HHS Public Access

Author manuscript

Nat Protoc. Author manuscript; available in PMC 2021 June 01.

Published in final edited form as:

Nat Protoc. 2020 December ; 15(12): 3777–3787. doi:10.1038/s41596-020-00403-2.

Genetically modified mouse models to help fight COVID-19

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Abstract

The research community is in a race to understand the molecular mechanisms of SARS-CoV-2 infection, to repurpose currently available antiviral drugs, and to develop new therapies and vaccines against COVID-19. One major challenge in achieving these goals is the paucity of suitable preclinical animal models. Mice constitute around 70 percent of all of laboratory animal species used in biomedical research. Unfortunately, SARS-CoV-2 only infects mice if they have been genetically modified to express human ACE2. The inherent resistance of wild-type mice to SARS-CoV-2, combined with a wealth of genetic tools that are only available for modifying mice, offer a unique opportunity to create a versatile set of genetically engineered mouse models (GEMMs) useful for COVID-19 research. We propose three broad categories of these models and more than two dozen designs that may be useful for SARS-CoV-2 research and for fighting COVID-19.

EDITORIAL SUMMARY

In this Perspective, the authors discuss strategies for creating a versatile set of 30 genetically engineered mouse models (GEMMs) useful for COVID-19 research. Additionally, they provide the genetic blueprints needed for developing these GEMMs.

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Author Contributions: C.B.G and M.O conceived the overall idea and wrote the initial manuscript. R.M.Q, G.P.R, L.P and S.L.M contributed additional conceptual ideas and revised the manuscript. C.B.G, R.M.Q and M.O developed the model designs (supplementary file information).

Competing interests: University of Nebraska Medical Center has filed a provisional patent on the proposed mouse model designs (inventors: C.B.G, R.M.Q and M.O).

Introduction

The research community is in desperate need of animal models for testing therapeutics and vaccines against COVID-19, and for studies aimed at understanding the molecular mechanisms of SARS-CoV-2 infection and pathogenesis. A wide spectrum of laboratory and domestic animal species have been challenged with SARS-CoV-2 in an effort to find suitable models, but only a few, such as hamsters, cats, ferrets, and monkeys are susceptible[1–3]. Unfortunately, many of these species are neither accessible nor practical for the vast majority of researchers to make rapid progress.

Mice are the most commonly used laboratory animals in biomedical research, and they have helped scientists develop diagnostics, therapeutics, and vaccines for many human diseases. As such, it would have been easy to use mice for COVID-19 research if they were susceptible to SARS-CoV-2 infection, like humans, but that is not the case. One way to render mice susceptible to SARS-CoV-2 is to genetically modify them.

Evaluating the utility of current GEMMs previously developed for SARS-CoV research

Several GEMMs that express the h*ACE2* gene were created over a decade ago for use in SARS virus studies[4–7]. Like SARS-CoV, SARS-CoV-2 uses the hACE2 receptor for entry into cells. Therefore some of these transgenic models have been investigated for their suitability for SARS-CoV-2 studies[8,9]. Repurposing of previously made GEMMs and non-GEMMs models (discussed in the next section), including animals other than mice, for the study of novel coronaviruses has been extensively reviewed by Yuan et al., 2020[10], and Singh et al., 2020[11]. Even though the existing h*ACE2* transgenic mice fully support SARS-CoV-2 replication, the pathogenesis does not accurately model the disease course seen in humans[12] and they also have other limitations. *First*, because these models still express the mouse orthologue of ACE2 (mAce2), this will occupy a portion of the cell surface pool of total ACE2, leading to non-physiological amounts of the human ACE2 available for virus interactions. *Second*, the transgenic expression cassettes in current models are driven by different promoters, and often expressed in different types of cells and at levels that are not similar to endogenous mACE2 expression. Furthermore, constitutively expressing transgenic mouse models are not suited to experiments requiring spatiotemporal regulation of hACE2 expression (*i.e.*, in specific cell types, such as lung epithelial cells, and/or at specific time points). Finally, these models will not be ideal for researchers wanting to simulate comorbid conditions such as obesity, hypertension and diabetes, in which affected tissues will have altered ACE2 expression[13,14].

Mouse models developed using non-GEMM approaches

Because the scientific community has such an urgent need for preclinical models, some groups explored non-GEMM strategies that were also previously used for SARS-CoV studies, such as (i) adapting the virus by serial passage through mice so that infectious mutant strains emerge[15], and (ii) delivering hACE2-encoding DNA through Adenovirus (AdV) vector systems followed by infection of the mice with SARS-CoV. Both of these

strategies were demonstrated recently as proof-of-principle for SARS-CoV-2[12,16–18]. Adaptation of SARS-CoV-2 virus to mice[4] did not cause lethality, but the approach seems useful for testing vaccine efficacy[12,16]. Delivering h*ACE2* into mice through an AdV vector system demonstrated the utility of the approach for pathogenicity studies, testing vaccines, and therapeutics[17,18]. Some limitations of the AdV vector approach, however, include the requirement for large quantities of vector and mouse-to-mouse variability in the response to infection. As most mice do not develop severe disease[19], they do not develop the extrapulmonary manifestations seen in humans[17], and some animals show lung pathology resulting from AdV administration itself[18]. Direct comparisons of GEMMs versus non-GEMMs show that the GEMMs provide a stable system for testing the ability of vaccines and antivirals to protect against disease, whereas the non-GEMMs offer the flexibility to be used across multiple genetic backgrounds[19].

Versatility of GEMMs

While the previously generated transgenic h*ACE2* mice and the non-GEMM approaches have successfully established that mice can be re-tooled as preclinical models for COVID-19 studies, sophisticated GEMM approaches provide a much more versatile suite of COVID-19 models than can be envisioned for any other species. The natural non-permissiveness of mice to SARS-CoV-2 infection combined with the wide variety of available genetic tools and molecular switches offers a unique opportunity to make this species even more useful for COVID-19 research.

