TITLE OF THE INVENTION

Cell line for producing coronaviruses

FIELD OF THE INVENTION

The invention relates to medicine. In particular the invention relates to the production of coronaviruses such as human SARS-CoV.

BACKGROUND OF THE INVENTION

Severe acute respiratory distress syndrome (SARS) is a new respiratory disorder in humans that is caused by the SARS coronavirus (SARS-CoV). The disease emerged at the beginning of 2003 in China and various other portions of South East Asia and has since then rapidly spread throughout the world. Although the disease had disappeared by June 2003 its re-emergence cannot be excluded. Therefore, much effort is currently being put into the development of therapeutic and prophylactic treatments for SARS-CoV.

Although SARS-CoV is phylogenetically distinct from all previously known human and animal coronaviruses, significant progress has been made in understanding the molecular and cell biology of SARS-CoV. Next to the complete sequence of the SARS-CoV genome (see Marra *et al.* (2003); Rota *et al.* (2003)), Li *et al.* (2003) have recently identified a zinc metallopeptidase, angiotensin-converting enzyme 2 (ACE2 protein), as a functional receptor for SARS-CoV. This and other knowledge regarding the molecular and cell biology of SARS-CoV have offered avenues for developing anti-viral as well as vaccine strategies.

The development of a vaccine protecting against SARS-CoV has mainly focused on two strategies, *i.e.* the use of

inactivated whole SARS-CoV (Tang *et al.* (2004); Takasuka *et al.* (2004)) and the use of SARS-CoV proteins (Zhang *et al.* (2004); Yang *et al.* (2004); Kim *et al.* (2004)). Inactivated whole virus vaccines are usually prepared by producing large amounts of virus in cell tissue culture and then rendering the virus harmless without destroying its immunological properties. For optimal virus production in cell culture, it is pivotal that the respective virus is capable of infecting the cells and replicating in the cell. To date only a limited number of cells have been reported to be susceptible to SARS-CoV infection and to support SARS-CoV replication in culture (see Mossel *et al.* (2005)). The most frequently used cells in that respect are kidney cells derived from African Green Monkeys such as Vero or Vero E6 cells. A disadvantage associated with these cells is *inter alia* that they require the presence of serum and/or the adherence to a solid support for growth resulting in purification and safety issues as well as a laborious system for large-scale production. Furthermore, the cells are not human.

Recently, it was shown that cells refractory to SARS-CoV infection could be rendered permissive for SARS-CoV replication by expressing a functional receptor, *i.e.* the human ACE2 receptor. In WO 2005/032487 it was shown that human 293T cells transfected with the ACE2 protein supported SARS-CoV replication and were suitable for the production of SARS-CoV. However, the yields obtained with these cells were low making production methods using them economically unattractive. Taken together, there is still a need in the art for a method of producing SARS-CoV in a host cell system that improves on the existing cell culture systems, specifically on the yields obtained.

The present invention addresses this need by providing primary human retina cells (HER cells) expressing the ACE2 protein. These cells give unexpectedly high SARS-CoV yields. They have as a further advantage that they are extensively documented and better behave in the process of upscaling, suspension growth and growth factor independence compared to the cells in the art. Especially the fact that the cells can be brought in suspension in a highly reproducible manner is something that makes them very suitable for large scale production. Moreover, the cells of the present invention can advantageously be used for the replication of various isolates of human SARS-CoV and are further not only suitable for the production of SARS-CoV, but also for production of other human coronaviruses that make use of the ACE2 protein as a functional receptor.

DESCRIPTION OF THE FIGURES

Figure 1 shows binding of SARS-CoV S protein fragments (amino acids 318 to 510) to PER.C6® cells expressing ACE2 protein. Variant F comprises mutation N479S and variant H comprises mutations K344R, F360S, L472P, D480G, T487S compared to wild-type S318-510 fragment.

