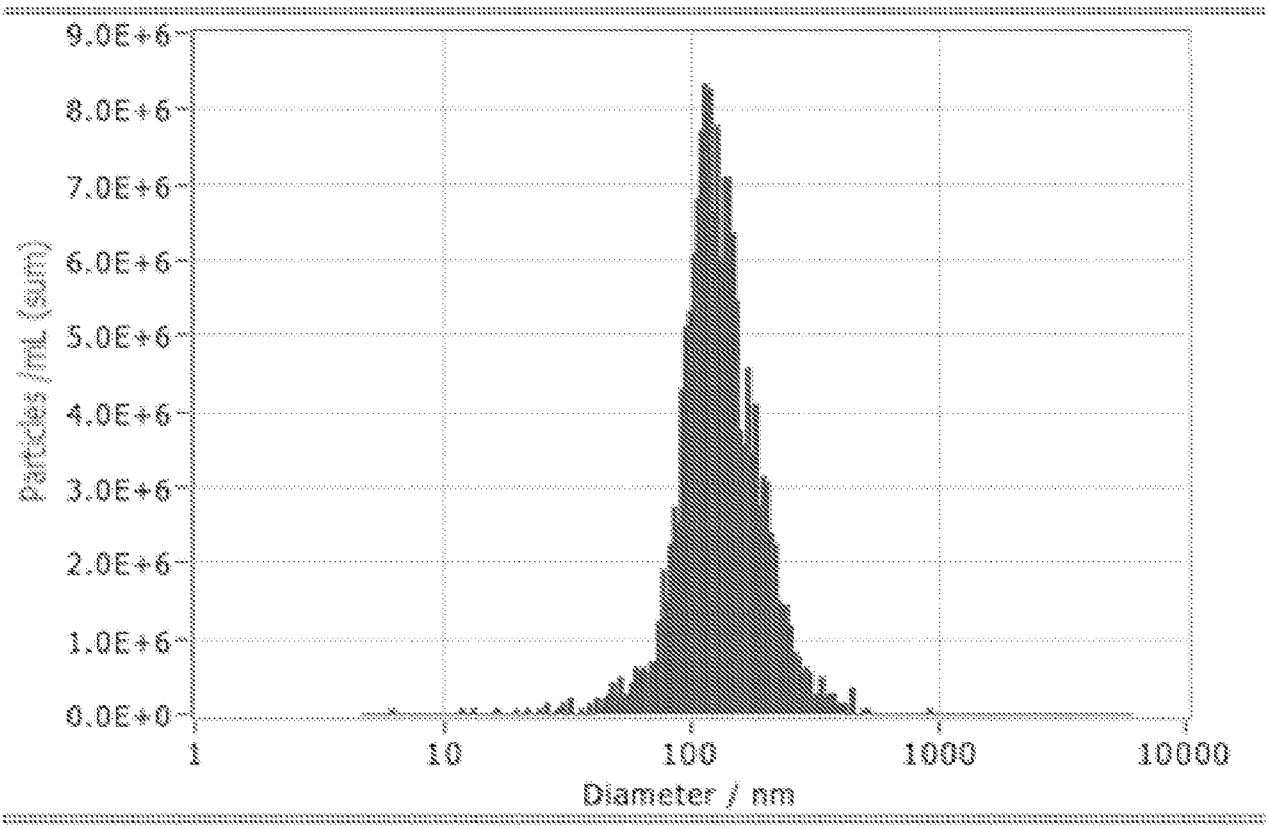
9.0E+6
8.0E+6
7.0E+6
6.0E+6
5.0E+6
4.0E+6
3.0E+6
2.0E+6
1.0E+6
0.0E+0
Particles /mL (sum)
1
10
100
1000
10000
Diameter / nm

