Swine acute diarrhea syndrome coronavirus replication in primary human cells reveals potential susceptibility to infection

Caitlin E. Edwards¹,², Boyd L. Yount¹, Rachel L. Graham¹,², Sarah R. Leist¹,², Yixuan J. Hou¹,², Kenneth H. Dinnon III, Amy C. Sims², Jessica Swanstrom³, Kendra Gully², Trevor D. Scobey¹,², Michelle R. Cooley², Caroline G. Currie², Scott H. Randell³, and Ralph S. Baric¹,b,f,1

¹Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; ²Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; ³Chemical and Biological Signatures Division, Pacific Northwest National Laboratory, Richland, WA 99354; ⁴Department of Comparative Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; ⁵Marsico Lung Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; and ⁶Rapidly Emerging Antiviral Drug Discovery Initiative, University of North Carolina, Chapel Hill, NC 27599

Edited by Peter Daszak, EcoHealth Alliance, New York, NY, and accepted by Editorial Board Member Diane E. Griffin August 18, 2020 (received for review January 22, 2020)

Zoonotic coronaviruses represent an ongoing threat, yet the myriad of circulating animal viruses complicate the identification of higher-risk isolates that threaten human health. Swine acute diarrhea syndrome coronavirus (SADS-CoV) is a newly discovered, highly pathogenic virus that likely evolved from closely related HKU2 bat coronaviruses, circulating in Rhinolophus spp. bats in China and elsewhere. As coronaviruses cause severe economic losses in the pork industry and swine are key intermediate hosts of human disease outbreaks, we synthetically resurrected a recombinant virus (rSADS-CoV) as well as a derivative encoding tomato red fluorescent protein (trFP) in place of ORF3. rSADS-CoV replicated efficiently in a variety of continuous animal and primate cell lines, including human liver and rectal carcinoma cell lines. Of concern, rSADS-CoV also replicated efficiently in several different primary human lung cell types, as well as primary human intestinal cells. rSADS-CoV did not use human coronavirus ACE-2, DPP4, or CD13 receptors for docking and entry. Contemporary human donor sera neutralized the group I human coronavirus NL63, but not rSADS-CoV, suggesting limited human group I coronavirus cross protective herd immunity. Importantly, remdesivir, a broad-spectrum nucleoside analog that is effective against other group 1 and 2 coronaviruses, efficiently blocked rSADS-CoV replication in vitro. rSADS-CoV demonstrated little, if any, replicative capacity in either immune-competent or immunodeficient mice, indicating a critical need for improved animal models. Efficient growth in primary human lung and intestinal cells implicate SADS-CoV as a potential higher-risk emerging coronavirus pathogen that could negatively impact the global economy and human health.

SADS | coronavirus | One Health | emerging infectious disease

One Health recognizes that human, animal, and environmental health are tightly interconnected (1). In the 21st century, three novel human and three novel swine coronaviruses (CoVs) have emerged suddenly and spread globally, demonstrating a critical need for strategies that identify higher risk zoonotic coronaviruses (2). Contemporary human coronaviruses include four isolates (e.g., HCoV NL63, HCoV 229E, and HCoV OC43, HCoV HKU1) that reside within the group 1b and group 2a subgroups, respectively, and cause significant upper and lower respiratory infections in children and adults (3). These viruses likely originated from strains in bats, rodents, and bovine before the beginning of the 20th century (3). More recently, highly pathogenic human coronaviruses include the betacoronavirus subgenus Sarbecovirus severe acute respiratory syndrome coronavirus (SARS-CoV) strains that emerged in China in 2003 and the Merbecovirus Middle East respiratory syndrome coronavirus (MERS-CoV) strains that emerged in the Middle East in 2012. SARS-CoV and MERS-CoV cause an atypical pneumonia that rapidly progresses to acute respiratory distress syndrome, with fatality rates of 10% and 35%, respectively (4, 5). While the MERS-CoV outbreak is still ongoing throughout the Middle East and Sub-Saharan Africa, heterogeneous SARS- and MERS-like CoVs with human epidemic potential are circulating in bat species in Southeast Asia and elsewhere (6-8). As these data forecast, a new Sarbecovirus recently emerged in Wuhan, China in 2019 (SARS-CoV-2). As of September 2020, the rapidly expanding outbreak has surpassed 31 million cases, many of whom have progressed to respiratory failure, resulting in more than 972,000 deaths worldwide in the last 9 mo (see The Johns Hopkins University Dashboard, https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6) (9). Clearly, the cross-species transmission potential of zoonotic CoVs to humans and other important domesticated species remains high as global pathogens of concern (2, 10).