Researchers are now facing a familiar COVID-19 narrative: trying to make sense of a mystifying illness[20] affecting not only the respiratory system, but also many other organs, including those of the cardiovascular[21], digestive[22] and nervous systems[23]. If a researcher wants to study the pathophysiological and molecular events occurring only in the heart, intestine or brain, it would be difficult to study such events in an animal model in which many other organs are also infected (for example, lung or kidney). If the animal model allowed productive infection of only one type of tissue or cell (cardiac or endothelial or neuronal), and if the model allowed regulation of the dose and timing of infection, it would be very helpful in answering a variety of research questions relevant to cardiac or endothelial or neuronal manifestations. Related to this, a recent report described the expression pattern of *ACE2* in human tissues. Although somewhat intriguing, expression of ACE2 seemed to be very low in lung cells while it was detected at high levels in >150 cell types corresponding to 45 tissues[24]. This observation further highlights the value of the conditional and inducible expression models where researchers working in a wide range of tissue types can use the models to ask specific questions by limiting h*ACE2* expression and viral infection to only subset of tissue types.

This type of controlled experimental design is possible only in mice because the genetic tools and molecular switches (*e.g., Cre-LoxP* and Tetracycline-inducible systems) are readily available for multiple cell types and tissue systems. For example, a CRE-activatable h*ACE2* knock-in mouse model, once created, could be bred to any of the thousands of already available *Cre* driver lines, thus allowing researchers from a range of biomedical fields, from cardiac physiologists to neuroscientists, to use this model for probing their

particular research questions of interest. Additionally, individual gene knockout (KO) mouse models are available for thousands of genes. The molecular pathways of viral infection can be further dissected through classical mouse genetic studies by breeding the COVID-19 models with a number of different gene KO models. Thus, mice can be very useful models in the fight against COVID-19. In this Perspective, we propose concepts for developing more than two dozen GEMMs suitable for a variety of research on COVID-19 and SARS-CoV-2.

We propose the following three broad categories of GEMMs for COVID-19 research. 1) Knocking-in expression cassettes, or point mutations, into the endogenous mouse *Ace2* locus. 2) Knocking-in CRE-activatable- or Tetracycline-inducible-h*ACE2* expression cassettes into safe-harbor loci, by re-engineering the existing reporter or inducer lines. 3) Knocking-in CRE-activatable cassettes into the mouse *Ace2* locus. The proposed GEMMs are listed in Table 1; the schematics of the GEMM designs are shown in Figures 1–3.

GEMM Category 1: Knocking-in expression cassettes or point mutations into endogenous mouse *Ace2* loci

One of the ideal GEMM*s* for COVID-19 research is a Knock-Out:Knock-In (KO:KI) design in which the mouse *Ace2* gene is deleted (knocked-out [KO]), and the human *ACE2* cDNA is inserted in its place, termed Model **#1** herein. Both mouse and human *ACE2* genes are located on the X chromosome, and each contains 19 exons. Although all corresponding exons are the same size, the mouse introns are larger, with a total gene size of 49kb and 41kb in mice and humans, respectively. The KO:KI model can be generated by deleting the region between the start and the stop codons of mouse *Ace2* (*i.e.*, all coding exons and the introns between) and inserting the 2.4 kb h*ACE2* cDNA. In the KO:KI design, because the mouse counterpart of *ACE2* (m*Ace2*) is deleted, any potential confounding effects on the response to virus infection will not occur, unlike in the h*ACE2* transgenic mice with random insertions. In this model, the mouse *Ace2* promoter is expected to drive human *ACE2* cDNA expression at physiological levels, although the effect of deleting the mouse introns cannot be ascertained, as they may contain regulatory regions. While our manuscript was under preparation, a model similar to this design was reported in which the h*ACE2* cDNA was inserted near the start codon of the mouse *Ace2*[25]. Although the m*Ace2* genomic region is still retained in this model, m*Ace2* is not likely to be expressed because the insertion cassette contains a poly (A) sequence upstream of the m*Ace2* coding exons. This design also includes additional regulatory elements such as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), which enhances mRNA stability and translation efficiency and may produce higher than physiological hACE2 protein levels. The ideal model design would be one that can achieve near-physiological levels of hACE2 expression. Such a model design would require precise replacement of all of the 19 mouse exons with the corresponding human exons, leaving the mouse introns, which may contain regulatory sequences, intact. Developing such a model would, however, be quite difficult. One other feature included in the published model[25] is a fluorescent reporter (tdTomato [tdT]) expressed as a separate protein via an internal ribosomal entry site (IRES) element, with the reporter serving as a handy tool to mark the hACE2-expressing cells. We propose to include

a self-cleaving viral peptide P2A, instead of the IRES element, fused to the C terminus of hACE2, followed by the EGFP coding sequence in model **#2**.

Ten to fifteen years ago, generating a KO:KI model like the one described above would have required multiple lengthy steps of complex gene targeting in embryonic stem cells, single-cell cloning, and chimera breeding. The process would typically take over a year just to develop a founder mouse, and may be the reason why such a model was not created. In addition, it is possible that when SARS-CoV research declined, scientists were not motivated to invest the necessary time and resources. Now, the situation is quite different; the COVID-19 pandemic has increased the demand for any GEMM available for SARS-CoV-2 infection, even though the existing models are suboptimal for certain COVID-19 research designs. On the bright side, GEMM technologies have improved tremendously during the last decade due to the advent of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology[26–35]. Thus, more sophisticated GEMM*s* (e.g., the KO:KI model) can be created within the timespan of a few months.