FIG. 1

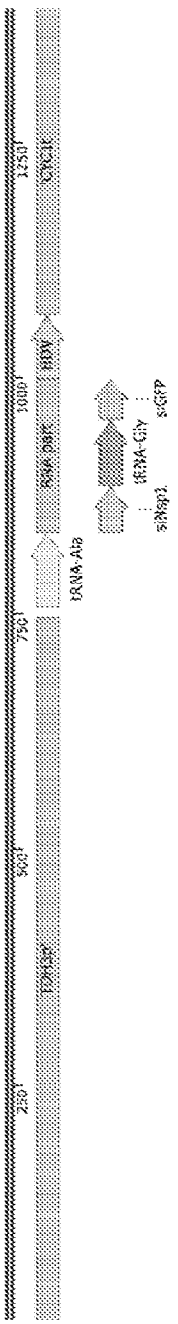
250
500
750
1000
1250
tRNA-Ala
siNsp1
tRNA-Gly
sfGFP
NTS-start
ETS-2
NTS-stop
nts-2
Start

FIG. 2A

FIG. 2B

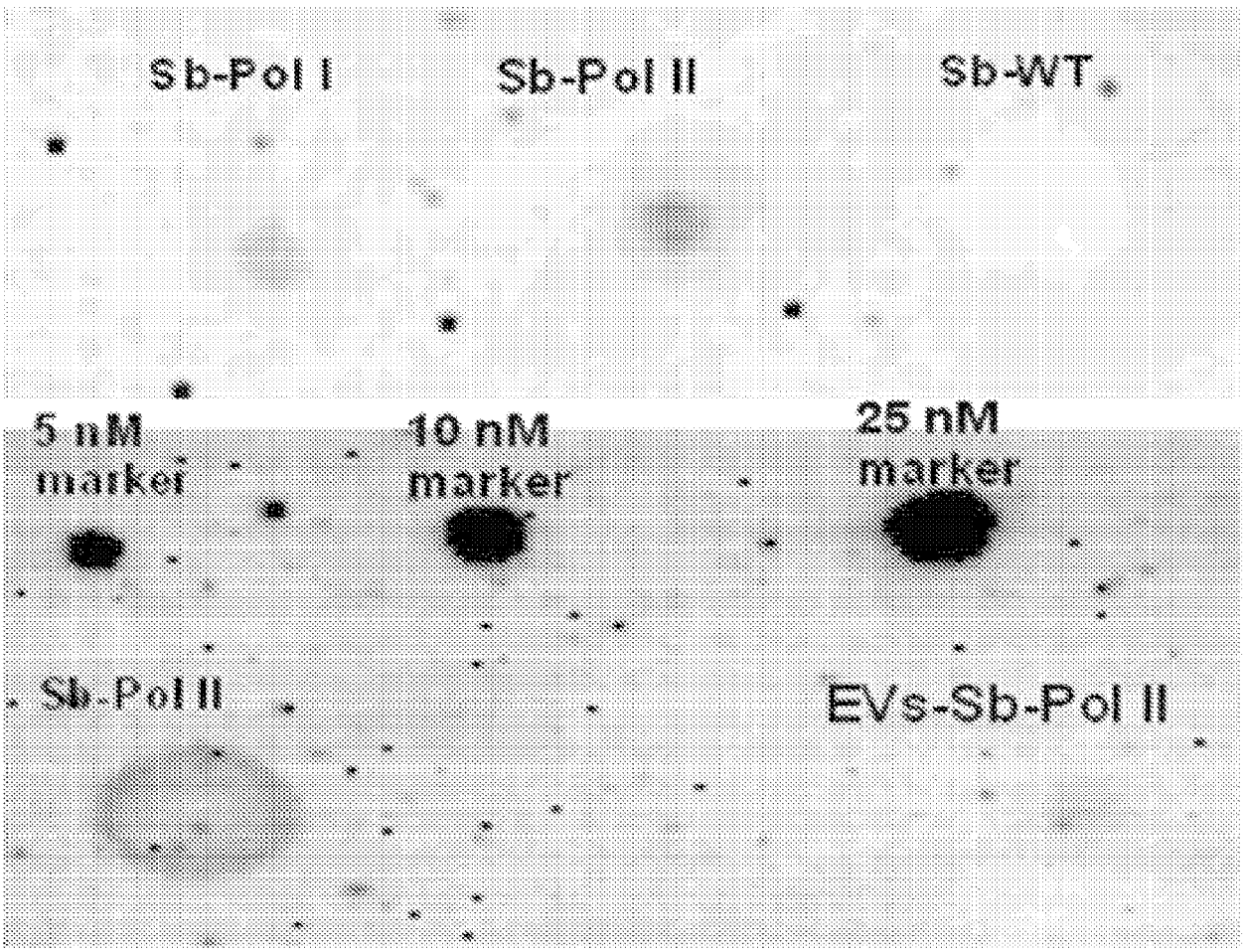
Sb-Pol I
Sb-Pol II
Sb-WT
5 nM marker
10 nM marker
25 nM marker
Sb-Pol II
EVs-Sb-Pol II