Over the past 80 y, several novel coronaviruses have caused extensive outbreaks and economic losses in swine, including transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea coronavirus (PEDV), porcine hemagglutinating encephalomyelitis virus (PHEV), and porcine deltacoronavirus (PDCoV) (11-14). Between October 2016 and 2019, highly pathogenic human coronaviruses included the betacoronavirus subgenus Sarbecovirus severe acute respiratory syndrome coronavirus (SARS-CoV) strains that emerged in China in 2003 and the Merbecovirus Middle East respiratory syndrome coronavirus (MERS-CoV) strains that emerged in the Middle East in 2012. SARS-CoV and MERS-CoV cause an atypical pneumonia that rapidly progresses to acute respiratory distress syndrome, with fatality rates of 10% and 35%, respectively (4, 5). While the MERS-CoV outbreak is still ongoing throughout the Middle East and Sub-Saharan Africa, heterogeneous SARS- and MERS-like CoVs with human epidemic potential are circulating in bat species in Southeast Asia and elsewhere (6-8). As these data forecast, a new Sarbecovirus recently emerged in Wuhan, China in 2019 (SARS-CoV-2). As of September 2020, the rapidly expanding outbreak has surpassed 31 million cases, many of whom have progressed to respiratory failure, resulting in more than 972,000 deaths worldwide in the last 9 mo (see The Johns Hopkins University Dashboard, https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6) (9). Clearly, the cross-species transmission potential of zoonotic CoVs to humans and other important domesticated species remains high as global pathogens of concern (2, 10).

Over the past 80 y, several novel coronaviruses have caused extensive outbreaks and economic losses in swine, including transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea coronavirus (PEDV), porcine hemagglutinating encephalomyelitis virus (PHEV), and porcine deltacoronavirus (PDCoV) (11-14). Between October 2016 and 2019, several novel coronaviruses have caused extensive outbreaks and economic losses in swine, including transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea coronavirus (PEDV), porcine hemagglutinating encephalomyelitis virus (PHEV), and porcine deltacoronavirus (PDCoV) (11-14). Between October 2016 and 2019, several novel coronaviruses have caused extensive outbreaks and economic losses in swine, including transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea coronavirus (PEDV), porcine hemagglutinating encephalomyelitis virus (PHEV), and porcine deltacoronavirus (PDCoV) (11-14). Between October 2016 and 2019, several novel coronaviruses have caused extensive outbreaks and economic losses in swine, including transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea coronavirus (PEDV), porcine hemagglutinating encephalomyelitis virus (PHEV), and porcine deltacoronavirus (PDCoV) (11-14). Between October 2016 and 2019,

Significance

The emergence of new human and animal coronaviruses demands novel strategies that characterize the threat potential of newly discovered zoonotic strains. We synthetically recovered recombinant wild-type and derivative swine acute diarrhea syndrome coronaviruses (SADS-CoVs) that express indicator genes and characterized their growth, macromolecular biosynthesis, and replication efficiency in a variety of mammalian cell lines, including primary human cells. The data demonstrate that SADS-CoV has a broad host range and has inherent potential to disseminate between animal and human hosts, perhaps using swine as an intermediate species.


The authors declare no competing interest.

This article is a PNAS Direct Submission. P.D. is a guest editor invited by the Editorial Board.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

To whom correspondence may be addressed. Email: rbaric@email.unc.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2001046117/-/DCSupplemental.

First published October 12, 2020.

www.pnas.org/cgi/doi/10.1073/pnas.2001046117

PNAS | October 27, 2020 | vol. 117 | no. 43 | 26915–26925
several novel coronavirus outbreaks were described in swine herds throughout China. Infection with the novel swine acute diarrhea syndrome coronavirus (SADS-CoV) was associated with acute diarrhea and vomiting with 90% mortality rates in piglets less than 5 d of age (10, 15–17). SADS-CoV is an alphacoronavirus most closely related to bat coronavirus HKU2, while also being distinctly related to other coronaviruses, such as HCoV 229E, HCoV NL63, and swine coronavirus PEDV (15). Rhinolophus spp. bats in the vicinity of local outbreaks had viruses (HKU2) with high sequence similarity to SADS-CoV strains, demonstrating that SADS-CoV likely originated from bats (10). The recent and rapid global dissemination of highly pathogenic variants of PEDV and PDCoV highlights the critical One Health threat associated with a newly emerged swine coronavirus (18, 19), and demonstrates a need for resources to understand the virus and its pathogenic potential in mammals.