Additionally, CRISPR tools offer many more technical options than were previously possible[36]. For example, GEMMs can be created in any strain background. While we propose that models #1 and #2 be made in the C57BL/6 reference mouse strain, which is immunocompetent, we also propose that these design strategies are implemented in an immunocompromised mouse strain background, such as NOD/scid-γ_c^{null} (NSG/NOG). NSG mice lack mature T cells, B cells, and natural killer (NK) cells[37]. These GEMMs (#3 and #4) will be useful in many ways. First, due to the immunosuppressed background, there may be uncontrolled SARS-CoV-2 replication, which would make this strain particularly suitable for screening antiviral drugs. Second, the NSG/NOG strain is very commonly used for transplantation of human hematopoietic stem cells, which leads to the development of a humanized immune system[38–41]. These models will be suitable for pathogenesis studies involving interactions of the human immune system with the virus and can lead to better understanding of the role of human macrophages permissive for SARS-CoV-2 and mechanisms of human immunopathogenesis[42]. *Third*, some COVID-19 patients exhibit cytokine storm syndromes and immunosuppression[43], which are the result of active viral replication in an immune deficient host. Therefore, GEMMs allowing SARS-CoV-2 infection in immunocompromised backgrounds can serve as valuable tools for such immunological studies. *Lastly*, humanization of NSG/NOG mice with hACE2 will provide the ability to study the reaction of human immune cells in viral replication. This can be achieved by creating additional genetic modifications in GEMMs, which will allow more sophisticated immunological studies involving transplanting and infecting human cells into the mice, similar to studies with HIV[44,45]. Considering that the BALB/c is one of the most commonly used mouse strains used for SARS and MERS virus research[46,47], we also propose developing the KO:KI model in the BALB/c background strain (Models #5 and #6). For more information on additional strain considerations see the ‘Future Directions” section.

As an alternative to the KO:KI design, one could mutate the amino acids of mACE2 into those required for hACE2 binding to the SARS-CoV-2 spike protein. Crystal structures[48,49] showed that five residues (K31, E35, D38, M82 and K353) are critical for receptor binding[50]. Of these, residues K31, M82, and K353 differ in mice. Therefore, changing just

these three residues could allow mACE2 to bind effectively to the SARS-CoV-2 spike protein and permit infection of expressing cells. This strategy would retain any regulatory elements within the mouse introns, and it should result in nearly physiological levels of a functional mouse ACE2 receptor. These models are termed #7, #8, and #9 in C57BL/6, NSG/NOG and BALB/c strains, respectively. A similar point mutation knock-in strategy in which two amino acids (288 and 300) of the DPP4 receptor for MERS-CoV were mutated to render mice susceptible to MERS-CoV was reported previously[51].

Transmembrane protease serine 2 TMPRSS2 is a protease important in cleaving hACE2 and SARS-CoV-2 spike proteins, enabling efficient viral entry into cells[52]. It is possible h*ACE2* expression alone in mice will be insufficient to permit infection and viral pathogenesis at a level similar to humans. However, co-expression of h*TMPRSS2* may achieve this level. Models #10 to #15 (and #25 to #30: see below in category 3) are proposed for the co-expression of h*TMPRSS2*. KI designs for the purpose of this co-expression would involve either fusing TMPRSS2 to the C terminus of the hACE2 KI cassette via a self-cleavable P2A peptide, or expressing TMPRSS2 as a separate polypeptide via an IRES. These designs are similar to models #1, #3, and #5 but contain P2A-hTMPRSS2 for C-terminus fusion (models #10 to #12) or IRES elements (models #13 to #15). In certain cases, fusion of additional peptide sequences (*e.g.*, P2A) may affect protein folding and function, which can be avoided by using IRES elements. However, the gene downstream of the IRES element may not express as efficiently as the gene placed upstream of it. A strategy of co-expressing hACE2 and hTMPRSS2, similar to the ones we proposed here, was also proposed by Soldatov et al., 2020 (ref. 53), although these authors suggested inserting the expression cassettes into the m*TMPRSS2* locus instead of the m*Ace2* locus. Co-expression of hTMPRSS2 may not be necessary in the case of the key amino acid KI mutant models (#7 to #9) because the mACE2 will likely retain its endogenous cleavage site for mouse TMPRSS2 proteins. It is unknown, however, whether mTMPRSS2 will cleave SARS-CoV-2 spike proteins.

GEMM Category 2: Knocking-in CRE-activatable or tetracycline-inducible expression cassettes into safe-harbor loci by re-engineering existing reporter or inducer mouse lines

Models #1 to #15 will express h*ACE2* alone or in conjunction with h*TMPRSS2* in all cells in which the m*Ace2* promoter is active. There will, however, be many situations in which scientists require GEMMs for answering very specific questions. For example, what molecular events are perturbed when only cardiac, intestinal, or lung epithelial cells in the mouse are infected with SARS-CoV-2? Such a scenario would require a GEMM expressing h*ACE2* in only one of the respective cell types, and this expression could be switched on or off when needed. Models #1 to #15 do not offer flexibility to control the timing or tissue specificity of expression.

There are sophisticated molecular genetic switches, however, such as Cre-*LoxP* and tetracycline-inducible systems that can be included in GEMM designs to allow such defined experimental scenarios. Leveraging the vast numbers of the available *Cre* driver lines created by the mouse genetics community, CRE-activatable KI mice can be bred to those driver lines

to remove the stop cassette and to express the insertion cassette. For example, a KI mouse containing a *LoxP*-Stop-*LoxP* cassette between the promoter and h*ACE2* cassette will express h*ACE2* only after the removal of the stop cassette, and this can be achieved by breeding the KI mouse with a *Cre* driver line. Similarly, a KI mouse in which a tetracycline response element drives h*ACE2* expression could be used for turning on or off expression of h*ACE2* in any desired cell type at any given time point. Numerous tetracycline-inducible models that express the tetracycline transactivator (tTA) or reverse tTA (rtTA) have been generated by the community and can be bred with the KI model to turn on or off h*ACE2* expression simply by administering/withdrawing doxycycline. The conditional and inducible expression systems described below will also offer a degree of biosafety compared to constitutively expressing models, as they will likely not produce potentially infectious virus particles before the start of the experiments.