FIG. 3

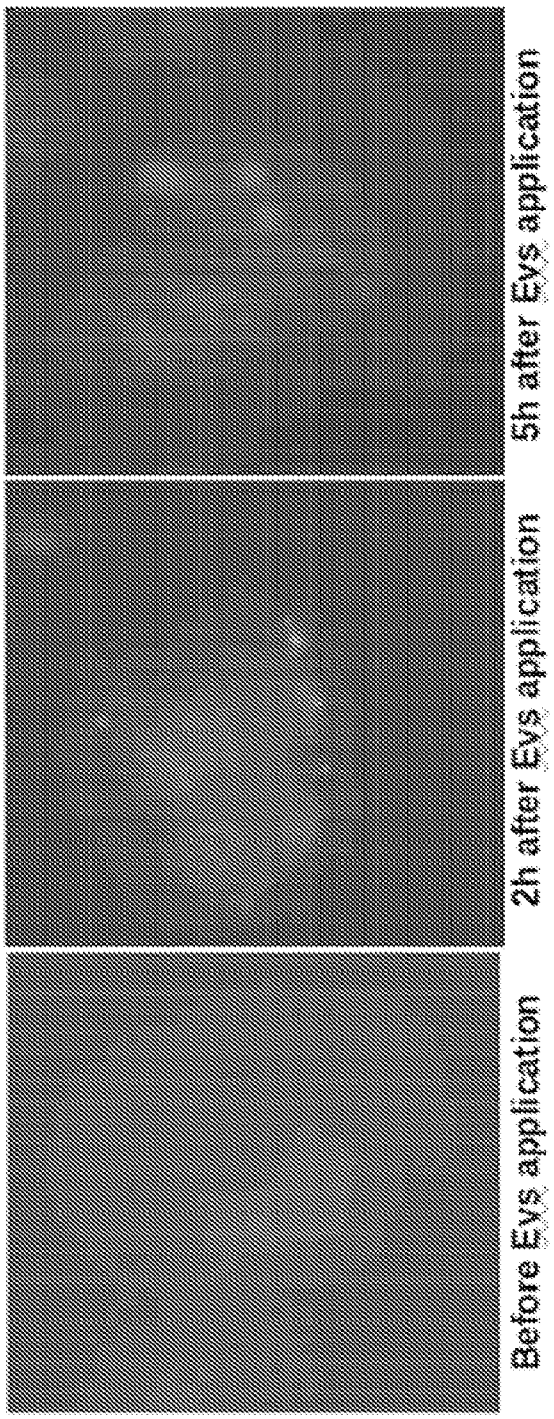
Before Evs application
2h after Evs application
5h after Evs application