The goal of this study was to evaluate human susceptibility for SADS-CoV cross-species transmission and replication. To address this question, we synthesized a full-length infectious clone and recovered wild-type and derivative recombinant (r)SADS-CoV that expresses tomato red fluorescent protein (rSADS-CoV tRFP). We used these viruses to study virus replication, transcription programs, and gene expression in vitro. We also demonstrated that SADS-CoV replicated efficiently in primary human cells derived from both the lung and intestine, highlighting an intrinsic potential for cross-species transmission and human susceptibility to infection. Although wild-type and IFN receptor (IFNRL)-immunodeficient mice were not susceptible, we demonstrated the availability of a small-molecule inhibitor that efficiently block SADS-CoV replication in vitro. While revealing the threat potential of SADS-CoV to humans and the global community, the reagents and models provide a critical infrastructure to study the molecular and evolutionary programs that promote virus cross-species transmission while providing for potential intervention strategies designed to control the pandemic spread of SADS-CoV in swine and potentially humans.

**Results**

**Assembly of SADS-CoV Full-Length cDNAs.** SADS-CoV likely emerged after multiple independent virus introductions from heterologous bat coronavirus strains circulating in bat populations into swine in China (10). We focused on the prototype SADS-CoV isolate (GenBank, accession no. MG557844) (Fig. S1). Importantly, and like several other zoonotic coronaviruses (25), one-step growth curves demonstrated efficient virus growth in swine LLC-PK1 and primate Vero CCL-81 kidney cultures with virus titers exceeding 10⁶ plaque forming units (PFU)/mL, in the presence, but not absence, of low levels of trypsin (10 μg/mL) (Fig. 2A and B).

Recombinant viruses that encode indicator genes provide tools to study virus host range, species specificity, and sensitivity to small-molecule inhibitors. To generate recombinant SADS-CoV indicator viruses, we replaced the accessory ORF encoding nonstructural protein 3a (ORF3a) with the gene encoding tRFP, noting that many downstream ORFs encode luxury functions in the Coronaviridae, including the ORF3 proteins of PEDV and HCoV NL63 (Fig. 1D) (23, 26, 27). Integrating tRFP into the SADS-CoV genome (rSADS-CoV tRFP) allows for real-time visualization of virus replication via fluorescence microscopy in living cells, providing a strong marker for productive virus infection, subgenomic transcription, and replication. Despite the loss of ORF3a, rSADS-CoV tRFP replicated to titers similar to wild-type rSADS-CoV, approaching ~10⁶ PFU/mL, in LLC-PK1 cells and Vero CCL-81 cells following a single-cycle growth curve over a 48-h time course (Fig. 2A and B). tRFP expression observed under a fluorescent microscope demonstrated productive infections of LLC-PK1 and Vero CCL-81 cells with SADS-CoV (Fig. 2D). Additionally, Northern blot analysis was utilized to detect the predicted mRNAs that should be expressed during SADS-CoV infection in vitro (Fig. 2E). These data demonstrate that ORF3 is likely not essential for efficient virus replication in vitro.

Alphacoronaviruses like the highly attenuated, nonelect Venezuelan horse encephalitis virus strain 3526 (VEE 3526) have been engineered as replcics to express high concentrations of foreign transgene proteins in cell culture and in animals (28). To evaluate SADS-CoV structural protein synthesis following infection, we produced VEE 3526 replicon particles (VRP) expressing the SADS-CoV N and spike (S) genes. Using mouse antisera from mice infected with the VEE VRP expressing the SADS-CoV N gene, we demonstrated extensive N protein expression in LLC-PK1 cells via Western blotting (Fig. 2C). Additionally, CCL-81 cells infected with rSADS-CoV tRFP were stained using both N and S antisera from vaccinated mice and images observed via immunofluorescence assay (Fig. 3D and E). Both N and S proteins are clearly evident in the infected cultures.