GEMMs #16 and #17 offer constitutive expression with a reporter capability, and models #18 and #19 offer CRE-activatable features. These models will contain hACE2 fused to a fluorescent reporter, such as EGFP or tdT, either through a self-cleavable P2A peptide or expressed as a separate protein via an IRES. The four models will be useful for many different scenarios, including constitutive expression, tissue-specific expression or for turning on or off expression of the receptor as needed, simply by breeding them with *Cre* driver lines (in the case of models #18 and #19), which are available for thousands of promoters. Fluorescent reporters in animal models will offer a useful tool for histological and biochemical experiments. Models #20 and #21 offer tetracycline-inducible features wherein the expression cassettes are placed downstream of tetracycline response elements. As above, these models will contain hACE2 fused to a fluorescent reporter either through a self-cleavable P2A peptide or expressed as a separate protein via IRES. The expression of the transgenes can be achieved in an inducible fashion by breeding the models with tTA/rtTA driver lines and through doxycycline administration.

These KI models (#16 to #21) can be developed by re-engineering the genomes of some of the previously developed GEMMs, using CRISPR approaches. This re-engineering approach will allow the use of available GEMMs harboring proven genetic elements such as *LoxP*, tetracycline response elements, polyA signals and built-in reporters. For generating models #16 to #19, for example, a reporter mouse model called mT/mG offers a suitable model for re-engineering. The mT/mG double-fluorescent *Cre* reporter mouse contains a floxed cassette of membrane-targeted tdT (mT) followed by a membrane-targeted green fluorescent protein (mG), targeted into the *ROSA26* locus, one of the most commonly used safe harbor sites in the mouse genome. The mT reporter is expressed in every mouse cell. Once the Cre recombinase is introduced via breeding, the mT cassette is deleted, allowing the CAG promoter to express the mG reporter[54]. This mouse strain is available for purchase from The Jackson Laboratory (JAX Stock No. 007676). To create tetracycline-inducible KI models (#20 and #21), the mouse strain Ai63 can be re-engineered to insert the h*ACE2* cDNA near the start codon of the tdT sequence, either as a fusion via a P2A peptide or with an IRES. The Ai63 mouse was developed in Dr. Hongkui Zeng's laboratory at Allen Brain Research Institute in Seattle and expresses tdT under the Tetracycline Response Element (TRE) promoter[55].

GEMM Category 3: Knocking-in CRE-activatable cassettes into mouse *Ace2* locus

Another advance in developing models for COVID-19 would be to combine the features of knocking-in expression cassettes with conditional potential. In models #22, #23 and #24, CRE-activatable inversion cassettes expressing h*ACE2*, flanked by a combination of mutant *LoxP* elements (*Lox2272* and *5171*)[56] are inserted into the intron 1 of mouse *Ace2*. The coding sequences are placed in the opposite orientation to the m*Ace2* promoter. Upon CRE-mediated recombination, the coding sequences will be inverted, thereby placing them in the correct orientation to be expressed from the *mAce2* promoter, enabling the promoter to drive expression of h*ACE2*. In models #25 to #30, the inversion cassettes express both hACE2 and hTMPRSS2 (either fused to P2A, or as separate proteins using an IRES in between). We call this design, used in models #22 to #30, a conditional KI (cKI) strategy. These GEMMs are expected to express near-physiological levels of hACE2 along with the required amounts of hTMPRSS2 upon CRE-mediated recombination. These models will be particularly suitable for research projects that address well-defined questions requiring selective expression of the two key proteins (hACE2 and hTMPRSS2) only in certain cell types of the mouse at a specific time point of interest. While numerous Cre driver lines developed in the C57BL/6 strain (or the ones backcrossed to this strain) are readily available, such strains are not available for the NSG/NOG and BALB/c strains. Considering that Cre driver lines can be generated using robust technologies like *Easi*-CRISPR[30,32] quite easily, such models can be generated as needed very rapidly (~2 months' time to generating and identifying the G0 founder mice)[32].

Details of all the 30 GEMM designs are given in Table 1. The full sequences of the targeting constructs and the guide RNA sequences, and additional notes of the models are given in Supplementary Note 1. The targeting constructs for these models, except the ones containing h*TMPRSS2* coding sequences, are ~3 kilobases or shorter in length. These models can be generated using the *Easi*-CRISPR approach, which uses long single-stranded DNA as a donor for highly efficient insertion of sequences into the mouse genome[30,32]. The models containing h*TMPRSS2* sequences are longer than 4kb and could also be generated using the *Easi*-CRISPR approach, but the efficiency may be lower and require the injection of more zygotes to achieve the faithful insertion of a full-length cassette. Alternative strategies of using plasmid DNA constructs (circular dsDNA) containing much longer homology arms[25,28,57,58] or ssODN mediated knock-in of large cassettes[31] can be attempted in the event that the *Easi*-CRISPR method is not successful in generating the desired models containing h*TMPRSS2*.

Concluding remarks

Mice are the most commonly used laboratory animals in biomedical research, and they have helped scientists develop diagnostics, therapeutics and vaccines for many human diseases. While the previously generated transgenic h*ACE2* mice and non-GEMM approaches (such as adaptation of virus to mice and adenovirus mediated delivery of h*ACE2*) have been successfully validated as potential preclinical models for COVID-19 studies, more

sophisticated GEMM approaches will render mice much more versatile COVID-19 models than can be envisioned for any other species. The availability of a wide variety of genetic tools and molecular switches available only in mice offers a unique opportunity to make this species useful for COVID-19 research. We described three broad categories of GEMMs and about 30 different model designs, in three important mouse strains that may be useful for SARS-CoV-2 research and for fighting COVID-19. By using robust CRISPR-based strategies such as *Easi*-CRISPR[30,32] and/or *i*-GONAD[33,34], the founder mice for the majority of these models, particularly those involving only insertion of h*ACE2* and point mutation knock-ins can be generated within ~2 months. The designs involving both h*ACE2* and h*TMPRSS2* insertion are more complex because of the longer insertion sizes. They may require the injection of larger numbers of zygotes to create knock-in models because as the size of the insertion cassettes become longer, the efficiency of full-length cassette insertion becomes lower. Nevertheless, even the simpler models will rapidly provide the opportunity for significant advances. Furthermore, the use of GEMM mice together with SARS-CoV-2 mouse adapted virus[12,16] may provide the ideal mouse model for COVID-19.

