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Construction of a CRISPR based cloning vector targeting ACE2.

by

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DECLARATION

I hereby declare that the thesis is based on my originality work expect for quotations and citation which have been duty acknowledged.

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
°C/min	Degree Celsius per minute
°C/s	Degree Celsius per second
kb	Kilo base
µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolar
M	Molar
min	Minute
mL	Milliliter
mM	Millimolar
ng/µL	Nanogram per microliter
ng	Nanogram
nm	Nanometer
rpm	Revolutions per minute
V	Volt

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Cas	CRISPR associated systems
CRF	Circulating recombinant forms
CRISPR	Clustered regularly interspaced palindromic repeats
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DSB	Double strand break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FI	Fusion inhibitor
GFP	Green fluorescent protein
gRNA	guide RNA
HDR	Homology-directed repair
mRNA	Messenger RNA
NHEJ	Non-homologous end joining
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PCR	Polymerase chain reaction
PAM	Protospacer Adjacent Motif
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TALEN	Transcription activator-like effector nuclease
TAR	Trans-activation response element
TNF-α	Tumor necrosis factor α
ZFN	Zinc finger nuclease

ABSTRAK

COVID-19, yang disebabkan oleh coronavirus sindrom pernafasan akut teruk 2 (SARS-CoV-2) pada akhir 2019, menimbulkan ancaman besar kepada kesihatan dan keselamatan awam. Usaha untuk menguruskan wabak, termasuk imunisasi, telah dihadkan oleh kemunculan varian novel SAR-CoV-2 dan penghakisan imuniti. Rangkaian interaksi Protein-Protein untuk ACE2 telah dibina menggunakan Cytoscape 3.10.0 untuk mengkaji interaksi ACE2 dengan gen atau protein lain yang terlibat dalam laluan patogenesis SARS-CoV-2. Analisis rangkaian ACE2 menunjukkan interaksi dengan sembilan gen (ACE2, TMPRSS2, CLEC4M, DPP4, ADAM 17, ANEP, CCL2, NPC1, & MEPLA) yang terlibat dalam proses jangkitan virus. Selain itu, analisis ekspresi gen ACE2, TMPRSS2, Arg 1 & IL6 ditentukan menggunakan RT-PCR. Analisis menunjukkan bahawa ACE2 mempunyai tahap ekspresi tertinggi berbanding dengan tiga gen lain dalam talian sel HEK 293 dan memberikan pemahaman yang lebih baik tentang ekspresi ACE2 dalam talian sel buah pinggang dan laluan isyarat yang berkaitan. Selain itu, pembinaan plasmid CRISPR kalah mati ACE2 telah berjaya dilaksanakan dengan mereka bentuk gRNA dan mengklonkannya menjadi plasmid CRISPR, pX458.

ABSTRACT

- COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in late 2019, poses a significant threat to public health and safety. Efforts to manage the pandemic, including immunizations, have been limited by the emergence of novel SAR-CoV-2 variants andthe erosion of immunity. The Protein-Protein interaction network for ACE2 was constructed using Cytoscape 3.10.0 to study the interaction of ACE2 with other genes or proteins involved in the pathogenesis pathway of SARS-CoV-2. Analysis of the ACE2 network showed interaction with nine genes (ACE2, TMPRSS2, CLEC4M, DPP4, ADAM 17, ANEP, CCL2, NPC1, & MEPLA) involved in virus infection process. Besides that, the gene expression analysis of ACE2, TMPRSS2, Arg 1 & IL6 was determined using RT-PCR. The analysis indicated that ACE2 had the highest expression level compared to other three genes in HEK 293 cell line and provided a better understanding of expression of ACE2 in kidney cell line and its related signaling pathway. Apart from that, the construction of an ACE2-knockout CRISPR plasmid was successfully executed by designing gRNA and cloning it into a CRISPR plasmid, pX458.

CHAPTER 1: INTRODUCTION

1.1. Research Overview

Severe Acute Respiratory Syndrome-Related Coronavirus (SARS-CoV-2) is responsible for the Coronavirus disease (COVID-19) which emerged in 2019 in Wuhan, China. To date, more than 636 million people have been infected with COVID-19 while more than 6 million people have died from the disease (WHO, 2023).

Several effective measures were taken to control this public health crisis, including vaccines, which controlled the severity of the infection. However, the emergence of new variants of SAR- CoV-2 and the warning of immunity leads to the question of the long-term effectiveness of vaccines. This also highlights the problem statement of the research where the need to study potential therapies may prepare us to combat Covid-19. The most recent genome editing method, CRISPR/Cas9, functions as a molecular pair of scissors to knock out the targeted gene by causing a double strand break (DSB). The non-homologous end joining (NHEJ) cell repair method would randomly repair the DSB by introducing indels at the break shift. According to Perez et al. (2008), Mussolini et al. (2014), and Kistler et al. (2015), this causes frameshift and reduces the function of the relevant gene.

Pathogenic virus, including SARS-CoV-2, can cause cells to create both cellular and humoral immunity, both of which are essential for treating viral infection. In any case, an uncontrolled or insufficient immune response could lead to immunopathology, which wouldbe exceedingly damaging to patients (Kruse et al., 2020). The infection of virus can create excessive inflammation to immune response which can leads pathophysiological changes in virus infected tissues or organs. For an infection to occur, SARS-CoV-2 would need to bind to angio-converting enzyme 2 (*ACE2*) prior to entry into the human cells. Because epithelial cells have *ACE2* receptors, SARS-CoV-2 primarily affects both the respiratory and gastrointestinal systems (Zhang et al.,2020).

Learning from past successful therapeutics such as the delta 32 mutation on CCR5 which prevents HIV from binding to the receptor, similarly, inhibiting the expression of *ACE2* might also serve as a proof-of-concept to prevent Covid-19 infection. In this study, a CRISPR-mediated knock-out of *ACE2* will be done to evaluate the suitability of this approach as a potential therapeutic against Covid-19.

1.2. Hypothesis

Successful construction of CRISPR/ACE2 clones, combined with protein-protein interaction and gene expression analysis as insight.

1.3. General Objective

To construct an ACE2-knockout CRISPR plasmid as a proof-of-concept approach to study SARS-CoV-2 progression.

1.4. Specific objectives

1.4.1. Mapping out protein-protein interactions with *ACE2* to elucidate host pathways crucial for SARS-CoV-2 progression.

1.4.2. Determining basal level expression of genes involved in SARS-CoV-2 progression in HEK 293

1.4.3. Constructing an ACE2- knockout CRISPR plasmid.

CHAPTER 2: LITERATURE REVIEW

2.1. Epidemiology of SARS COV2

On February 5, 2020, Malaysia reported the first local case of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The nation saw its first two coronavirus disease 2019 (COVID-19) fatalities on March 17, 2020, just one week after the World Health Organization (WHO) formally declared the growing outbreak to be a pandemic (Cucinotta & Vanelli,2020). Two years after the COVID-19 illness initially surfaced in Malaysia, 32,063 fatalities have been recorded there. Men and women 80 and older had the highest mortality rates (13 and 18%, respectively), and the year 2021 had the highest death rate(31,059 out of 32,063). (Wyper et al.,2021). According to estimates, COVID-19 caused 683,903 Years of life lost (YLL) over the previous two years, or around 21 years lost for every COVID-19 fatality and 1,998 years for every 100,000 individuals. Men lost more YLL per 100,000 people thanwomen (2,132 vs. 1,853). YLL was higher in the age categories of males aged 55–59 and females aged 45–49 (Figure 1) (Raveendran et al,2021)