**SADS-CoV Species Specificity.** Using the rSADS-CoV tRFP indicator virus, we examined recombinant virus growth in different hosts, including immunomalted monkey, pig, cat, and human cells. At 48 hpi, rSADS-CoV tRFP produced robust tRFP expression that was detected in African green monkey Vero CCL-81, swine LLC-PK1, and feline AK-D lung cells (Figs. 2D and 3A). Vero CCL-81 and LLC-PK1 cells also displayed increased tRFP fluorescence in the presence of trypsin, which was required for adequate viral propagation (SI Appendix, Fig. S2). At 48 hpi, viral infections were also detected in human cells via red fluorescence, including liver (HuH7.5), intestine (CaCo2), stomach-intestine (ST-INT), and colorectal tumor cells (HRT) (Fig. S4A), including limited overlap with cell lines reported by others (29). Importantly, rSADS-CoV tRFP growth in HuH7.5 cells was robust even in the absence of exogenous trypsin treatment, replicating to titers of 10⁷ PFU/mL (Fig. 3B).

Imortalized cells are often derived from cancers or encode aberrant numbers of chromosomes, including extensive deletions and duplications, resulting in the loss of many key innate immune and other antiviral genes (30–32). Consequently, evaluating zoonotic coronavirus infection potential and growth in primary cells derived from humans provides a more realistic assessment of potential human susceptibility (7, 8, 33). Importantly, primary human lung cells, including microvascular endothelial cells (MVE), fibroblasts (FB), human nasal epithelial (HNE), and human airway epithelial cells (HAE) were all susceptible to productive SADS-CoV infection (Fig. 4 A–C and SI Appendix, Fig. S4A). While SADS-CoV replicated most efficiently in MVE and FB cultures,
Fig. 1. Spike phylogeny of representative coronaviruses and organization of the SADS-CoV wild-type and RFP infectious clones. (A) The S protein sequences of selected coronaviruses were aligned and phylogenetically compared. Coronavirus genera are grouped by classic subgroup designations (1b, 2a–d, 4). Sequences designated as 1b* (including SADS-CoV and related viruses) group with 1b viruses in proteins other than S. Sequences were aligned using free end gaps with the Blosum62 cost matrix in Geneious Prime. The tree was constructed using the neighbor-joining method based on the multiple sequence alignment, also in Geneious Prime. Numbers following the underscores in each sequence correspond to the GenBank accession number. The radial phylogram was rendered for publication using Adobe Illustrator CC 2019. (B) The general arrangement of the SADS-CoV genome. Blue represents nonstructural proteins ORF1a and ORF1b. Red represents structural proteins spike, envelope, membrane, and nucleocapsid. Green represents accessory proteins 3a, 7a, and 7b. Yellow represents the untranslated region. (C) The full-length infectious clone was divided into six contiguous cDNAs flanked by either BsmBI (SADS-CoV A–C), SapI (SADS-CoV D), BglI (SADS-CoV E–F) to allow for efficient assembly of the full-length SADS cDNA. BsmBI and SapI are not present in the viral genome sequence but are introduced externally in the fragment plasmid sequence. SADS-CoV A (nucleotides 1 to 4,496), SADS-CoV B (nucleotides 4,497 to 8,996), SADS-CoV C (nucleotides 8,997 to 13,496), SADS-CoV D (nucleotides 13,497 to 17,997), SADS-CoV E (nucleotides 17,998 to 22,892), and SADS-CoV F (nucleotides 22,893 to end). (D) General organization of SADS-CoV tRFP depicting the insertion of tRFP in place of NS3a.
growth in HNE and HAE cultures approached 10^5 PFU/mL (Fig. 4 B and C and SI Appendix, Fig. S44). Importantly, SADS-CoV also replicated efficiently in primary human intestinal cells (Fig. 4D and SI Appendix, Fig. S4B), replicating to titers of 10^5 PFU/mL over 96 hpi (Fig. 4F). As seen with other coronaviruses, such as SARS-CoV-2 (34), SADS-CoV infection in primary cells is donor dependent with replication efficiency varying between patients. SADS-CoV replication was validated by RT-PCR evidence of N gene mRNA transcripts and for N gene protein expression by Western blot in several primary cell lines (Figs. 2C, 3C, and 4E and G and SI Appendix, Fig. S5). These data demonstrate that the host range of SADS-CoV is very broad, providing potential opportunities for spillover from bats to swine or to other economically important intermediate or companion hosts, including humans (35–37).