Future Directions

COVID-19 is a new disease. There are many unknowns concerning SARS-CoV-2, including its pathogenicity. Nevertheless, the research community is making advances at a remarkable speed. At this stage, the proposed GEMMs suitable for COVID-19 and SARS-CoV-2 research are based on the available information. Additional models could include expressing soluble h*ACE2*[59] along with FURIN protease[60], with the latter cleaving and producing mature proteins required for viral entry. A recent study identified polymorphisms in the human *ACE2* and *TMPRSS2* genes that may explain variability in susceptibility to COVID-19[61]. These polymorphisms could be engineered into mice for comparison with the proposed GEMMs expressing the standard human genes.

In addition, over the last century, the laboratory mouse has been bred to produce an extensive catalogue of inbred, hybrid and outbred strains, which collectively provide an elegant system suitable for studying many human disease conditions. This wealth of strain resources could be leveraged for SARS-CoV-2 disease research. For example, the GEMM designs proposed here can be generated in additional mouse strains taking into consideration SARS-CoV-2 co-morbidities and human genetic diversity. The New Zealand Obese (NZO) mouse strain could be useful for simulating SARS-CoV-2 co-morbidities[62] of obesity and diabetes because of its natural insulin resistance and susceptibility to developing these conditions[63]. Hybrids of the New Zealand Black (NZB) and New Zealand White (NZW) strains could be considered for simulating autoimmune conditions, as they have been used as models of human systemic lupus erythematosus[64]. Several inbred and hybrid strains have been tested for their suitability for performing studies of cardiac function[65], vascular properties[66], hypertension[67] and arrythmia[68]. These demonstrate a wide range of variability in assay performance among genetic backgrounds, however, suggesting that careful considerations should be made in choosing strain backgrounds for a given assay in cardiovascular disease research.

During the last decade, a novel set of strains called Collaborative Cross (CC) strains were also added to the vast array of mouse genetic backgrounds. CC strains are specially designed to overcome the narrow genetic diversity of inbred strains by crossing many inbred mouse lines together[69]. The complex mix of human genetic diversity is expected to be reflected in the mouse CC strains. These strains have been used for a wide range of studies, including identifying genetic modifiers affecting the susceptibility for the pathogenicity of infectious diseases affecting human lung[70]. Genome-wide studies have been performed by infecting CC strains with mouse adapted SARS-CoV to identify polymorphic host genes that contribute to pathogenesis, and these have identified genes such as *Trim55*[71] and *Ticam2*[72]. Related to this, human genome-wide association studies (GWAS), have identified genomic regions that may be important for SARS-CoV-2 susceptibility and severity. Interestingly, preliminary studies have identified a region of chromosome 3p21.31 comprising six genes[73,74], one of which is *SLC6A20*, a gene that encodes a transporter interacting with hACE2.

Incorporating factors like FURIN protease, or polymorphic versions of either *ACE2*, TMPRSS2 or SLC6A20 similar to our proposed GEMM designs, or producing our proposed GEMMs in additional strain backgrounds including collaborative cross strains can extend the COVID-19 GEMM list from #31 onward and vastly increase opportunities to understand COVID-19. Furthermore, using mouse as the model system allows us to leverage the results of research into over three dozen genes that have already been investigated with respect to coronavirus disease (see Figure 2 of a recent review by LoPresti et al)[75]. Systematically breeding the 30 knock-in models described here with each of the knockouts for those three dozen genes will serve as a valuable tool to understand more about coronaviruses.

The list of 30 GEMMs proposed herein offers a foundation for the research community to generate the first set of small animal models for COVID-19 and SARS-CoV-2 research. These are expected to prove valuable preclinical models for evaluating COVID-19 therapeutics and vaccines, which may continue to be important for studying the next coronavirus that may jump from animals to people.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We thank Hongkui Zeng, Bosiljka Tasic, and Tanya Daigle (Allen Institute for Brain Science) for advice and discussions on the design of re-engineered alleles. We thank Melody A. Montgomery and Douglas D. Meigs (University of Nebraska Medical Center) for professional editing. C.B.G is funded by NIH grants R35HG010719, R21GM129559, R21AI143394, and R21DA046831. S.L.M is funded by NIH grants R01DC011819, R01DC014470. M.O is funded by JSPS (16KK0189) for the Promotion of Joint International Research (Fostering Joint International Research)