SUMMARY OF THE INVENTION

The invention provides cells suitable for producing coronaviruses. In a preferred embodiment the cells are HER cells expressing the human ACE2 protein. The invention further provides methods for producing coronaviruses, in particular SARS-CoV, making use of the cells.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect the present invention encompasses cells expressing the human ACE2 protein. As it has recently been found that ACE2 gene polymorphisms do not affect outcome of severe acute respiratory syndrome (see Chiu *et al.* (2004)), cells expressing a variant of the ACE2 protein are also part of the present invention. Said variant should of course still be capable of functioning as a receptor for SARS-CoV. The cells of the invention are E1-immortalized retina cells. They have been derived from retina cells by immortalization with adenovirus E1 sequences, *e.g.* E1A and E1B sequences. The E1A sequences may be under influence of their endogenous adenovirus E1A promoter, but may also be controlled by a heterologous promoter, such as for instance a PGK promoter. E1A protein has transforming activity, while E1B protein has anti-apoptotic activities. Furthermore, E1A may aid in increasing expression levels from the cells. Preferably, the cells according to the invention are derived from primary cells. They may be cells of any origin, and are preferably of human origin. In one preferred aspect, the cells are derived from primary human embryonic retina cells, in other words, the cells of the invention are derived from primary human embryonic retinoblast (HER cells) and comprise in their genome sequences that encode E1A and E1B of an adenovirus. Primary HER cells can be isolated from fetuses (see Byrd *et al.* (1982); Byrd *et al.* (1988)). Immortalization of the cells with adenoviral E1 sequences has for instance been described in US patent 5,994,128. Accordingly, an embryonic retina cell that has been immortalized with E1 sequences from an adenovirus can be obtained by that method. Other cells expressing E1A and E1B of an adenovirus can be prepared accordingly.

The most preferred HER cells for the methods and uses of the invention are cells as deposited at the ECACC on 29 February 1996 under number 96022940 or a derivative thereof. One E1-immortalized cell line useful for the invention, and having the characteristics of the cells deposited at the ECACC under number 96022940, is marketed under the trade mark PER.C6® by Crucell Holland B.V. PER.C6® cells for the purpose of the present application means cells from an upstream or downstream passage or a descendent of an upstream or downstream passage of cells as deposited under ECACC no. 96022940. PER.C6® behaves better in handling than for instance transformed human 293 cells that have also been immortalized by the E1 region from adenovirus. Furthermore, PER.C6® cells have been fully characterized and have been documented very extensively, while they behave significantly better in the process of upscaling, suspension growth and growth factor independence. Especially the fact that PER.C6® cells can be brought in suspension in a highly reproducible manner is something that makes them very suitable for large-scale production. Moreover, the fact that they can grow in defined serum-free medium, devoid of any human or animal serum proteins, and their growth is compatible with roller bottles, shaker flasks, spinner flasks and bioreactors, with doubling times of about 35 hrs makes them suitable as hosts for growing viruses.

The E1A and E1B sequences can be derived from any adenovirus serotype including adenovirus serotypes 2, 5, 12 and 35 (for other suitable adenovirus serotypes see for instance Table 1 in EP 1 054 064).

A cell according to the invention may comprise a polynucleotide encoding the human ACE2 protein stably

integrated into the genomic material or as part of an autonomously replicating vector, *i.e.* the human ACE2 protein may be transiently expressed, but for long-term, high-yield expression of the human ACE2 protein stable expression is preferred. In other words, a cell according to the invention is engineered to express the human ACE2 protein. For example, the cells of the invention may be transformed using expression vectors that may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells that successfully express the ACE2 protein. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cells of the invention. A cell culture comprising a multitude of cells according to the invention is likewise part of the present invention and may be used in the methods below.

In another aspect, the invention provides a method of producing a coronavirus, *e.g.* a human SARS-CoV, by infecting a cell according to the invention with a coronavirus and harvesting the coronavirus from the medium or the cell. In an embodiment the production method comprises the steps of a) providing a human cell as described above, *e.g.* a human cell that is derived from a primary human embryonic retinoblast, said cell comprising in its genome sequences that encode E1A and E1B of an adenovirus, with a nucleic acid molecule encoding a human ACE2 protein,

b) culturing the cell under conditions suitable for expression of the human ACE2 protein, c) infecting the cell with a coronavirus, and d) harvesting the coronavirus from the medium or the cell. The cells are cultured for optimal expression of the ACE2 protein. This may be achieved in conventional media of the cells. If necessary, the media may be modified *e.g.* for appropriate selection, amplification or induction of transcription. The culture conditions for the cells such as temperature, pH, nutrients etc. are well known to those ordinary skilled in the art. The engineered cells are cultured under conditions conducive to the production of the coronavirus. Harvesting may start as soon as CPE is observed. The produced coronavirus can be recovered/harvested from the cell free extract, but also from the culture medium. Methods to recover viruses from cell free extracts or culture medium are well known to the man skilled in the art and may include centrifugation or chromatographic steps. Preferably, the human cell used in the method is PER.C6® as deposited under ECACC no. 96022940 or a derivative thereof. In a preferred embodiment the human cell is capable of growing in suspension and/or can be cultured in the absence of serum.