For many virus families, herd immunity to contemporary human viruses can cross-neutralize and minimize the potential for zoonotic virus cross-species transmission and emergence in nature (38–43). To provide some guidance for potential future outbreak control, we next tested whether human convalescent sera to related group 1 coronavirus HCoV NL63 could cross-neutralize SADS-CoV tRFP. Treatment with several human coronavirus sera provided little if any cross-neutralization of SADS-CoV in Huh7.5 cells. Under identical conditions, the sera effectively neutralized another group 1b CoV, HCoV NL63 (Fig. 5A and B). Additionally, we evaluated the capacity of a broad-spectrum coronavirus nucleoside antiviral, remdesivir, to inhibit rSADS-CoV tRFP replication in Huh7.5
In a concentration-dependent manner, remdesivir efficiently inhibited SADS-CoV replication, as evidenced by reduced tRFP expression and reduced virus titers after treatment (Fig. 5C and SI Appendix, Fig. S5).

SADS-CoV Receptor Interactions. During SARS-CoV and HCoV NL63 infections, the S glycoprotein binds the human angiotensin converting enzyme 2 (ACE-2) receptor to direct entry into the cell (44). During MERS-CoV infection, the virus binds human dipeptidyl peptidase 4 (DPP4) as a receptor to mediate infection of human cells (45), while TGEV and some related group I coronaviruses use aminopeptidase N (APN or CD13) (46). Consequently, we treated Huh7.5 cells with polyclonal antibodies against human DPP4 or human APN (CD13) under conditions that block MERS and HCoV 229E infection (47, 48). After 1 h, the cultures were inoculated with SADS-CoV tRFP. Additionally, DBT cells expressing human ACE-2 were tested for their ability to support SADS-CoV tRFP replication. Using tRFP as a readout, we found no evidence of altered viral infectivity and replication in Huh7.5 in the presence of high concentrations of either antireceptor antibody, and SADS-CoV did not replicate in DBT–ACE-2 cells (Fig. 6). These data suggest that SADS-CoV does not use any of these known coronavirus receptors for docking and entry into human cells.

In Vivo Mouse Model Availability. Previous studies have reported that SADS-CoV replicates to low levels in wild-type laboratory mice (29). To enhance this model, identify host functions that may modulate SADS-CoV infection severity, and evaluate drug performance, we inoculated type I/II IFNR-immunodeficient mice with 1 × 10^5 PFU of SADS-CoV by either intraperitoneal injection or oral gavage. Upon using qRT-PCR to detect genome-length mRNA and the most abundant single-guide RNA 6, we noted little, if any, evidence of reproducible or robust virus replication in the liver, spleen, or various sections of the intestine at day 2 (SI Appendix, Fig. S6A). In fact, only 1 of 12 animals had evidence of low-level virus gene expression in multiple tissues. While a few other samples contained sporadic, low-level SADS mRNA, the positive tissues were not consistent across organs or mice and indicate a nonproductive infection that fails to replicate, at best. In wild-type BALB/c mice, we also saw little, if any, evidence of measurable...
replication in vivo at day 2 postinfection (SI Appendix, Fig. S6B). While IFN-γ-deficient mice have been shown to enhance the replication of other coronaviruses in vivo (49, 50), this defect had no substantial positive effect on SADS-CoV growth under our treatment conditions. These data are consistent with the interpretation that SADS-CoV does not replicate efficiently in Mus musculus domesticus mice and that most, if not all, of the positive signal is arising from abortive infections or input virus. Thus, our data argue that standard laboratory mouse models are clearly insufficient to provide opportunities for studying viral pathological mechanisms or in evaluating the performance of antivirals and vaccines.