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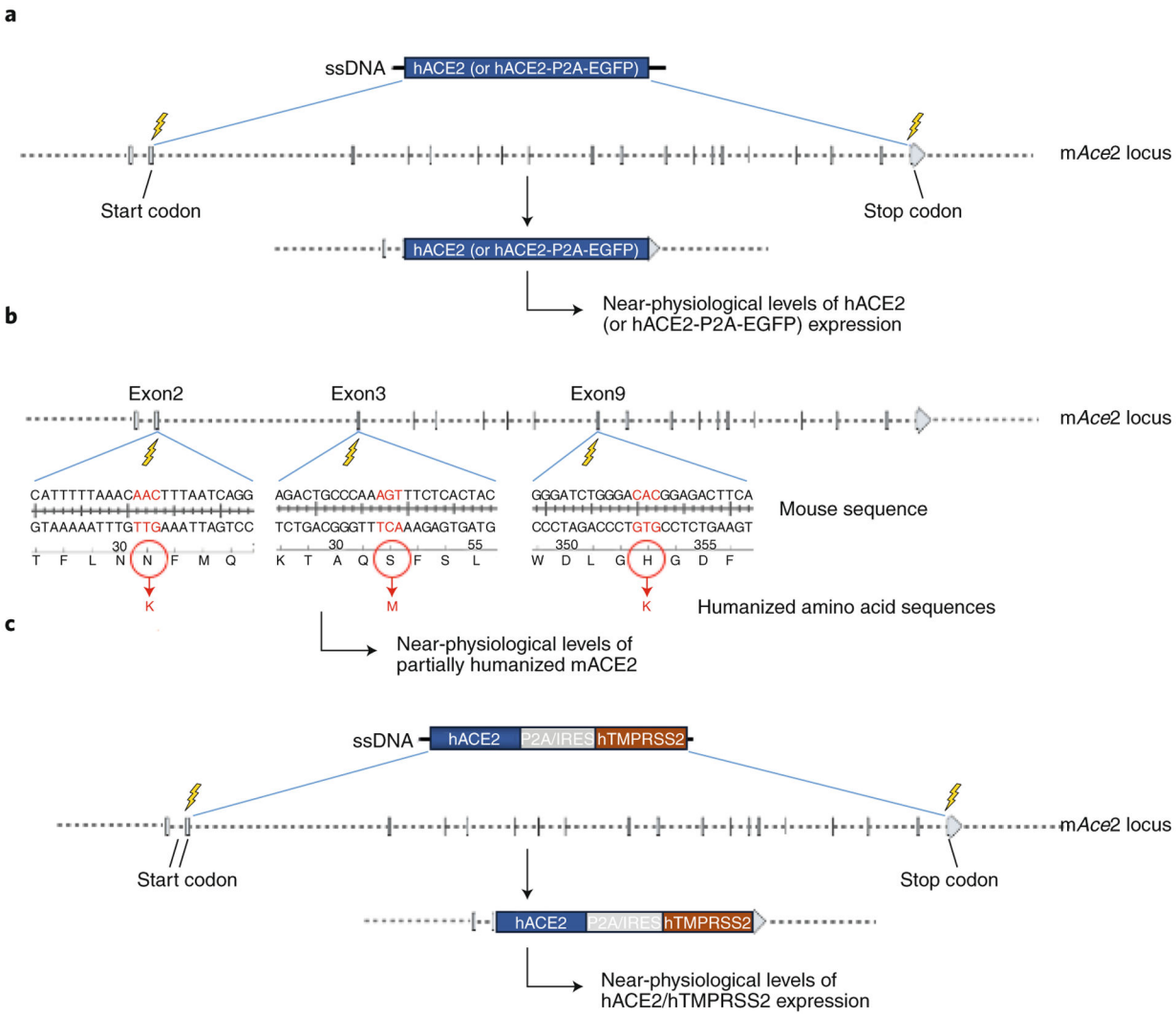

Figure 1. Schematics of COVID-19 GEMM designs, category 1: Knocking-in expression cassettes or point mutation changes into the endogenous mouse *Ace2* locus.

(**A**) KO:KI design; human *ACE2* cDNA (GEMMs #1, #3 and #5) or human *ACE2* cDNA fused to P2A-EGFP GEMMs #2, #4 and #6) will be inserted in place of mouse *Ace2* by deleting about 48kb of genomic sequence between the start and stop codons of m*Ace2*. (**B**) Key amino acids KI design (GEMMs #7 to #9); only the key amino acids that differ between mouse and human and are responsible for binding to the spike protein, will be replaced by knocking-in an ssODN donor containing the humanized codons. The locations of amino acids N31K, S82M and H353K are shown. (**C**) KO:KI designs to co-express h*ACE2* and h*TMPRSS2* (GEMMs #10 to #15). P2A: a self-cleavable viral peptide used for expressing two fusion proteins. IRES: Internal Ribosomal Entry Site.

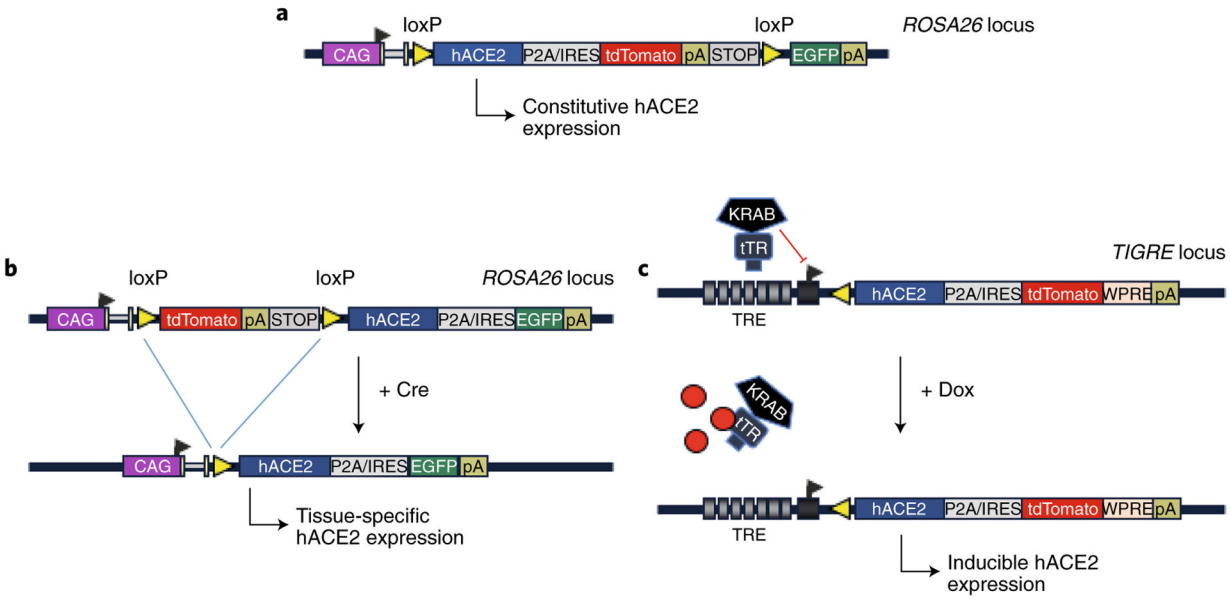

Figure 2. Schematics of COVID-19 GEMM designs, category 2: Knocking-in CRE-activatable or tetracycline-inducible expression cassettes into safe-harbor loci by re-engineering existing reporter or inducer mouse lines.