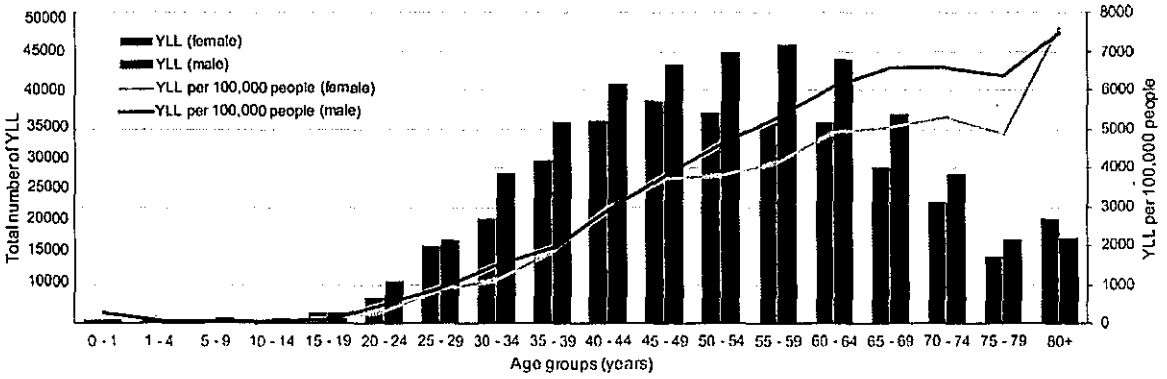

Figure 1: Years of life lost (YLL) attributable to COVID-19 in Malaysia over two full years beginning on February 5, 2020, broken down by age and sex. (Adapted from:DOI: 10.1016/j.lanwpc.2022.100456).

Apart from this, with 3,473 years per 100,000 people in the Federal Territory of Labuan and 3,457 years per 100,000 in the state of Selangor, respectively, these regions lost the most YLL (Figure 2) (Cucinotta & Vanelli,2020).

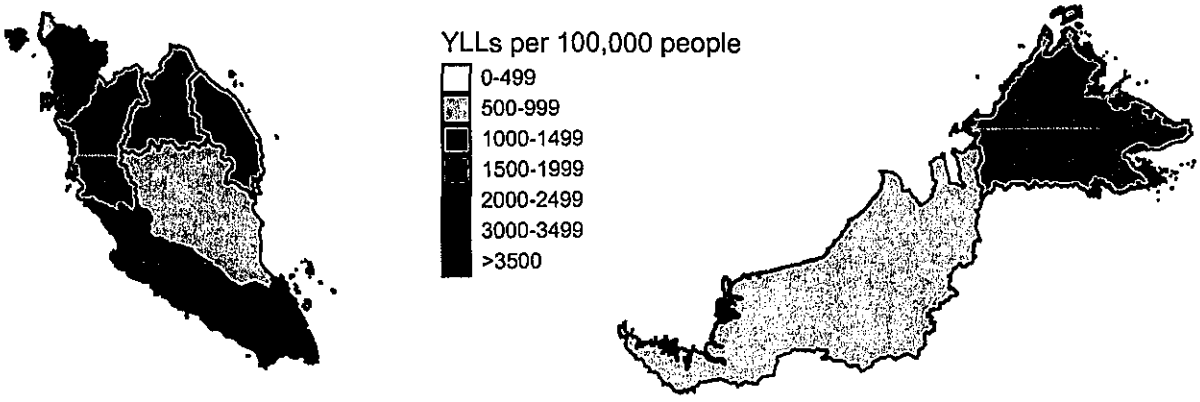

Figure 2: Shows the estimated years of life lost (YLL) for every state and federal territory in Malaysia, per 100,000 persons. (Adapted from: DOI: 10.1016/j.lanwpc.2022.100456).

2.2. Classification of SAR-Cov-2

The four primary coronavirus subgroups that can be distinguished based on their genetic makeup are alpha, beta, delta, and gamma (Wang & Zhang,2016). The *Coronaviridae* family, *Orthocoronavirinae* subfamily, and *Nidovirales* order all contain the SARS-CoV-2 virus (Mudgal,2014). Figure 3 shows that Beta coronaviruses, including SAR-CoV-2, belong to the subgenus *betacoronavirus. Embecovirus*, *Sarbecovirus*, *Merbecovirus*, and *Norbecovirus* are the four subgenera of the genus Betacoronavirus (Wilson & Preiser ,2021). SARS-CoV and SARS-CoV- 2 belong to the *Sarbecovirus* genus, while MERS-CoV is a member of the *Merbecovirus* genus.A vast variety of species, including humans, rats, cattle, horses, antelopes, camelids, ducks, geese, other birds, dolphins, whales, and aquatic mammals, are susceptible to infection by representative coronaviruses (Peiris,2016). The coronavirus genus is extensive.

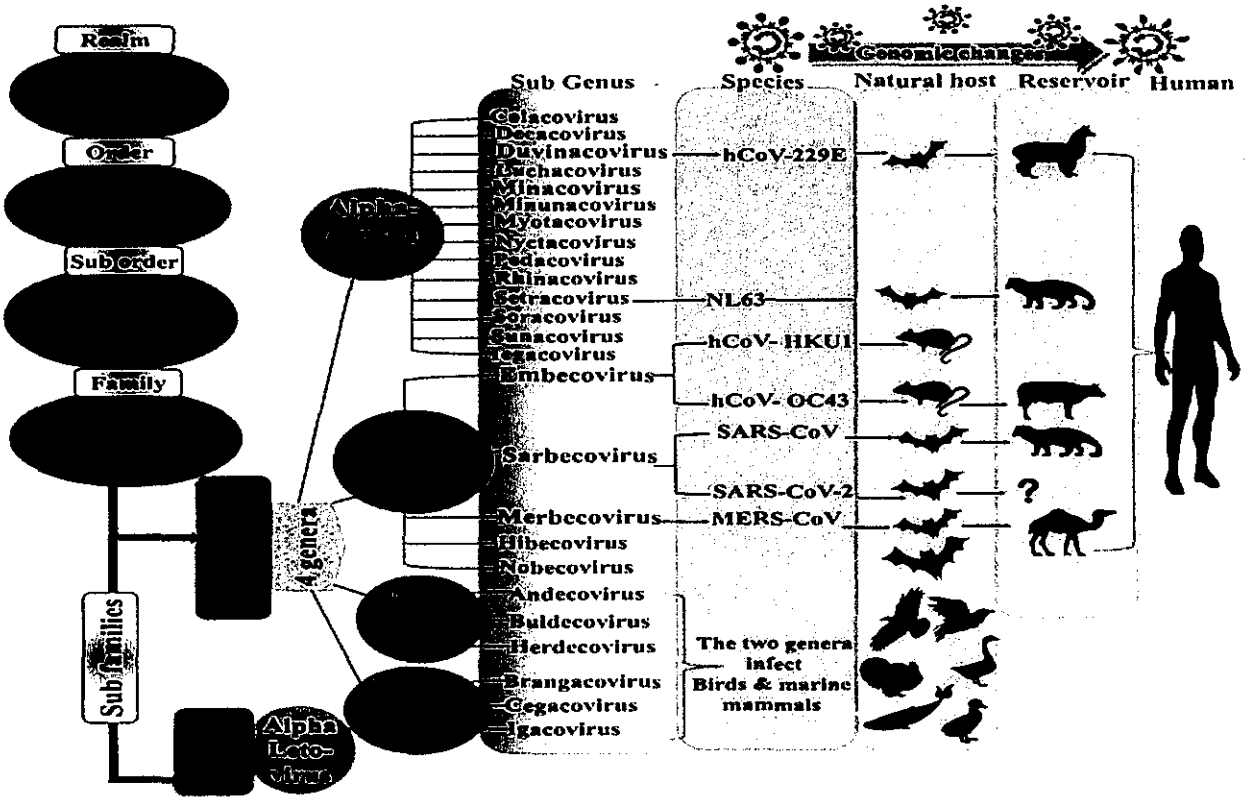

Figure 3: The International Committee on Taxonomy of Viruses (ICTV) has classified the SARS-CoV-2 coronavirus. (Hussein et al., 2020).