In a preferred embodiment the coronavirus is selected from the group consisting of coronaviruses using the ACE2 protein as a receptor for infectious entry. Such coronaviruses include, but are not limited to, human coronaviruses including human SARS-CoV isolates and human CoV-NL63 (see Hofmann *et al.* (2005)). In an embodiment the engineered cells may be suitable for producing all human SARS-CoV isolates (for a list of known human SARS-CoV isolates see Table 1).

The human ACE2 protein can be transiently expressed but is preferably stably expressed. The nucleic acid molecule encoding the human ACE2 protein can be provided to the cell by a suitable nucleic acid construct, *e.g.* a vector. Vectors can be derived from plasmids such as inter alia F, R1, RP1, Col, pBR322, TOL, Ti, etc; cosmids; phages such as lambda, lambdoid, M13, Mu, P1, P22, Qβ, T-even, T-odd, T2, T4, T7, etc; plant viruses; or animal viruses. The choice of the vector is dependent on the recombinant procedures followed and the cells used. Introduction of vectors in host cells can be effected by *inter alia* calcium phosphate transfection, virus infection, DEAE-dextran mediated transfection, lipofectamin transfection or electroporation. Vectors may be autonomously replicating or may replicate together with the chromosome into which they have been integrated. Preferably, the vectors contain one or more selection markers. The choice of the markers may depend on the host cells of choice, although this is not critical to the invention as is well known to persons skilled in the art. They include, but are not limited to, kanamycin, neomycin, puromycin, hygromycin, zeocin, thymidine kinase gene from Herpes simplex virus (HSV-TK), dihydrofolate reductase gene from mouse (dhfr). If necessary, vectors may comprising a nucleic acid molecule encoding the ACE2 protein operably linked to one or more nucleic acid molecules encoding proteins or peptides that can be used for isolation purposes. These proteins or peptides include, but are not limited to, glutathione-S-transferase, maltose binding protein, metal-binding polyhistidine, green fluorescent protein, luciferase and beta-galactosidase. The nucleic acid construct may comprise an expression-regulating nucleic acid sequence. This term as used herein refers to polynucleotide

sequences necessary for and/or affecting the expression of an operably linked coding sequence in a particular host organism. The expression-regulating nucleic acid sequences, such as *inter alia* appropriate transcription initiation, termination, promoter, enhancer sequences; repressor or activator sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*e.g.* ribosome binding sites); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion, can be any nucleic acid sequence showing activity in the host organism of choice and can be derived from genes encoding proteins, which are either homologous or heterologous to the host organism. The identification and employment of expression-regulating sequences is routine to the person skilled in the art. Expression and/or transfection vectors frequently used include plasmid vectors and retroviruses. Plasmid vectors are preferred in the present invention as retroviruses have the disadvantage that they infect and integrate only dividing cells. Other problems include cumbersome preparation and relatively low titer, size constraints on inserted genes, difficulties in controlling or ensuring expression, and the potential for genetic damage due to random integration in the host genome. Safety issues that arise from the use of retroviruses in the laboratory involving potential toxicities, particularly from viruses that can infect human cells, are a further disadvantage of the use of retroviruses.

In another aspect the invention provides a method further comprising the step of inactivating or attenuating the harvested coronavirus. The inactivated or attenuated

coronavirus may be purified before, during or after the inactivation or attenuation step. Purification may be performed by means of purification methods suitable for viruses such as for instance centrifugation through a glycerol cushion and other methods well known to a person of ordinary skill in the art. Inactivation may be performed by methods well known to the skilled artisan such as gamma- or UV irradiation, heat treatment or treatment with chemicals such as formaldehyde, acetone, alcohol and alkylating agents like ethylene oxide, ethyleneimine, acetylehtyleneimine and B-propiolactone. Following the inactivation step the viruses may be tested for the absence of infectivity in cell culture. Methods to test if a virus is still infective or partly or completely inactivated are also well known to the person skilled in the art. Once absence of infectivity is established, the thus obtained inactivated virus preparation can be used for further purposes *e.g.* vaccine preparation.

Attenuation reduces the virulence of the virus so that, although it is still alive, it can no longer cause disease. The most common method of attenuation involves adapting organisms to growth in unusual conditions so that they lose their adaptation to their usual host. The most commonly used method of virus attenuation is prolonged tissue culture growth. Prolonged tissue culture growth involves infecting tissue culture plates with the virus for many generations. Due to the virus not having to be virulent in tissue culture there is no selection for virulence and the virus loses its ability to cause disease. The tissue culture that is used for production of attenuated vaccines is preferably from the same species that will be vaccinated with the attenuated vaccine in order to decrease the chance

of immune reaction to the tissue. In that respect human cells are preferred as tissue culture system.