FIG. 4

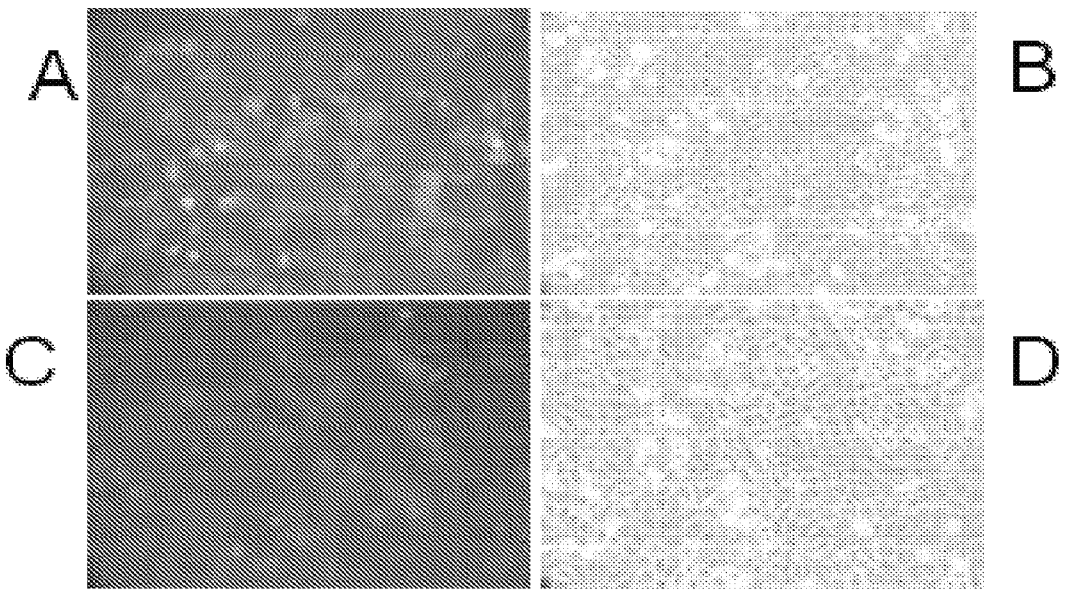

FIG. 5A-D

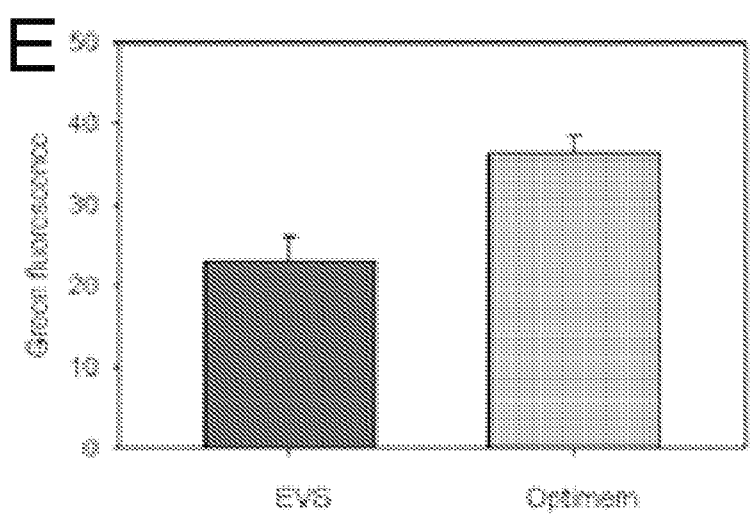

FIG. 5E

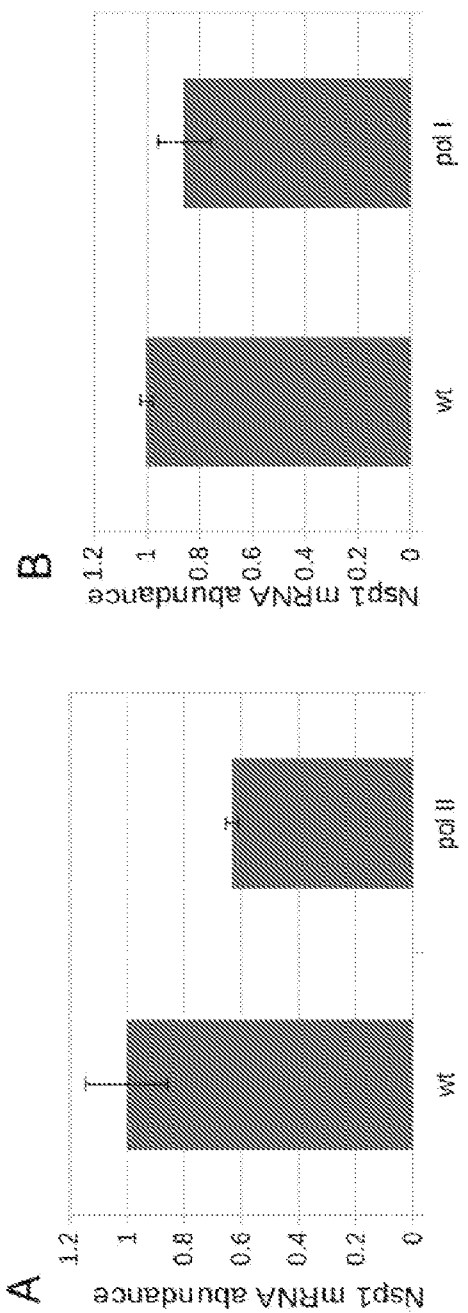
A
Nsp1 mRNA abundance
1.2
1
0.8
0.6
0.4
0.2
0
wt
ppi II
B
Nsp1 mRNA abundance
1.2
1
0.8
0.6
0.4
0.2
0
wt
ppi I