2.3. Morphology of SAR-Cov-2

Spike (S), envelope (E), membrane (M), and nucleocapsid (N) are the four structural proteins that the virus genomes encode (Figure 4) (Cabezas et al., 2012). Due to their remarkable sequence similarities, SARS-CoV-2 and SARS-CoV are believed to share the same structure. Spike, a viral surface protein produced by SARS-CoV-2, interacts with the cell surface ACE2 receptor. The virus's primary aim for entry into human cells is binding to receptors on host cell membranes (Goldsmith et al., 2004).

The result is fusion of the membrane. The protruding spike protein S that bears its name makes it easy to identify the crown-shaped virion after which it was called. The abnormally thick SAR CoV 2 envelope is due to the prolonged C-terminal endodomain of the M protein, which binds to the inner membrane flap and forms a thick matrix-like lattice. M protein is thought to carry out additional vital roles in viral assembly, including the packaging of genomic RNA into nucleocapsids (Naqvi et al.,2020). The SARS-CoV 2 envelope protein E is a virulence factor required for virion morphogenesis and assembly. To build the virion, the SAR-CoV-2 N protein interacts with the RNA genome and facilitates viral assembly and budding. It has two domains (NTD) and a C-terminal domain (Lalchhandama et al,2020).

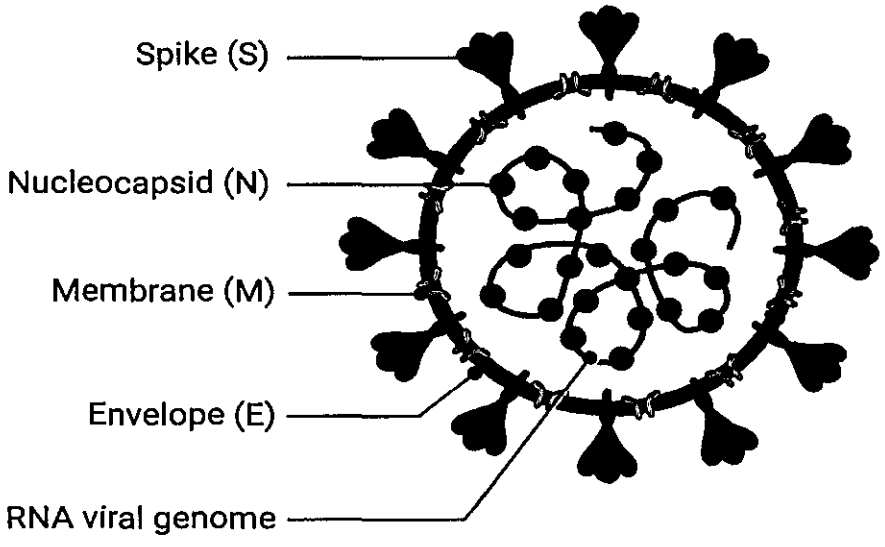

Figure 4: Structure of SARS -CoV-2 (Rothan et al.,2020).

2.4. Genome structure of SARS CoV 2

In January 2020, researchers in China successfully isolated and sequenced the first SARS-CoV-2 genome, which they termed Wuhan-Hu-1 and is designated as "NCBI reference sequence NC_045512". According to Ortiz et al. (2020), the SARS-CoV-2 virus belongs to the beta coronavirus family and is an enveloped, positive-sense, unsegmented virus. SARS-CoV-2 has some genomic similarities with the bat coronavirus RaTH13 (91% similarity), SARS-CoV (91% similarity), and MERS-CoV (Figure 5).

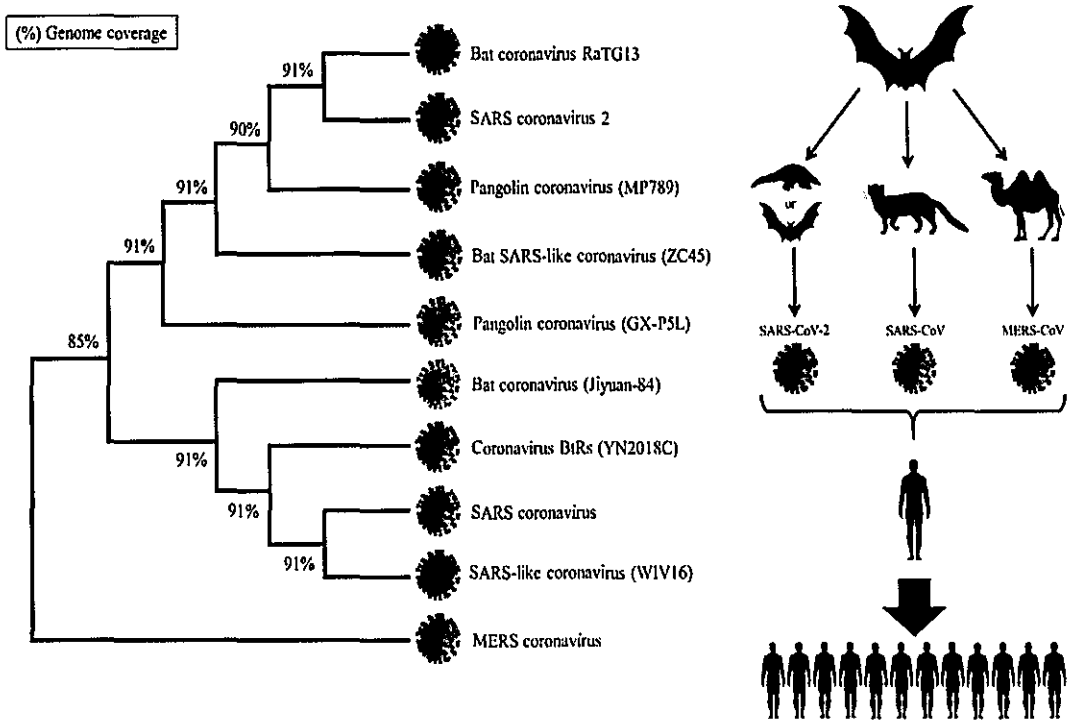

Figure 5: SARS-CoV-2 evolutionary analysis. The investigation shows that bat coronavirus RaTG13 is the most closely related species to SARS-CoV-2.

The SARS-CoV-2 genome is between 29 844 and 29 891 nucleotides long , 38% GC, 11 protein-coding genes that encode for roughly 9860 amino acids, and 14 open reading frames ORFs that code for 27 distinct proteins, according to whole-genome sequencing of the virus. (Naqvi et al.,2020).This organism possesses a poly (A) tail, many unknown non-structural ORFs, a 5′ untranslated region (UTR), replication complex "ORF1a and ORF1b," spike (S) and envelope (E) genes, membrane (M) gene, nucleocapsid (N) gene, and 3′ polyprotein pp1a, which has 10.

Nonstructural proteins (NSP) are encoded by the ORF1a gene, which is found at the 5′UTR (Ortiz et al,2020).

The polyprotein pp1ab, which contains 16 NSPs, is made by the ORF1b gene, which is located close to ORF1a. pp1b is cleaved by autoproteolytic proteases to generate the viral replication complex with ORF1a.

Eight auxiliary genes are interspersed between the four structural genes in the 3′UTR; their functions are largely unclear (Rastogi et al.,2020). More information is provided in Figure 6.