The inactivated or attenuated coronaviruses may be used in vaccines. Vaccines may be formulated by means known in the art. Usually this may involve the addition of an adjuvant and/or a suitable carrier.

In an embodiment the virus titer of the coronavirus harvested is at least 4.00, 4.25, 4.50, 4.75, 5.00, 5.25, 5.50, 5.75, 6.00, 6.25, 6.50, 6.75, 7.00, 7.25, 7.50, and preferably at least 7.75 log10 $TCID_{50}$/ml after 24 hours post infection.

Furthermore, the present invention provides the use of a human cell according to the invention for the production of a coronavirus, preferably a human coronavirus such as human SARS-CoV or HCoV-NL63. The human cell according to the invention can also be used for the screening of antiviral agents against a coronavirus. The antiviral agents can be molecules or compounds that affect the binding of the virus to the receptor or affect the receptor function of the ACE2 protein in any other way. They can for instance be obtained by measuring the binding of a candidate molecule to the cells or membranes of the cells bearing the ACE2 protein and may include chemical compounds, peptides, polypeptides, antibodies or fragments thereof

In yet another aspect, the invention provides a method for identifying a molecule capable of inhibiting coronavirus infection and/or replication, the method comprising the steps of a) incubating a cell according to the invention with a coronavirus in the presence and absence of the candidate molecule, and b) determining whether the presence of the candidate molecule inhibits coronavirus infection and/or replication. A person skilled in the art is aware

that several steps of the methods of the invention including washing steps and incubation conditions need optimization. The virus and the candidate molecule may be mixed together before being contacted with the cells. The invention also provides a method for selecting an antiviral molecule that is capable of reducing infection of a cell by a coronavirus, wherein the method comprises the steps of a) contacting a cell according to the invention with a surface protein of a coronavirus in the presence or absence of a candidate antiviral molecule, said surface protein being involved in binding of the coronavirus to the human ACE2 protein expressed by the cell, such as a coronavirus S protein, b) measuring the binding interaction between the cell and the surface protein, and c) selecting a candidate antiviral molecule whereby the binding interaction in the presence of the candidate antiviral molecule is reduced or decreased when compared to the binding interaction in the absence of the candidate antiviral molecule. Membranes bearing the ACE2 protein may also be used in the above selection method. The cells and membranes may also be used in a screening assay used in screening compound libraries for compounds that specifically bind to the ACE2 protein. Since the ACE2 protein plays a role in entrance of certain coronaviruses, such human SARS-CoV and HcoV-NL63, into cells, such compounds may be used in treating or preventing coronavirus infections. Thus, the present invention provides for a method for screening compounds, which affect this function of the human ACE2 protein. These compounds may inhibit the function of the receptor. Compounds and molecules that may be identified with the screening/identification/selection method of the invention may be derived from a variety of sources including chemical compound libraries or mixtures of

(natural) compounds. The methods may involve measuring the binding of a candidate molecule or compound to the cells of the present invention or membranes thereof bearing the ACE2 protein. Binding may be measured directly or indirectly. Binding may be measured directly, for example, by means of label associated with the candidate molecule. Binding may also be measured indirectly. For example, indirectly measuring the binding of a candidate molecule may involve competition with a (labeled) competitor. The measuring of binding of a candidate molecule may, for example, also be determined in a cell-based assay, wherein it can be determined whether a candidate molecule is able to block the coronavirus from entering a cell. In that case it can be determined whether, in the presence of the candidate molecule or compound, cells can still be infected with the coronavirus. Alternatively, labeled human SARS-CoV S protein or a fragment responsible for binding to ACE2 protein can be contacted with cells of the invention in the presence or absence of candidate compounds. Next, it can be determined if the candidate compounds decrease the amount of S protein or fragment bound to the cells. Candidate molecules or compounds can be chemical compounds or can likewise be other molecules, *e.g.* antibodies or antibody fragments. The candidate molecule or compound may be capable of binding to the ACE2 protein or may be capable of binding to a protein of the coronavirus involved in infection and/or replication such as the S protein. Alternatively, the candidate molecule or compound may in any other way decrease or inhibit/abolish virus entry and/or replication. The candidate molecules or compounds that do inhibit coronavirus infection and/or replication can be used in methods of treating or preventing coronavirus infection.

EXAMPLES

To illustrate the invention, the following examples are provided. The examples are not intended to limit the scope of the invention in any way.