FIG. 6A-B

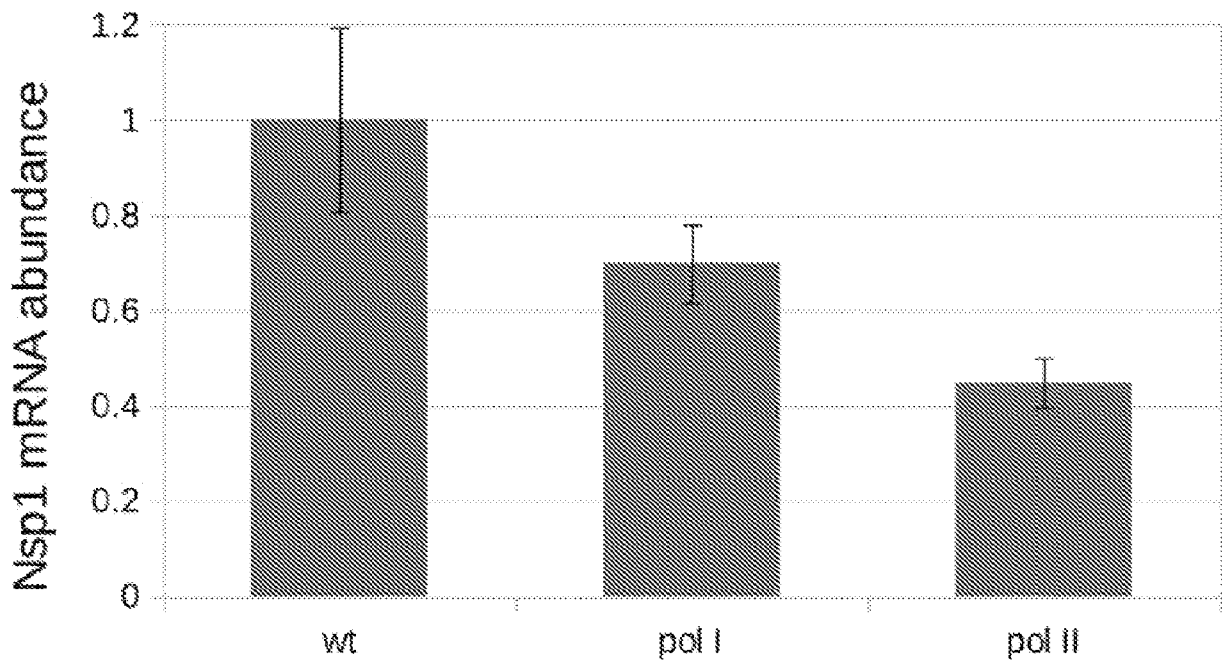

FIG. 7

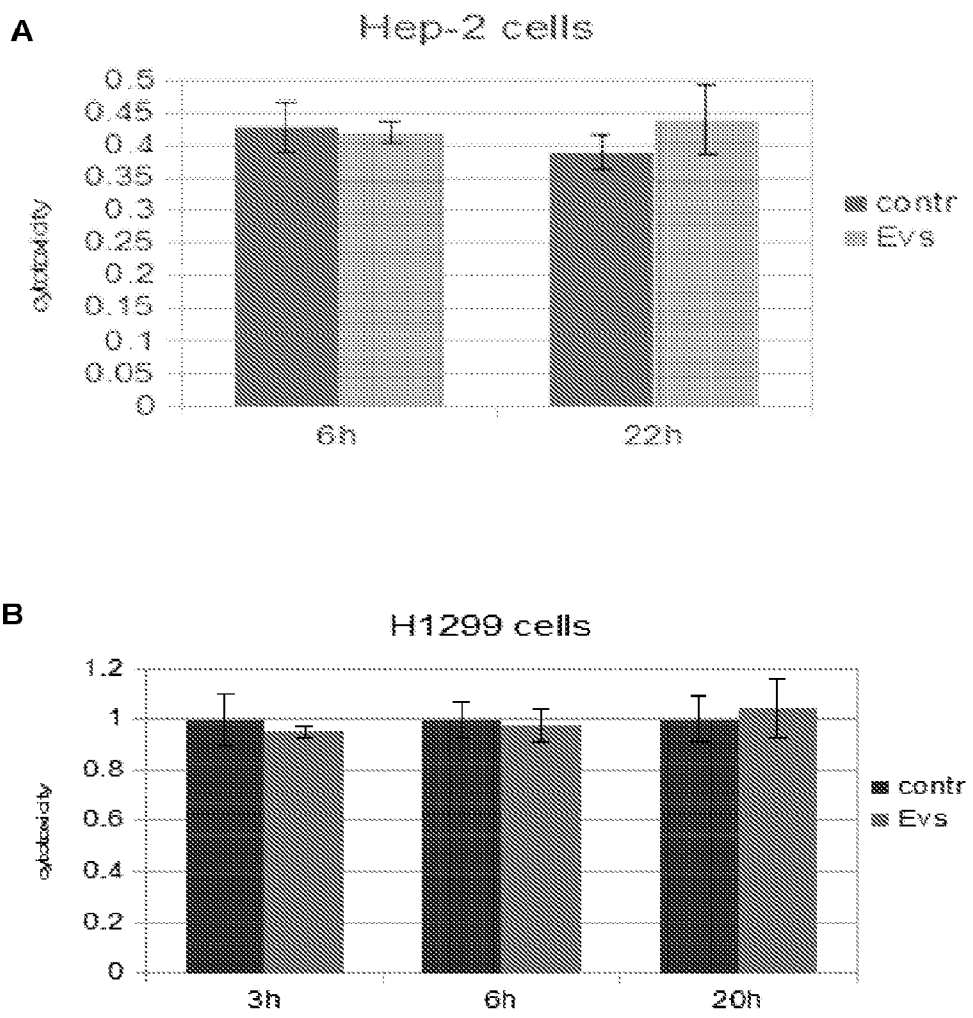
A
Hep-2 cells
cytotoxicity
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
6h
22h
contr
Evs
B
H1299 cells
cytotoxicity
1.2
1
0.8
0.6
0.4
0.2
0
3h
6h
20h
contr
Evs