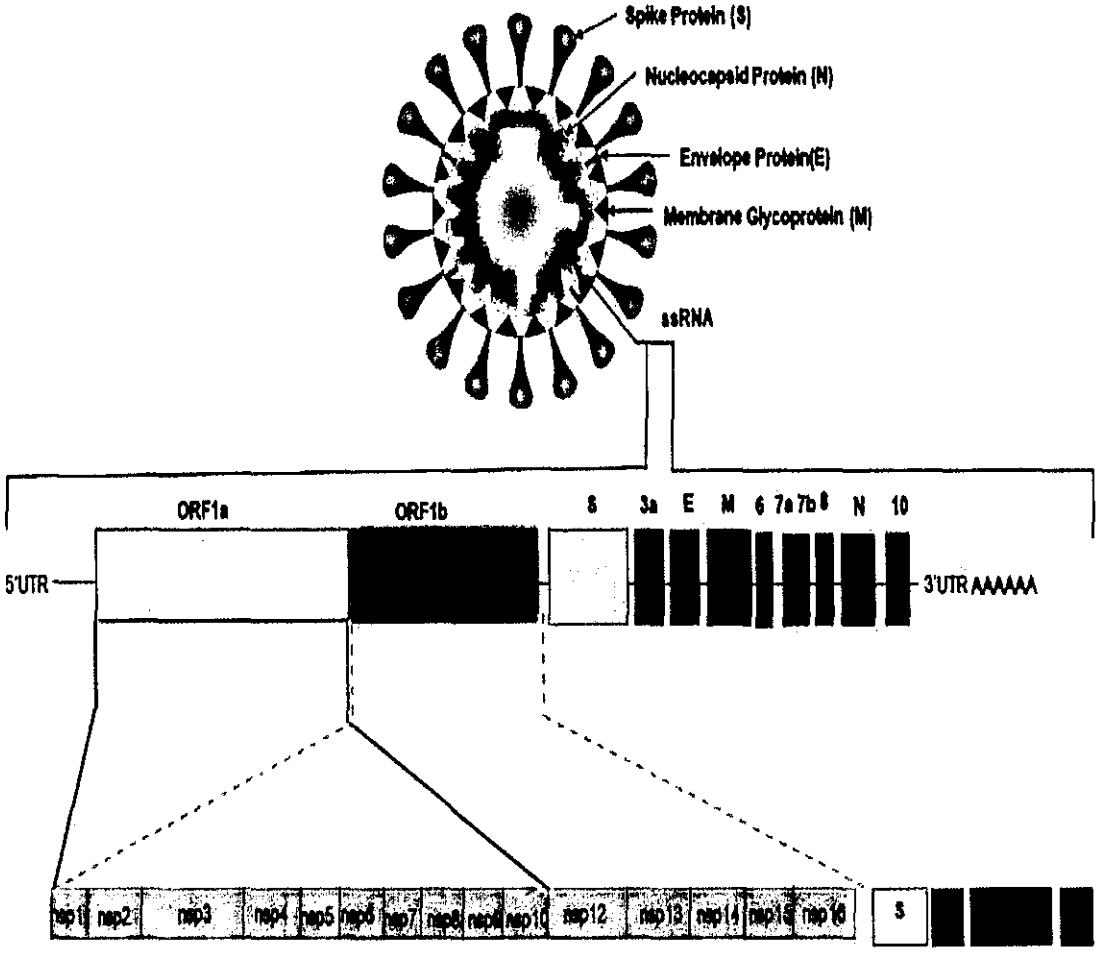

Figure 6: shows the SARS-CoV-2 genome structure schematically. About 29,903 nucleotides make up the full-length RNA genome, which also features a replicase complex at the 5′UTR.

made up of ORF1a and ORF1b. NSP1–NSP10 are encoded by ORF1a, while NSP1–NSP16 are encoded by ORF1b. Four genes—the spike gene, the envelope gene, the membrane gene, the nucleocapsid gene, and a poly (A) tail at the 3′UTR—encode the structural proteins.

2.5. *ACE2*

There is the chromosome X gene, which codes for the transmembrane protein *ACE2*. Initially recognized as an angiotensin converting enzyme homolog, *ACE2* has an active zincmetalloprotein domain in its carboxyl-terminal catalytic region in addition to an amino-terminal domain (Swertz et al.,2020). *ACE2* is divided into independent and peptidase-dependent groupsin terms of functionality. The levels of *ACE2* expression vary among the organs of the body and the expression of ACE2 is higher in lungs, heart, and kidneys (Figure 7). According to studies by Zou et al. who examined data from single-cell RNA sequencing of human systems cardia muscle cells, renal tubule cells, and other cells, type 2 alveolar cells in particular exhibit high levels of *ACE2* expression (Zhang etal.,2020). Similar conclusions were reached by Zhou et al., who found that the new SARS-CoV- 2 only infected cells expressing *ACE2* receptors and that the lack of this receptor prevented viral entry into host cells. This discovery lends credence to the idea that *ACE2* receptor is required for SARS-CoV-2 pathogenesis. Therefore, in this study we knockout the *ACE2* by using CRISPR Cas 9 system to investigate potential therapy that against covid.

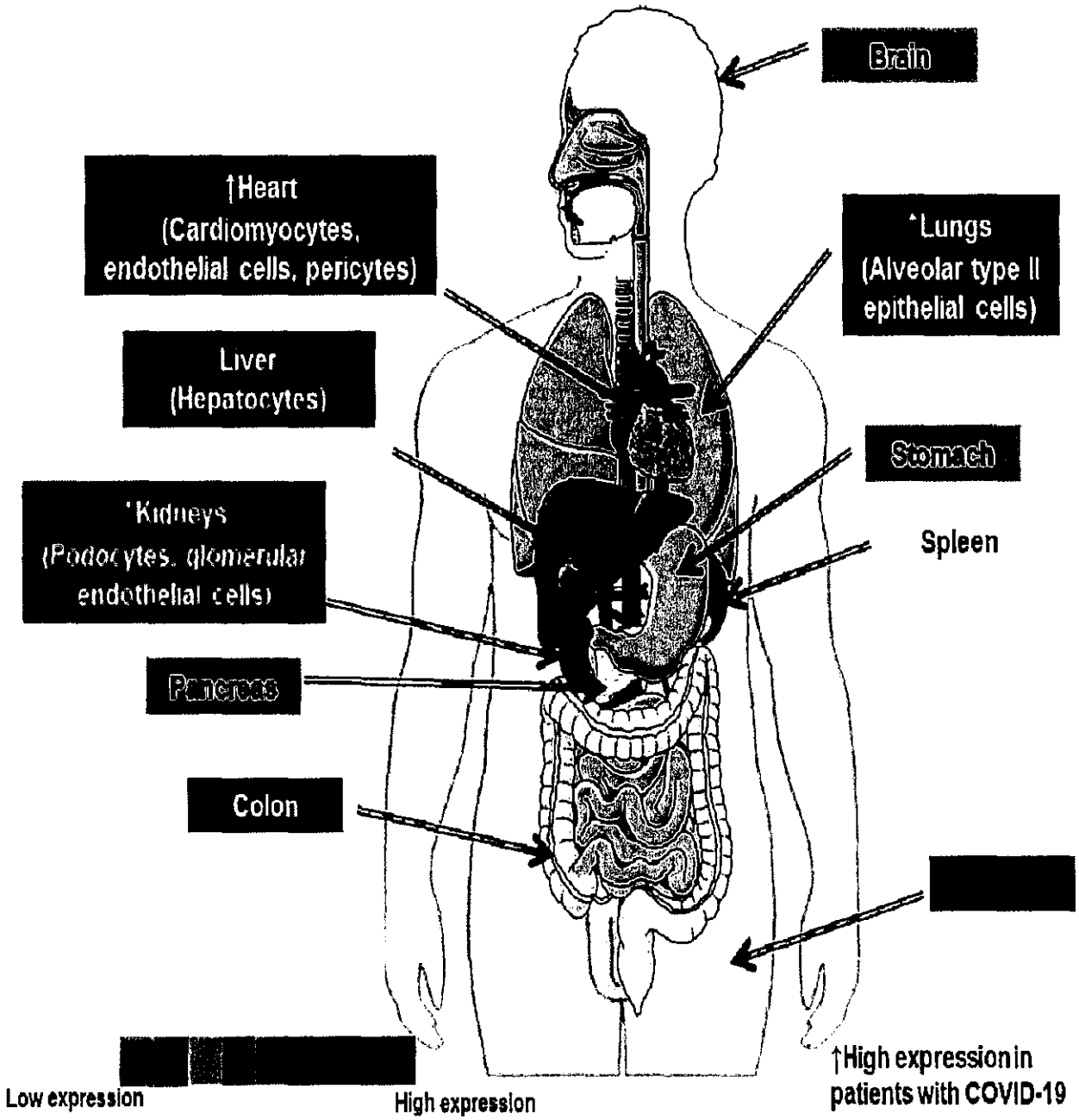

Figure 7: *ACE2* expression in different organs in healthy individuals and individuals with COVID-19. The figure represents *ACE2* expression in normal organs and cells, such as cardiomyocytes, podocytes, and hepatocytes, which are colored by its level of expression. The expression of *ACE2* is gradually increased from healthy spleen to the highest expression in colon. The up-arrow indicates high expression in kidneys, lungs and heart of patients with COVID-19 (Zhang et al.,2020).