Example 1

Generation of a stably transfected human ACE2 PER.C6® cell line

To evaluate the ability to grow SARS-CoV and other coronaviruses on PER.C6® cells that recombinantly express angiotensin-converting enzyme 2 (ACE2), a natural receptor for *inter alia* human SARS-CoV and HCoV-NL63, PER.C6® cells were transfected with a plasmid carrying the cDNA sequence encoding the ACE2 protein (see Donoghue *et al.* (2000) and Tipnis *et al.* (2000); see also GenBank numbers AAF78220 and AAF99721; and SEQ ID NO:1). Stable transfectants were selected using standard techniques known to a person skilled in the art (see Coligan JE, Dunn BM, Ploegh HL, Speicher DW and Wingfield PT (eds.) (2001) Current protocols in protein science, volume I. John Wiley & Sons, Inc., New York). The cDNA encoding the ACE2 protein was cloned as a *Hind*III-*Xba*I fragment in pcDNA2004neo(-) (SEQ ID NO:2). DNA transfections in PER.C6® cells were performed using standard techniques. Stable clones were selected in the presence of 0.5 mg/ml G418 (Gibco). Expression of ACE2 was monitored using flow cytometry. Transfected cells were incubated with goat anti-human ACE2 ectodomain polyclonal antibodies (R&D systems) for one hour at 4°C. Cells were washed three times with PBS containing 0.5% BSA, incubated for 45 minutes with phycoerythrin-conjugated F(ab')2 donkey anti-goat IgG, and

analyzed on a FACSCalibur using CELLQuest Pro software (Becton Dickinson). Analysis revealed that approximately 40% of the assayed clones expressed ACE2 protein. All clones expressing ACE2 protein bound the SARS-CoV S318-510 fragment (see Example 3 below).

Example 2

Production of human SARS-CoV on ACE2 protein expressing PER.C6® cells

To evaluate whether stably ACE2-transfected PER.C6® cells were permissive for human SARS-CoV and supported the growth of human SARS-CoV, three sets of ACE2 protein expressing PER.C6® cell cultures were infected in parallel with human SARS-CoV Frankfurt 1 strain at a multiplicity of infection (MOI) of 0.1. SARS-CoV permissive Vero cells were included as a positive control cell line. Supernatants of the infected cultures were harvested and snap-frozen at -80°C after 12, 24, 48 and 72 hours post infection (pi). After collection of all samples, the supernatants were thawed and cleared by centrifugation. Serial 10-fold dilutions were made and titrated on a confluent culture of Vero cells to determine the titer. The calculated titers indicated in Table 2 show that PER.C6® cells expressing human ACE2 protein are capable of producing SARS-CoV to levels similar as those observed for Vero cells and higher than those observed for other cells such as 293T cells engineered to express the ACE2 protein.

Example 3

Binding of spike protein fragments to ACE2 protein expressing PER.C6® cells

Flow cytometry analysis was used to assay binding of recombinant fragments of the S protein to ACE2 transfected PER.C6® cells. PER.C6® cells expressing ACE2 were incubated for 1 hour at 4°C with saturating concentrations of myc-tagged S318-510 fragments. Construction and expression of recombinant S fragments was performed essentially as described in van den Brink *et al.* (2005). Briefly, amino acids 318-510 of the S1 subunit of the spike glycoprotein of SARS-CoV strain Frankfurt 1 were transiently expressed as myc/His-tagged proteins in 293T cells and purified using Ni-chromatography (for amino acid sequence of wild-type Frankfurt 1 S318-510 fragment see SEQ ID NO:3; for amino acid sequence of S318-510 fragment including signal sequence, myc tag and his tag see SEQ ID NO:4).

Next, selected mutations derived from published human SARS-CoV S protein sequences were introduced in the S318-510 fragment. The mutations correspond to mutations found in strains BJ302 cl.2 (variant F; GenBank no. AY429073; mutation N479S) and GD03T0013 (variant H; GenBank no. AY525636; mutations K344R, F360S, L472P, D480G, T487S). After three washes, bound fragment were detected by flow cytometry analysis by using biotinylated anti-myc antibody (Santa Cruz Biotechnology Inc.) and streptavidin-conjugated phycoerythrin (Caltag). All incubations and washes were performed at 4°C in PBS, supplemented with 0.5% bovine serum albumin (BSA). Binding of the anti-ACE2 IgG and the recombinant S fragment revealed that no loss in ACE2 expression was observed after 18 passage numbers (data not shown). As shown in Figure 1, all fragments were capable of binding to the PER.C6® cells expressing ACE2 protein, with the strongest binding detected for wild-type S318-510

fragment and fragment variant F. The binding of S protein fragments of several human SARS-CoV isolates to PER.C6® cells expressing ACE2 protein shows that the cells can be used to produce human SARS-CoV isolates in general.

Table 1. List of human SARS-CoV isolates that can be grown on ACE2 protein expressing PER.C6® cells.