(A) Constitutive **(B)** CRE-activatable and **(C)** tetracycline-inducible designs (GEMMs #16 to #21). These models can be generated by re-engineering existing reporter and tetracycline-inducible models. P2A: a self-cleavable viral peptide used for expressing two fusion proteins. IRES: Internal Ribosomal Entry Site.

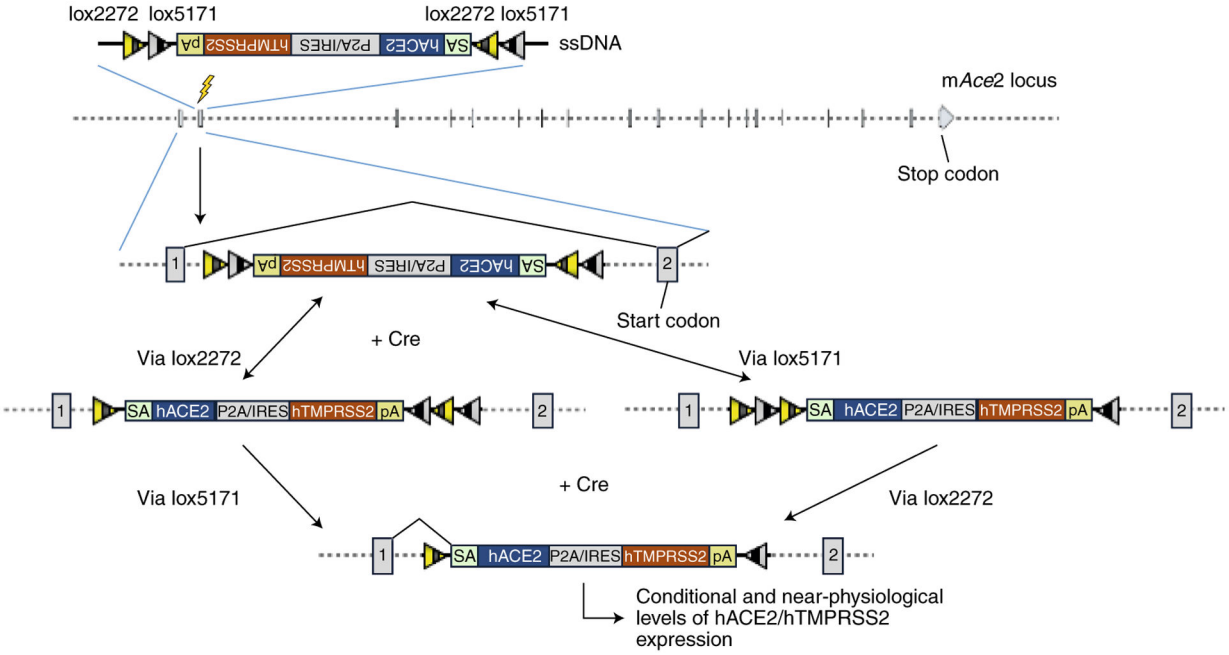

Figure 3. Schematics of COVID-19 GEMM designs, category 3: Knocking-in CRE-activatable cassettes into the mouse *Ace2* locus.

A CRE-invertible KI cassette is inserted in the opposite orientation to the m*Ace2* locus. Upon CRE-mediated inversion of the cassette through sequential recombinations of lox2272s and lox5171s, the transcript is spliced from exon1 to the inverted KI cassette and downstream transcription is terminated to prevent m*Ace2* function. This CRE-mediated inversion allows h*ACE2* expression in models #22 to #24 (not shown), or h*ACE2* and h*TMPRRS2* expression in models #25 to #30. SA: splice acceptor. P2A: a self-cleavable viral peptide used for expressing two fusion proteins. IRES: Internal Ribosomal Entry Site.

Table 1.

GEMM designs suitable for COVID-19 and SARS-CoV-2 research

Model #	Name	Locus/ promoter	Gene to express	Expression	Additional features
Category 1: Knocking-in expression cassettes or point mutations into endogenous mouse *Ace2* loci					
1	B6.mAce2^{KO}-hACE2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*	Constitutive	m*Ace2* gene is inactivated
2	B6.mAce2^{KO}-hACE2-P2A-EGFP^{KI}	m*Ace2*/ m*Ace2*	h*ACE2-P2A-EGFP*	Constitutive	m*Ace2* gene is inactivated, includes a reporter
3	NSG.mAce2^{KO}-hACE2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*	Constitutive	m*Ace2* gene is inactivated; immunocompromised mouse strain background, useful for studies involving interaction of human immune system
S4	NSG.mAce2^{KO}-hACE2-P2A-EGFP^{KI}	m*Ace2*/ m*Ace2*	h*ACE2-P2A-EGFP*	Constitutive	m*Ace2* gene is inactivated, includes a reporter; immunocompromised mouse strain background, useful for studies involving interaction of human immune system
5	BALB/c.mAce2^{KO}-hACE2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*	Constitutive	m*Ace2* gene is inactivated; mouse strain background commonly used for SARS and MERS virus research
6	BALB/c.mAce2^{KO}-hACE2-P2A-EGFP^{KI}	m*Ace2*/ m*Ace2*	h*ACE2-P2A-EGFP*	Constitutive	m*Ace2* gene is inactivated, includes a reporter; mouse strain background commonly used for SARS and MERS virus research
7	B6.mAce2$^{KI-31K,82M,353K}$	m*Ace2*/ m*Ace2*	Partially humanized m*Ace2*	Constitutive	Enables mACE2 to bind to the SARS-Co-V2 spike protein
8	NSG.mAce2$^{KI-31K,82M,353K}$	m*Ace2*/ m*Ace2*	Partially humanized m*Ace2*	Constitutive	Enables mACE2 to bind to the SARS-Co-V2 spike protein; immunocompromised mouse strain background, useful for studies involving interaction of human immune system
9	BALB/c.mAce2$^{KI-31K,82M,353K}$	m*Ace2*/ m*Ace2*	Partially humanized m*Ace2*	Constitutive	Enables mACE2 to bind to the SARS-Co-V2 spike protein; mouse strain background commonly used for SARS and MERS virus research
10	B6.mAce2^{KO}-hACE2-P2A-hTMPRSS2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Constitutive	m*Ace2* gene is inactivated; hTMPRSS2 fused to hACE2 via a self-cleavable P2A peptide
11	NSG.mAce2^{KO}-hACE2-P2A-hTMPRSS2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Constitutive	m*Ace2* gene is inactivated; immunocompromised mouse strain background, useful for studies involving interaction of human immune system; hTMPRSS2 fused to hACE2 via a self-cleavable P2A peptide
12	BALB/c.mAce2^{KO}-hACE2-P2A-hTMPRSS2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Constitutive	m*Ace2* gene is inactivated; mouse strain background commonly used for SARS and MERS virus research; hTMPRSS2 fused to hACE2 via a self-cleavable P2A peptide