2.6. Viral entry of SARS CoV 2

The plasma membrane or endosomes can fuse to allow SARS-CoV-2 to enter cells (Pample,2021). Coronaviruses' spike (S) protein makes it easier for the virus to enter its target cells. Entry requires binding of the S protein's S1 surface unit to a ACE2. Virus receptor that makes it easier for viruses to bind to target cells' surfaces (Pample,2021). The S protein must also be primed by cellular proteases to operate; this necessitates that the S proteinbe cleaved at the S1/S2 site. The S2 subunit mediates the process whereby the S2 site allows viral fusion in cellular membranes.

The effectiveness of ACE2 utilization was found to be a key determinant of SARS-CoV-2 transmissibility. The rapid spread of severe symptoms, and high mortality rate of the disease are all caused by these SARS- CoV-2 entry characteristics.

According to Figure 8, the nucleocapsid's spike S protein's interaction with the protease *ACE2* on the surface of the host cell affects the host cell's ability to enter. The S protein's attachment to *ACE2* is facilitated by the host cell protease TMPRSS2 (Hoffmann et al., 2020). The S protein binds, and then the virus is internalized. The internalized virus is uncoated, and the SARS-CoV-2 genome is then released into the cytoplasm.

The viral RNA is then copied and translated after that. As a result, two polyproteins—PP1A and PP1AB—are created. These proteins contain the papain-like protease PLpro and the coronavirus primary protease Mpro, which break the polyproteins into a variety of other functional proteins.

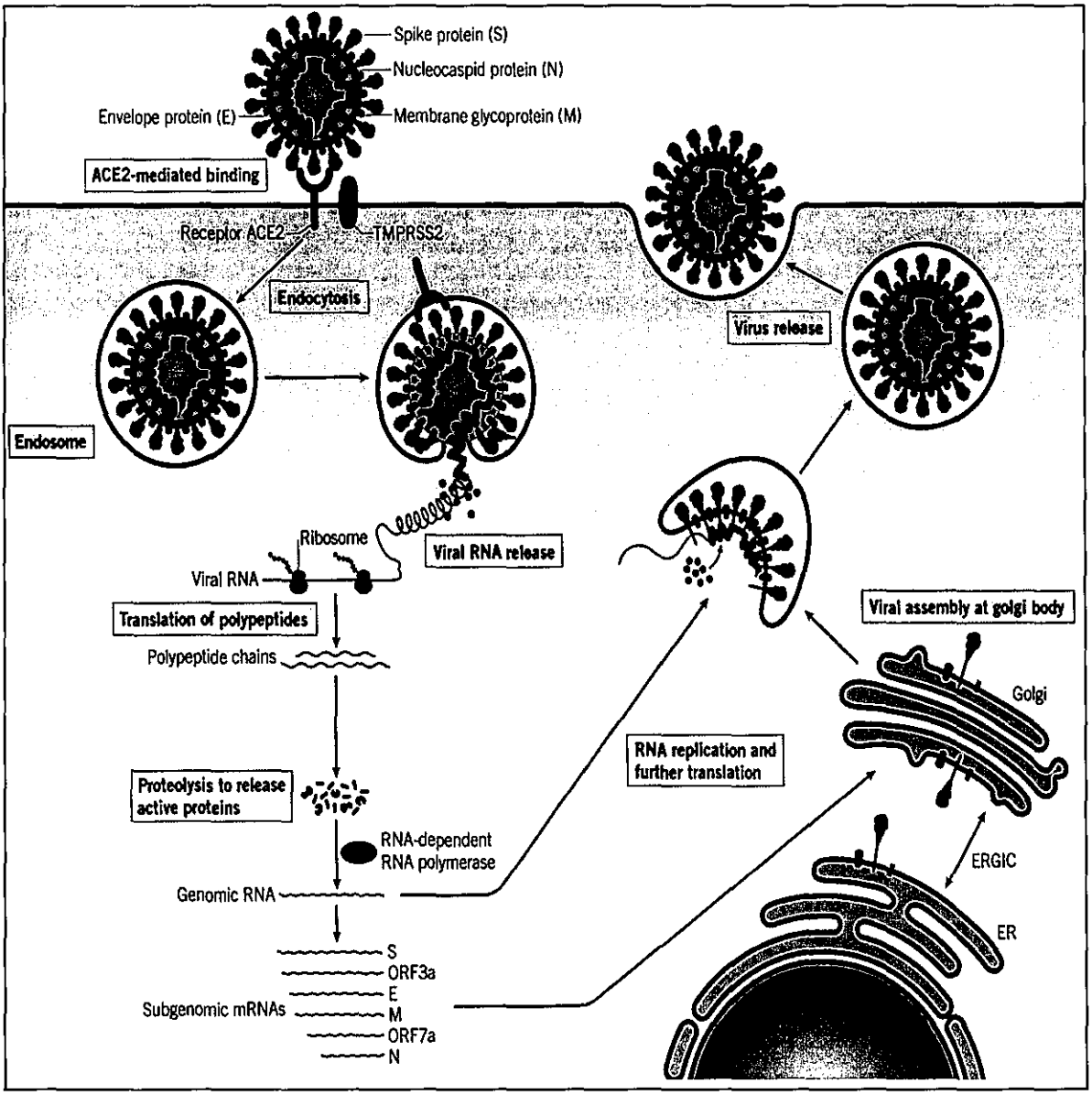

Figure 8: A diagram showing the viral entry into the host cell. (Hoffmann et al., 2020). The replication site generates viral structural proteins and genomic RNA, which are then transported to the ER-Golgi intermediate compartment (ERGIC), where virus assembly and budding take place, through an unidentified process. The proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) are all present in the virion. While the N protein, which is connected to the viral genomic RNA, is packed inside the virion, the structural proteins S, E, and M are incorporated into the membrane of the virion. When the S protein is arranged in a trimer, which resembles a crown (corona), it facilitates important entry events such receptor binding and

membrane fusion. During creation and maturation in the infected cell, the S protein is divided into the related S1 and S2 subunits by furin or a furin-like proprotein convertase in the Golgi apparatus.

2.7. Gene therapy

Gene therapy is defined as an experimental therapy used to help prevent or treat some diseases brought on by viruses or other pathogens by replacing or repairing damaged genes inside the body's cells (Akbulut et al., 2016). The relevant gene may be repaired, replaced, or eliminated to address infectious diseases. It is also possible to modify the genomes of pathogens to produce crippling mutations that can be utilized to cure infectious diseases. This potential therapy can treat a wide range of human illnesses, including Covid19.

The development of human gene therapy has been aided by the use of zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) nucleases (Meissner et al., 2018). Double-strand breaks (DSBs), which are frequently repaired by non-homologous end joining (NHEJ) are the mechanism by which these programmable nucleases harm the targeted gene (Zhang et al., 2017).