Virus isolate	Gene/genome	Genbank	FASTA
SARS coronavirus AS	SARS coronavirus AS, complete genome.	AY427439	37576845
SARS coronavirus BJ01	SARS coronavirus BJ01, complete genome.	AY278488	30275666
SARS coronavirus BJ02	SARS coronavirus BJ02, complete genome.	AY278487	31416292
SARS coronavirus BJ03	SARS coronavirus BJ03, complete genome.	AY278490	31416305
SARS coronavirus BJ04	SARS coronavirus BJ04, complete genome.	AY279354	31416306
SARS coronavirus BJ2232			
SARS coronavirus BJ302			
SARS coronavirus CUHK-AG01	SARS coronavirus CUHK-AG01, complete genome.	AY345986	33114190
SARS coronavirus CUHK-AG02	SARS coronavirus CUHK-AG02, complete genome.	AY345987	33114202
SARS coronavirus CUHK-AG03	SARS coronavirus CUHK-AG03, complete genome.	AY345988	33114214
SARS coronavirus CUHK-L2			
SARS coronavirus CUHK-Su10	SARS coronavirus CUHK-Su10, complete genome.	AY282752	38304867
SARS coronavirus CUHK-W1	SARS coronavirus CUHK-W1, complete genome.	AY278554	30027610
SARS coronavirus cw037			
SARS coronavirus cw049			
SARS coronavirus FRA	SARS coronavirus FRA, complete genome.	AY310120	33578015
SARS coronavirus Frankfurt 1	SARS coronavirus Frankfurt 1, complete genome.	AY291315	31581502
SARS coronavirus GD01	SARS coronavirus GD01, complete genome.	AY278489	31416290
SARS coronavirus GD03T0013	SARS coronavirus GD03T0013 spike glycoprotein gene, complete cds.	AY525636	41764105
SARS coronavirus GD69	SARS coronavirus GD69, complete genome.	AY313906	37960831
SARS coronavirus GZ-A	SARS coronavirus GZ-A, partial genome.	AY394977	37624320
SARS coronavirus GZ-B	SARS coronavirus GZ-B, complete genome.	AY394978	37624321
SARS coronavirus GZ-C	SARS coronavirus GZ-C, complete genome.	AY394979	37624322
SARS coronavirus GZ-D	SARS coronavirus GZ-D, partial genome.	AY394980	37624323

SARS coronavirus GZ02	SARS coronavirus GZ02, complete genome.	AY390556	41323719
SARS coronavirus GZ43	SARS coronavirus GZ43, partial genome.	AY304490	34482141
SARS coronavirus GZ50	SARS coronavirus GZ50, complete genome.	AY304495	34482146
SARS coronavirus GZ60	SARS coronavirus GZ60, partial genome.	AY304491	34482142
SARS coronavirus HB			
SARS coronavirus HGZ8L1-A	SARS coronavirus HGZ8L1-A, partial genome.	AY394981	37624324
SARS coronavirus HGZ8L1-B	SARS coronavirus HGZ8L1-B, partial genome.	AY394982	37624325
SARS coronavirus HGZ8L2	SARS coronavirus HGZ8L2, complete genome.	AY394993	37624336
SARS coronavirus HKU-36871	SARS coronavirus HKU-36871, partial genome.	AY304492	34482143
SARS coronavirus HKU-39849	SARS coronavirus HKU-39849, complete genome.	AY278491	30023963
SARS coronavirus HKU-65806	SARS coronavirus HKU-65806, partial genome.	AY304493	34482144
SARS coronavirus HKU-66078	SARS coronavirus HKU-66078, partial genome.	AY304494	34482145
SARS coronavirus Hong Kong/03/2003			
SARS coronavirus HPZ-2003			
SARS coronavirus HSR 1	SARS coronavirus HSR 1, complete genome.	AY323977	33115118
SARS coronavirus HSZ-A	SARS coronavirus HSZ-A, partial genome.	AY394984	37624327
SARS coronavirus HSZ-Bb	SARS coronavirus HSZ-Bb, complete genome.	AY394985	37624328
SARS coronavirus HSZ-Bc	SARS coronavirus HSZ-Bc, complete genome.	AY394994	37624337
SARS coronavirus HSZ-Cb	SARS coronavirus HSZ-Cb, complete genome.	AY394986	37624329
SARS coronavirus HSZ-Cc	SARS coronavirus HSZ-Cc, complete genome.	AY394995	37624338
SARS coronavirus HSZ2-A	SARS coronavirus HSZ2-A, complete genome.	AY394983	37624326
SARS coronavirus HZS2-Bb	SARS coronavirus HZS2-Bb, partial genome.	AY395004	37624347
SARS coronavirus HZS2-C	SARS coronavirus HZS2-C, complete genome.	AY394992	37624335
SARS coronavirus HZS2-D	SARS coronavirus HZS2-D, complete genome.	AY394989	37624332
SARS coronavirus HZS2-E	SARS coronavirus HZS2-E, complete genome.	AY394990	37624333
SARS coronavirus HZS2-Fb	SARS coronavirus HZS2-Fb, complete genome.	AY394987	37624330
SARS coronavirus HZS2-Fc	SARS coronavirus HZS2-Fc, complete genome.	AY394991	37624334
SARS coronavirus JMD	SARS coronavirus JMD, partial genome.	AY394988	37624331
SARS coronavirus LC1	SARS coronavirus LC1, complete genome.	AY394998	37624341
SARS coronavirus LC2	SARS coronavirus LC2, complete genome.	AY394999	37624342
SARS coronavirus LC3	SARS coronavirus LC3, complete genome.	AY395000	37624343