Model #	Name	Locus/ promoter	Gene to express	Expression	Additional features
13	B6.mAce2^{KO}-hACE2-IRES-hTMPRSS2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Constitutive	m*Ace2* gene is inactivated; hTMPRSS2 expressed as a separate polypeptide via an IRES
14	NSG.mAce2^{KO}-hACE2-IRES-hTMPRSS2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Constitutive	m*Ace2* gene is inactivated; immunocompromised mouse strain background, useful for studies involving interaction of human immune system; hTMPRSS2 expressed as a separate polypeptide via an IRES
15	BALB/c.mAce2^{KO}-hACE2-IRES-hTMPRSS2^{KI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Constitutive	m*Ace2* gene is inactivated; mouse strain background commonly used for SARS and MERS virus research; hTMPRSS2 expressed as a separate polypeptide via an IRES
Category 2: Knocking-in CRE-activatable or tetracycline-inducible expression cassettes into safe-harbor loci by re-engineering existing reporter or inducer mouse lines					
16	ROSA26$^{(ACTB-Lox-hACE2-P2A-tdT-Lox-EGFP)}$	*ROSA26*/ pCAG	h*ACE2*	Constitutive	Constitutive expression of hACE2 with reporter capability
17	ROSA26$^{(ACTB-Lox-hACE2-IRES-tdT-Lox-EGFP)}$	*ROSA26*/ pCAG	h*ACE2*	Constitutive	Constitutive expression of hACE2 with reporter capability
18	ROSA26$^{(ACTB-Lox-tdT-Lox-hACE2-P2A-EGFP)}$	*ROSA26*/ pCAG	h*ACE2*	Tissue-specific	CRE-activatable expression of hACE2 with reporter capability.
19	ROSA26$^{(ACTB-Lox-tdT-Lox-hACE2-IRES-EGFP)}$	*ROSA26*/ pCAG	h*ACE2*	Tissue-specific	CRE-activatable expression of hACE2 with reporter capability.
20	Ai63-TIGRE-TRE-hACE2-P2A-tdT	*TIGRE*/ *TRE*	h*ACE2*	Tetracyclin-inducible	Tetracycline inducible expression of hACE2 with reporter capability
21	Ai63-TIGRE-TRE-hACE2-IRES-tdT	*TIGRE*/ *TRE*	h*ACE2*	Tetracyclin-inducible	Tetracycline inducible expression of hACE2 with reporter capability
Category 3: Knocking-in CRE-activatable cassettes into mouse *Ace2* locus					
22	B6.mAce2^{cKO}-hACE2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated allowing expression of h*ACE2*
23	NSG.mAce2^{cKO}-hACE2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated allowing expression of h*ACE2*; immunocompromised mouse strain background, useful for studies involving interaction of human immune system
24	BALB/c.mAce2^{cKO}-hACE2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated, allowing expression of h*ACE2*; mouse strain background commonly used for SARS and MERS virus research
25	B6.mAce2^{cKO}-hACE2-P2A-hTMPRSS2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated, allowing expression of h*ACE2* and h*TMPRSS2*; hTMPRSS2 fused to hACE2 via a self-cleavable P2A peptide

Model #	Name	Locus/ promoter	Gene to express	Expression	Additional features
26	NSG.mAce2^{cKO}-hACE2-P2A-hTMPRSS2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated, allowing expression of h*ACE2* and h*TMPRSS2*; immunocompromised mouse strain background, useful for studies involving interaction of human immune system; hTMPRSS2 fused to hACE2 via a self-cleavable P2A peptide, immunocompromised mouse strain background
27	BALB/c.mAce2^{cKO}-hACE2-P2A-hTMPRSS2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated, allowing expression of h*ACE2* and h*TMPRSS2*; mouse strain background commonly used for SARS and MERS virus research; hTMPRSS2 fused to hACE2 via a self-cleavable P2A peptide
28	B6.mAce2^{cKO}-hACE2-IRES-hTMPRSS2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated, allowing expression of h*ACE2* and h*TMPRSS2*; hTMPRSS2 expressed as a separate polypeptide via an IRES
29	NSG.mAce2^{cKO}-hACE2-IRES-hTMPRSS2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated, allowing expression of h*ACE2* and h*TMPRSS2*; immunocompromised mouse strain background, useful for studies involving interaction of human immune system; hTMPRSS2 expressed as a separate polypeptide via an IRES
30	BALB/c.mAce2^{cKO}-hACE2-IRES-hTMPRSS2^{cKI}	m*Ace2*/ m*Ace2*	h*ACE2*, h*TMPRSS2*	Tissue-specific expression of h*ACE2* at physiological levels	m*Ace2* gene is conditionally inactivated, allowing expression of h*ACE2* and h*TMPRSS2*; mouse strain background commonly used for SARS and MERS virus research; hTMPRSS2 expressed as a separate polypeptide via an IRES