Cong et al. (2013) claim that in addition to human adult stem cells, induced pluripotent stem cells, and embryonic and pluripotent stem cells, CRISPR/Cas9-mediated genome editing has also been successfully used in a few model animals. Gene therapy provides an advantage over conventional medicines, according to Xiao-jie et al. (2015), because it may be applied locally and at a high therapeutic dose without the possibility of experiencing systemic side effects.

Additionally cost-effective because they are frequently only needed once, gene treatments. Meissner et al. (2018) claim that gene therapy is a cutting-edge technique with a promising future in clinical research.

2.8. CRISPR Cas 9

CRISPR, also known as Cluster Regularly Interspaced Short Palindromic Repeats, is an illustration of a prokaryotic adaptive immune system. Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-associated proteins (Cas) eliminate undesirablegenetic material via RNA-guided nucleases. In order to remove external genetic elements (EGEs), it has a brief repeating nucleotide that was first noticed in the genomes of bacteria and archaea (Zhang et al., 2017).

In order to categories CRISPR/Cas9 into three groups, different bacterial and archaeal hosts were used (Ran et al., 2013). The repetitive sequences, noncoding RNAs, and CRISPR- associated (Cas) genes were unique to each group. Type II is one of the CRISPR systems that has the finest characterization. The transactivating crRNA (tracrRNA), which enables the processing of the crRNA array into discrete units, the endonuclease Cas9, and the crRNA array that encodes the guide RNAs are included in it (Ran et al., 2013; Zhang et al., 2017).

The use of CRISPR/Cas9 for genome editing is made simpler by the ability to substitute the duplex structure made up of the crRNA and tracrRNA molecules, with a synthetic fused chimeric single gRNA (sgRNA) (Oude et al., 2016).

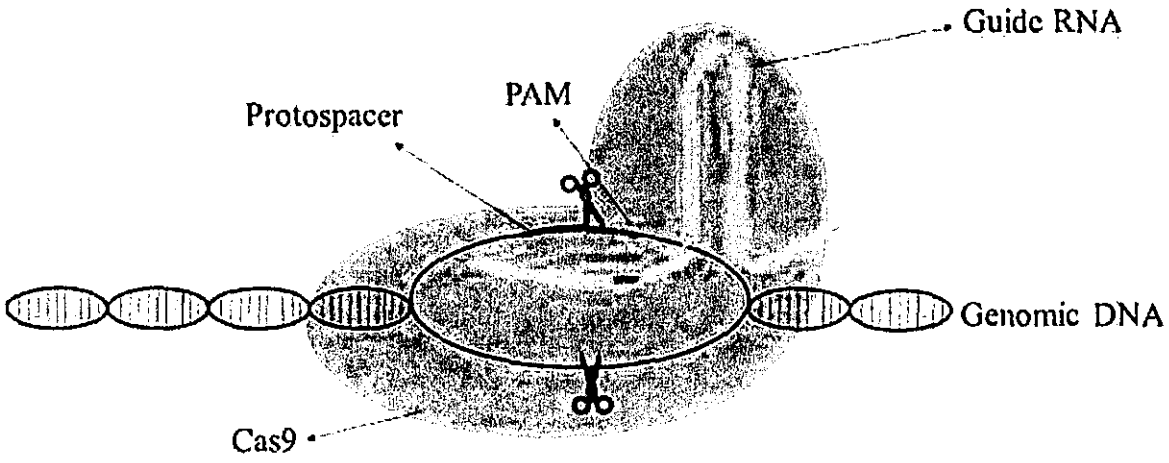

Figure 9: The mechanism of the CRISPR Cas9 system and its component (Ooi,2020)

The protospacer adjacent motif sequence (PAM), which is frequently NGG or NCC, is placed as a spacer to the CRISPR locus to discriminate between one's own DNA and foreign DNA. When cells transcribe this spacer to produce a CRISPR RNA (cRNA) with a complementary sequence to the foreign DNA invasion, a memory is produced.. The guide RNA (gRNA), which isproduced by the connection between the trans-activating RNA (tracRNA) and the cRNA, directs Cas9 to form an active gRNA/Cas9 complex that functions similarly to an antibody.

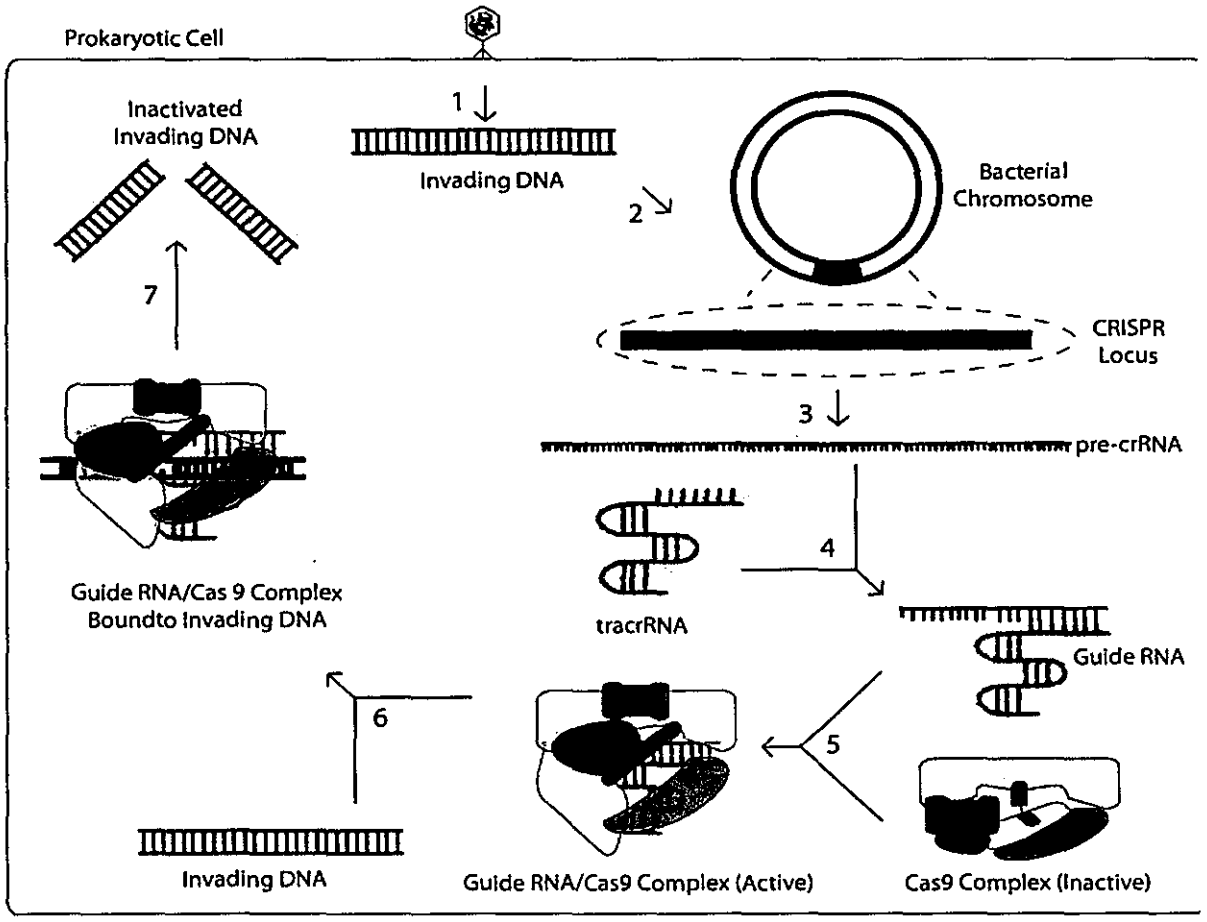

Figure 10: The natural CRISPR approach is shown 1) Invasion of foreign viral DNA. 2) Incorporating DNA from the area that was invaded into a CRISPR locus. 3) Translation of invading foreign DNA into pre-cRNA. 4) Making a duplex of cRNA and tracRNA to produce gRNA. 5) Cas9 and gRNA interact to form a functional gRNA/Cas9 complex. 6) The DNA of similar invaders is bound by the Cas9/gRNA complex. 7) The Cas9 protein annihilates the invader's DNA (Ran et al., 2013).