SARS coronavirus LC4	SARS coronavirus LC4, complete genome.	AY395001	37624344
SARS coronavirus LC5	SARS coronavirus LC5, complete genome.	AY395002	37624345
SARS coronavirus NS-1	SARS coronavirus NS-1, complete genome.	AY508724	40795744
SARS coronavirus PUMC01	SARS coronavirus PUMC01, complete genome.	AY350750	38231927
SARS coronavirus PUMC02	SARS coronavirus PUMC02, complete genome.	AY357075	38231932
SARS coronavirus PUMC03	SARS coronavirus PUMC03, complete genome.	AY357076	38231937
SARS coronavirus sf098			
SARS coronavirus sf099			
SARS coronavirus ShanghaiQXC1	SARS coronavirus ShanghaiQXC1, complete genome.	AY463059	40457433
SARS coronavirus ShanghaiQXC2	SARS coronavirus ShanghaiQXC2, complete genome.	AY463060	40457448
SARS coronavirus Shanhgai LY	SARS coronavirus Shanhgai LY spike glycoprotein gene, complete cds.	AY322205S3	32454341
SARS coronavirus Sin0409			
SARS coronavirus Sin2500	SARS coronavirus Sin2500, complete genome.	AY283794	30468042
SARS coronavirus Sin2677	SARS coronavirus Sin2677, complete genome.	AY283795	30468043
SARS coronavirus Sin2679	SARS coronavirus Sin2679, complete genome.	AY283796	30468044
SARS coronavirus Sin2748	SARS coronavirus Sin2748, complete genome.	AY283797	30468045
SARS coronavirus Sin2774	SARS coronavirus Sin2774, complete genome.	AY283798	37361915
SARS coronavirus Sin3408	SARS coronavirus Sin3408, complete genome	AY559083	45644998
SARS coronavirus Sin3408L	SARS coronavirus Sin3408L, complete genome	AY559097	45645024
SARS coronavirus Sin3725V	SARS coronavirus Sin3725V, complete genome	AY559087	45645004
SARS coronavirus Sin3765V	SARS coronavirus Sin3765V, complete genome	AY559084	45645000
SARS coronavirus Sin842	SARS coronavirus Sin842, complete genome	AY559081	45644994
SARS coronavirus Sin845	SARS coronavirus Sin845, complete genome	AY559093	45645019
SARS coronavirus Sin846	SARS coronavirus Sin846, complete genome	AY559094	45645021
SARS coronavirus Sin847	SARS coronavirus Sin847, complete genome	AY559095	45645022
SARS coronavirus Sin848	SARS coronavirus Sin848, complete genome	AY559085	45645001
SARS coronavirus Sin849	SARS coronavirus Sin849, complete genome	AY559086	45645003
SARS coronavirus Sin850	SARS coronavirus Sin850, complete genome	AY559096	45645023
SARS coronavirus Sin852	SARS coronavirus Sin852, complete genome	AY559082	45644996
SARS coronavirus Sin_WNV			
SARS coronavirus Sino1-11	SARS coronavirus Sino1-11, complete genome.	AY485277	38505482

SARS coronavirus Sino3-11	SARS coronavirus Sino3-11, complete genome.	AY485278	38505491
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SARS coronavirus SoD	SARS coronavirus SoD, complete genome.	AY461660	38385714
SARS coronavirus SZ1	SARS coronavirus SZ1, partial genome.	AY304489	34482140
SARS coronavirus SZ13	SARS coronavirus SZ13, partial genome.	AY304487	34482138
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SARS coronavirus Taiwan JC-2003			
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SARS coronavirus Tor2	SARS coronavirus TOR2, complete genome.	AY274119	30248028
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Human coronavirus NL63	Human Coronavirus NL63, complete genome	AY567487	49035964
Human group 1 coronavirus associated with pneumonia	Human group 1 coronavirus associated with pneumonia, complete genome	AY518894	46369870