FIG 8A-B

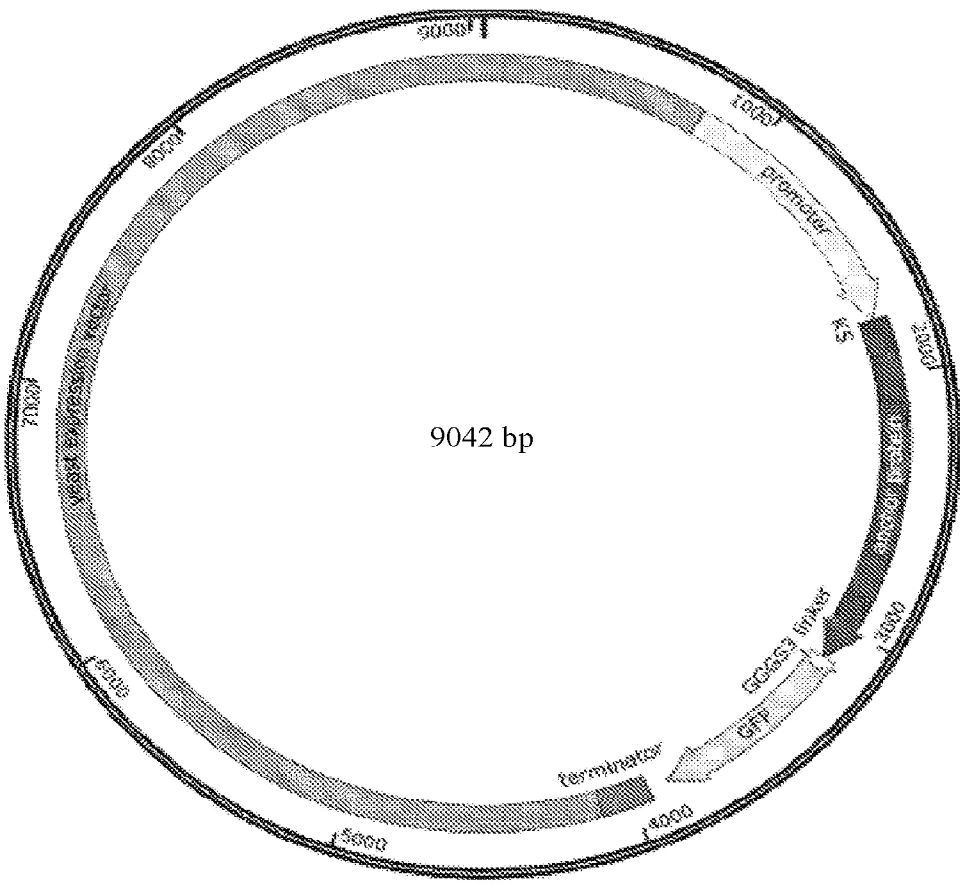
9042 bp
1000
promoter
KS
2000
GGGGS linker
3000
GFP
terminator
4000
5000
6000
7000
8000
9000

FIG. 9

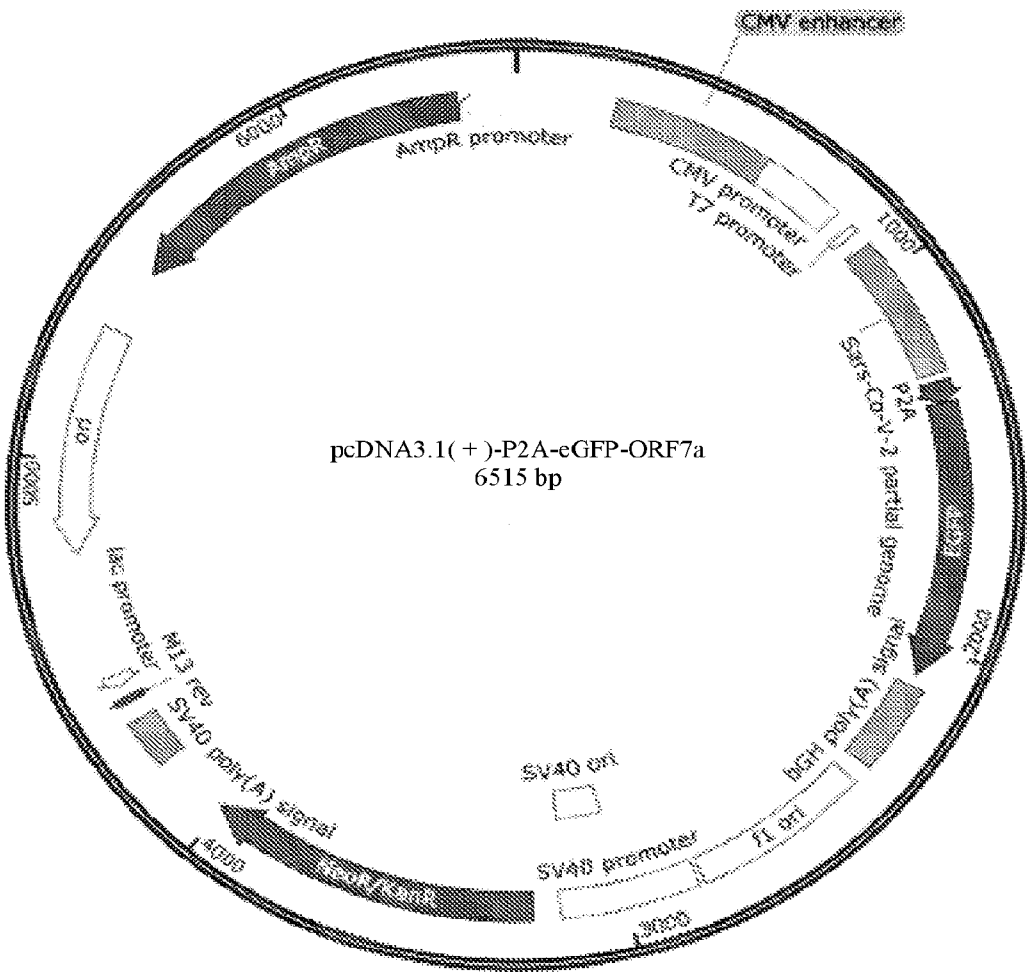
CMV enhancer
6000
AmpR promoter
CMV promoter
T7 promoter
1000
P2A
SARS-CoV-2 partial genome
2000
bGH poly(A) signal
SV40 ori
SV40 promoter
3000
f1 ori
4000
SV40 poly(A) signal
M13 rev
lac promoter
5000
ori
pcDNA3.1(+)-P2A-eGFP-ORF7a
6515 bp