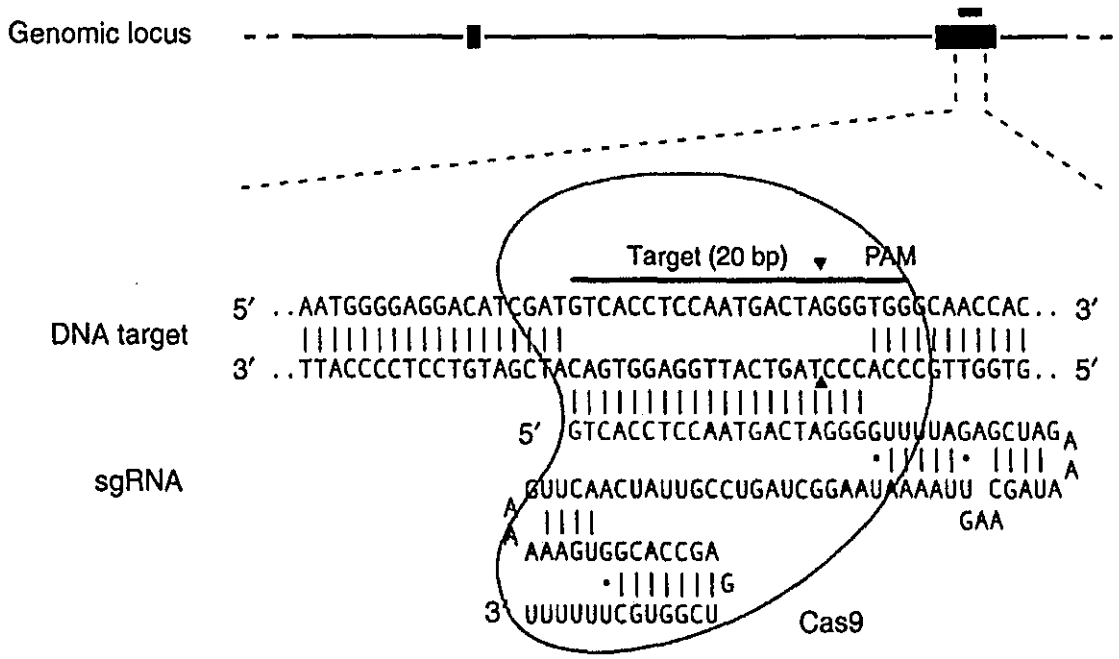

Figure 11: Schematic of the RNA-guided Cas9 nuclease. Genomic DNA is the target of a sgRNA that consists of a scaffold (red) and a 20-nt guide sequence (blue) for the S. pyogenes Cas9 nuclease. The necessary 5′-NGG adjacent motif (PAM; pink) and the DNA target's upstream guide sequence pair are shown. According to Ran et al. (2013), Cas9 mediates a DSB roughly 3 bp upstream of the PAM (red triangle).

Both a crucial variable region and a crucial scaffold are present in the gRNA. The 18–20 nucleotide gRNA iteration can attach to the target DNA because of the base complementation pairing rule. According to Oude et al. (2016), the latter is a large scaffold-like RNA that is used to bind Cas9 nuclease and form a gRNA/Cas9 complex. According to Zhang et al. (2017), the gRNA/Cas9 complex must recognize and bind a PAM (NGG) of three nucleotides for the genome-editing mechanism to result in double-strand breaks (DSBs).

DSBs are repaired by either non-homologous end joining (NHEJ) or homologous directed repair (HDR), according to Xiao-jie et al. (2015) if homologous sequences are available. Instead of the error prone NHEJ correction, which can result in minute inserts or deletions (indels), HDR produces precise gene correction or replacement. According to research by Xiao-jie et

al. (2015), these indels eventually cause a gene disruption or gene knockdown. These methods have a sizable potential for editing any gene in any organism, claim Ran et al. (2013).

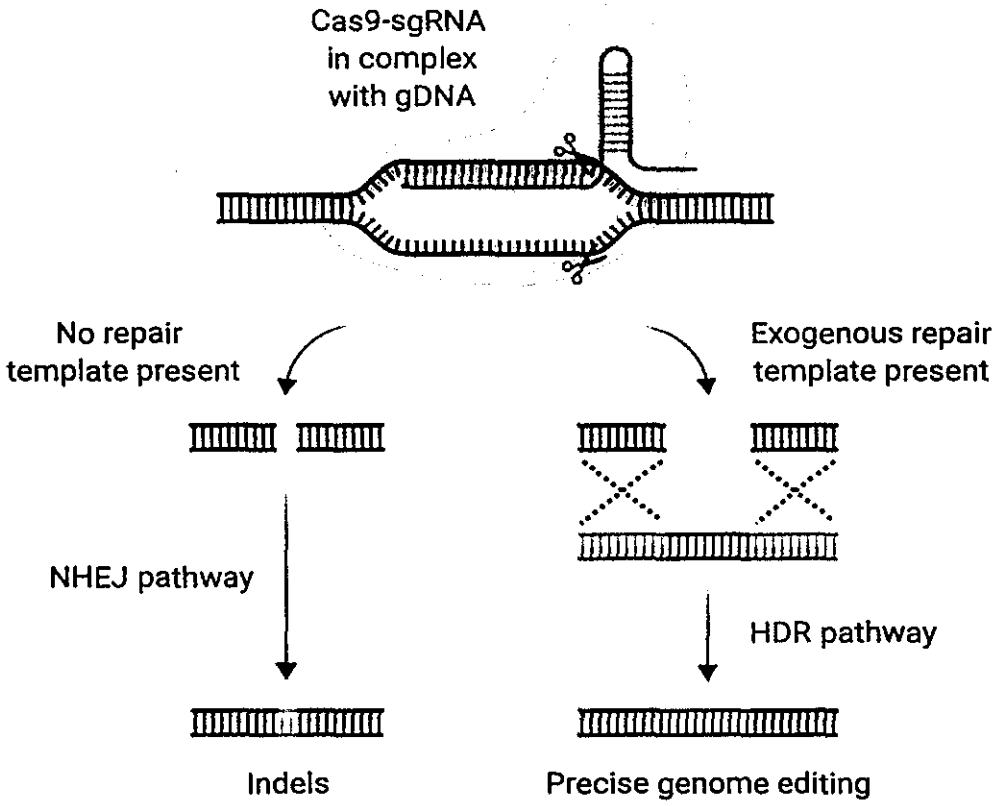

Figure 12: Mechanisms for CRISPR-Induced DSB Repair (Xiao-jie et al.,2015).

CRISPR/Cas9, which is considered more superior than zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) in a variety of aspects, including ease of modification, increased targeting effectiveness, and capacity for multiplex genome editing. Because new targets can be created as gRNAs from primers, CRISPR/Cas9 is more user-friendly than ZFN and TALEN (Zhang et al., 2017).

Since plasmid-mediated variants of CRISPR/Cas9 are less expensive and their genome-editing process may be completed in two weeks, it is more cost-effective (Zhang et al., 2017). By simultaneously targeting numerous genomic loci and co-delivering a variety of sgRNAs to the cells of interest, Cas9 is also very effective at editing a wide range of cell types and organisms (Ran et al., 2013). As a result, biological and biomedical research has made substantial use of

CRISPR/Cas9-based gene editing techniques, such as gene knockout, gene knock-in, gene interference or activation, and other chromosome-related applications.