Table 2. SARS-CoV titers measured in culture supernatants harvested after 12, 24, 48 and 72 hours post-infection of PER.C6® and Vero cultures at MOI 0.1. Titers were expressed in log10 ± log10 standard error.

hr pi	PER.C6®-ACE2			Vero		
12	5.05±0.16	5.05±0.16	5.30±0.19	7.30±0.23	7.05±0.16	7.05±0.16
24	7.93±0.13	7.55±0.25	6.93±0.13	7.43±0.18	7.18±0.21	7.05±0.16
48	6.05±0.16	6.18±0.18	6.43±0.18	7.05±0.18	6.80	7.43±0.18
72	6.80±0.29	6.18±0.30	5.93±0.21	6.80±0.18	6.55±0.16	6.68±0.13

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Van den Brink, Edward N.

Ter Meulen, Jan H.

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        35                  40                  45

Asn Tyr Asn Thr Asn Ile Thr Glu Glu Asn Val Gln Asn Met Asn Asn
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Ala Gly Asp Lys Trp Ser Ala Phe Leu Lys Glu Gln Ser Thr Leu Ala
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Ser Lys Arg Leu Asn Thr Ile Leu Asn Thr Met Ser Thr Ile Tyr Ser
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Thr Gly Lys Val Cys Asn Pro Asp Asn Pro Gln Glu Cys Leu Leu Leu
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Glu Pro Gly Leu Asn Glu Ile Met Ala Asn Ser Leu Asp Tyr Asn Glu
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REFERENCES

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CLAIMS

1. A human cell derived from a primary human embryonic retinoblast, said cell comprising in its genome sequences that encode E1A and E1B of an adenovirus, characterized in that the cell is engineered to express the human ACE2 protein.

2. A human cell according to claim 1, wherein the cell used in engineering to express the human ACE2 protein is a PER.C6 cell as deposited under ECACC no. 96022940.

3. A human cell according to claim 1 or 2, wherein the human ACE2 protein is stably expressed.

4. A method of producing a coronavirus, said method comprising the steps of:
   a) providing a human cell that is derived from a primary human embryonic retinoblast, said cell comprising in its genome sequences that encode E1A and E1B of an adenovirus, with a nucleic acid molecule encoding a human ACE2 protein,
   b) culturing the cell under conditions suitable for expression of the human ACE2 protein,
   c) infecting the cell with a coronavirus, and
   d) harvesting the coronavirus from the medium or the cell.

5. A method according to claim 4, characterized in that the coronavirus is selected from the group consisting of SARS-CoV and HcoV-NL63.

6. A method according to claim 4 or 5, characterized in that the human cell is PER.C6 as deposited under ECACC no. 96022940 or a derivative thereof.

7. A method according to any of the claims 4-6, characterized in that the human ACE2 protein is stably expressed.

8. A method according to any of the claims 4-7, characterized in that the nucleic acid molecule encoding the human ACE2 protein is provided by a vector.

9. A method according to any of the claims 4-8, characterized in that the human cell is capable of growing in suspension.

10. A method according to any of the claims 4-9, characterized in that human cell can be cultured in the absence of serum.

11. A method according to any of the claims 4-10 further comprising the step of inactivating or attenuating the harvested coronavirus.

12. Use of a human cell according to any of the claims 1 to 3 for the production of a coronavirus.

13. Use of a human cell according to any of the claims 1 to 3 for the screening of antiviral agents against a coronavirus.

14. A method for identifying a molecule capable of inhibiting a coronavirus infection and/or replication, the method comprising the steps of:
    a) incubating a cell according to any of the claims 1 to 3 with a coronavirus in the presence and absence of the candidate molecule, and
    b) determining whether the presence of the candidate molecule inhibits coronavirus infection and/or replication.

15. A method for selecting an antiviral molecule that is capable of reducing infection of a cell by a coronavirus, the method comprising the steps of:

a) contacting a cell according to any of the claims 1 to 3 with a surface protein of a coronavirus in the presence or absence of a candidate antiviral molecule, said surface protein being involved in binding of the coronavirus to the human ACE2 protein expressed by the cell,

b) measuring the binding interaction between the cell and the surface protein, and

c) selecting a candidate antiviral molecule whereby the binding interaction is reduced when compared to the binding interaction in the absence of the candidate antiviral molecule.