FIG. 10

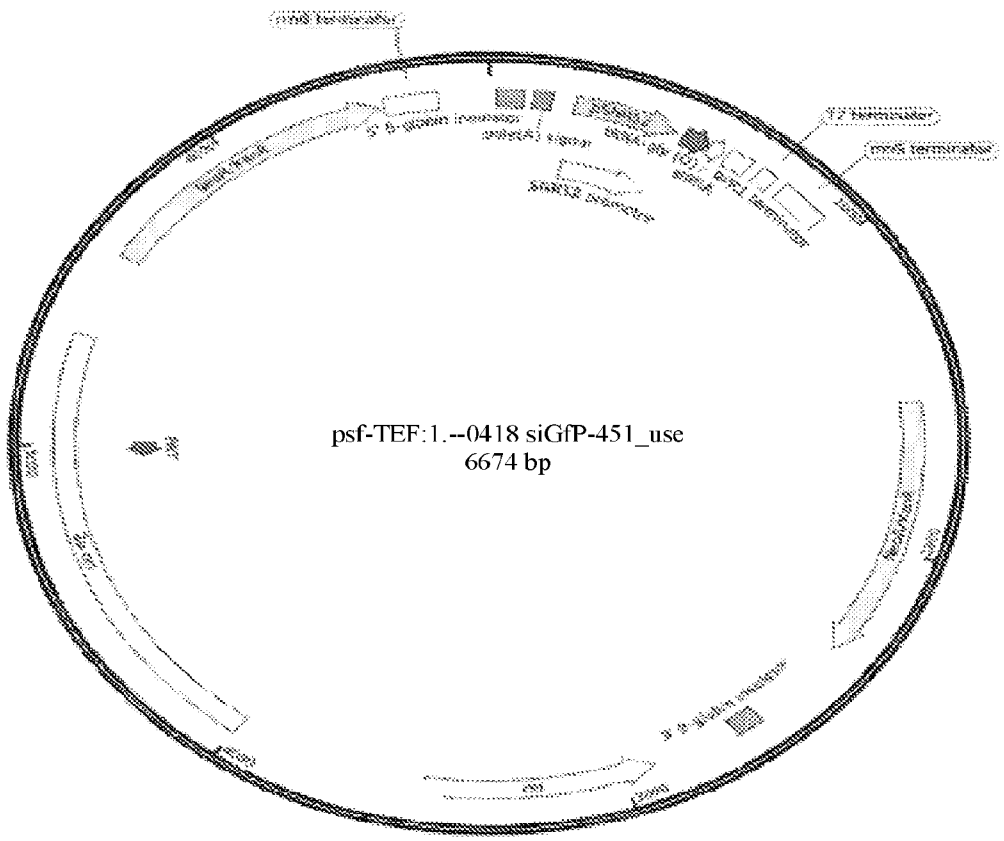

FIG. 11