We will use CRISPR/Cas9 because of the technique's adaptability to create a CRISPR-mediated ACE2 knockout to examine its function as a potential treatment target for Covid19. Even though several efficient vaccines have been approved and are accessible to the general public, new cases and rising mortality persist. The binding of the S protein to its receptor ACE2 is the first step in the entry, replication, and dissemination of the SARS-CoV-2 virus. Therefore, it has beenrationally considered could be a very promising therapy to address viral infection and maybe prevent the following effects to stop this binding event.

CHAPTHER 3: METHODOLOGY

3.1. Constructing Protein-Protein interaction network map of ACE2 with other major genes that related to covid.

In this study, a Protein-Protein Interaction PPI network was constructed to identify the connection between ACE2 and other genes and to study the role of ACE2 in SARS CoV 2. The Protein-Protein Interaction network was constructed using Cytoscape 3.10.0 from the DNEX database in the software. The search query is "ACE2" and "SARS CoV-2". The interaction network between the ACE2 and other genes were composed of a set of genes (nodes) connected by edges that represent the functional relationship between the genes. The complete network of ACE2 were obtained.

3.2. Determination of gene expression of ACE2, TMPRSS2, ARG1, IL6 involved in SARS-CoV-2 progression in HEK 293 via qRT-PCR.

3.2.1. Cell culture

The HEK 293 cell line were cultured Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cell lines are incubated at 37°C in a humidified environment with 5% carbon dioxide (CO2). The RNA extraction of the cells is carried out when the cells reached in between 70%-85% confluency which is when they are in logarithmic growth phase.

3.2.2. RNA Extraction

After the cell lines are ready, aspirated the media from the cell culture T25 flask and add 1ml of PBS solution to wash the cell culture Easy flask. After wash removed all the PBS solution and scraped the cell culture flask briefly. Trizol Reagent was used for extracted the total amount of RNA from the cell lines. Lysis was carried out by adding 1ml Trizol reagent to sample in PCR tube and homogenized the sample by shaking vigorously. The sample was incubated for 5 minutes at room temperature. Then 250 µl of chloroform were added, followed by 15 seconds of vigorous shaking, and incubated at room temperature for three minutes. After that, samples were centrifuged for 15minutes at 12000rpm at 4°C to get the phase separation. After the centrifugation, transferred the aqueous phase into fresh tube. Precipitated the RNA by mixing with 500 µl of isopropanol. The samples were incubated for 5 minutes and followed by centrifuged for 20 minutes at 12 000 rpmat 4°C. The sample is placed on ice. Then the supernatant was carefully removed, and pallet was washes in 1ml of 75% ethanol and the sample was centrifuged for 10 minutes at 3000 rpm at 4°C. Removed the supernatant pellet was air dried for 5-10 minutes. The RNA pellet was dissolved with nuclease free water. Mix gently and recentrifuged at 9500 rpm for 5min. Using a NanoDropTM spectrophotometer and gel electrophoresis, the quality and amount of the RNA extracted were evaluated. A 260/280 ratio of 1.8-2.0 was used to check the quality of the total RNA. To ensure the integrity of the RNA, it was electrophoresed on a 2% agarose gel with 0.2 g/mL ethidium bromide in 1X TBE buffer at 80 volts for 30 minutes. To use the whole RNA as a template for qRT-PCR (quantitative Real-Time PCR), the total RNA was then converted to cDNA.

3.2.3. CDNA synthesis from RNA sample

The TetroTM cDNA Synthesis Kit reverse transcription kit was used to convert RNA to cDNA in accordance with the manufacturer's instructions. The solutions were vortex and centrifuged briefly before use. The priming master mix was prepared on ice in a PCR tube by mixing following reagents.

Reagents	**Volume**
RNA sample (up to 5 µg)	1.0 µl
Primer: Oligo(dT)$_{18}$	1.0 µl
10mM dNTP mix	1.0 µl
5x RT Buffer	4.0 µl
RiboSafe RNase Inhibitor (10 U/ µL)	1.0 µl
Tetro Reverse Transcriptase (200u/µL)	1.0 µl
DEPC-treated water	11 µl
Total	**20.0µl**

The reaction was first incubated for 30 minutes at 45 °C, and then it was stopped by incubation at 85 °C for 5 minutes. The reaction was then put on ice right away.

3.2.4. Quantification of gene expression of ACE2, TMPRSS2, ARG1, IL6 inHEK 293 via qRT- PCR.

The expressions of the key genes *ACE2, TMPRSS2, ARG 1, and IL6* were measured using qRT-PCR, which measures the accumulation of fluorescence signal that is released when it intercalates with dsDNA (that is produced during the extension step of PCR). As a result, the amount of target DNA present in the sample is inversely correlated with the number of cycles required for the fluorescence signal to surpass the threshold (the background level) (CT value); the lower the CT value, the higher the amount of DNA in the sample.

To enable data normalization, the expression of the housekeeping (reference) gene GAPDH, which is constitutively expressed under any circumstance, was used as the sample. The primers specified in Table were used along with the Luna® Universal qPCR Master Mix kit to carry out the qRT-PCR. The manufacturer's method is described below and was used to make the target gene and housekeeping gene master mix solutions in PCR tubes.

Reagents	**Volume**
Luna Universal qPCR Master mix	10 µl
Forward primer (10µM)	0.5 µl
Reverse primer(10µM)	0.5 µl
Template DNA (CDNA)	1.0 µl
Nuclease free water	8.0 µl
Total	20 µl

The master solution was then divided into PCR tubes, briefly spined down, and subjected tothe following qRT-PCR conditions.

Table 1: The parameters for qRT-PCR conditions

Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95°C	60 seconds	1
Denaturation	95°C	15 seconds	45
Extension	60°C	30seconds	
Melt Curve	60-95°C	Various	1

Table 2: The qRT-PCR primers used for quantification of *ACE2, TMPRSS2, ARG1 & IL6* genes and housekeeping gene expression in HEK 293

Primers	**Sequence (5' →3')**	**Length**	**Tm**	**GC Content**
Qpcr_ACE2_F	TCCATTGGTCTTCTGTCACCCG	133	59.1°C	54.55 %
Qpcr_ACE2_R	AGACCATCCACCTCCACTTCTC		58.2°C	54.55 %
Qpcr_TMPRRS2_F	CCTCTAACTGGTGTGATGGCGT	121	59.0°C	54.55 %
Qpcr_TMPRRS2_R	TGCCAGGACTTCCTCTGAGATG		58.4°C	54.55 %
Qpcr_Arg1_F	TGATGTTGACGGACTGGACC	130	57.0°C	55.00 %
Qpcr_Arg1_R	ATCTAATCCTGAGAGTAGCCCTGT		56.6°C	45.8%
Qpcr_IL6_F	CCACCGGGAACGAAAGAGAA	77	57.2°C	55.00 %
Qpcr_IL6_R	TGTTACATGTTTGTGGAGAAGGA		54.2°C	39.1 %
Qpcr_GAPDH_F	GTCTCCTCTGACTTCAACAGCG	131	60.9°C	54.55
Qpcr_GAPDH_R	ACCACCCTGTTGCTGTAGCCAA		64.4°C	54.55

The primer sequences in table 2 were obtained by using Blast in National Biotechnology Information Centre (NCBI) (www.ncbi.com).

Each gene's average CT values (measured in triplicates) were identified, and they were then adjusted to be comparable to the average CT value for *GAPDH*. Further analysis of the computed CT values was conducted using the comparative threshold cycle and (CT) 2-(CT) calculation methods, and the results were shown as fold changes in gene expression.

3.2.5. Statistical analysis

The RT-PCR reactions were prepared in triplicates. The expression data were displayed using mean and standard deviation. By using a one-way ANOVA test, the means of the three replicated experiments were compared. The Grapad prism software was used to conduct all statistical analyses. In each experiment, statistical significance was set at $P < 0.05$.