**Discussion**

Coronaviruses, as well as other zoonotic virus families—such as orthomyxoviruses, paramyxoviruses, enteroviruses, and filoviruses—represent high-threat pathogens to One Health and the global economy (51, 52). The recent emergence of novel human and animal coronaviruses dictates the need for new strategies to identify higher-risk strains that may seed future disease outbreaks. Most emerging human and animal coronaviruses, including the recently emerged SARS-CoV-2, probably originated from bats (3, 27, 53–55) and have the capacity to evolve and spread rapidly across the globe (56–58). Contemporary human and swine strains have become fully adapted to their hosts, although some retain the capacity to replicate in bat cells (27). Using large numbers of bat CoV polymerase sequences from multiple bat species, recent
studies argue that the α-CoVs have a much higher likelihood to switch hosts within their natural bat reservoirs than the β-CoVs, implying high spillover risk to other mammalian species (59). In support, recent findings indicate that SADS-CoV swine outbreaks have likely originated from multiple spillover events from exotic bats in China, and may readily cycle between these species (10). In this report, we demonstrate human susceptibility potential by demonstrating efficient SADS-CoV infectivity and growth in primary human cells, derived from both the lung and intestine (33). Moreover, efficient SADS-CoV replication in the primary intestinal cells also support an earlier hypothesis that some emerging bat coronaviruses may initially replicate efficiently in the human alimentary track and stroma, before evolving efficient replication phenotypes in the lung (25).

With increased access to global travel and frequent human-to-human interaction, it is crucial to develop and utilize strategies to understand the risk potential of emerging viruses to One Health globally. In this study, we used metagenomics, bioinformatics, synthetic genome design, and reverse genetics to recover recombinant wild-type and derivative SADS-CoVs from in silico sequence databases. Using this model, we studied virus genome organization and expression patterns in infected cells and evaluated the capacity of human serum and small-molecule inhibitors to impede SADS-CoV growth in vivo. The molecular clone and work described herein expands upon a recently published swine enteric alphacoronavirus molecular clone (60) by: 1) Generating mutants that demonstrated a nonessential role for ORF3 is virus growth in vitro; 2) the development of indicator viruses, which revealed new insights into the broad host range; 3) studying cross-group 1b neutralization; and 4) identifying SADS-CoV susceptibility to a broad-spectrum nucleoside chain terminator, remdesivir, which has been used to treat lethal Ebola infections with some success (61). Additionally, remdesivir is effective at reducing SARS-CoV-2 infection and has been cleared for emergency use in treating COVID-19 patients (62, 63). Previous studies in our laboratory and others have shown that remdesivir was broadly active against alpha-, beta-, and gammacoronaviruses both in vitro and in vivo (64–66). As SADS-CoV is highly susceptible to remdesivir treatment in vitro, these findings support its potential use in the event of possible future SADS-CoV or related HKU2 disease outbreaks. In mouse models of human disease, remdesivir is very effective at treating lethal SARS-CoV2 infection (67) and MERS-CoV infection than combination lopinavir, ritonavir, and IFN-β (68). In contrast to other reports, SADS-CoV replication in wild-type mice was minimal at best. We noted little evidence of significant or reproducible replication across animals or tissues at day 2, indicating the need for a proper small-animal model for use in vivo antiviral or vaccine testing (SI Appendix, Fig. S5). Our inability to enhance SADS-CoV growth in immunodeficient IFNAR−/− mice provide further evidence that the mouse is not a suitable model for SADS-CoV pathogenesis, vaccine, or therapeutic studies, revealing a vulnerability in global preparedness.

The emergence of PEDV in the United States and China was associated with devastating economic losses in the swine industry (69, 70). SADS-CoV is closely related to HKU2 alphacoronavirus of bats, which was first identified in Hong Kong and Guangdong Province, China, in 2007 (15). Although SADS-CoV S glycoprotein variation is limited across outbreaks, HKU2-like strains are widely dispersed throughout Europe, Southeast Asia, and elsewhere and encode diverse S glycoprotein genes that vary by as much as 15% (Fig. L4) (10, 71). Although speculative, one or more of these diverse HKU2-related strains may also encode broad host range or transmission potentials, like SADS-CoV, enhancing the potential for animal and human outbreaks. The development of a stable molecular clone that can be used to recover wild-type and derivative recombinant viruses encoding different HKU2 S glycoprotein genes provides a robust platform to address this important One Health concern. Importantly, SADS-CoV has caused severe disease outbreaks in herds vaccinated with attenuated PEDV, illustrating the inability of existing swine PEDV vaccines to elicit protective immune responses against this emerging pathogen (17). The major component of protective immunity for coronaviruses usually targets the S glycoprotein and, to a lesser extent, the N protein (72). Given the extensive amino acid differences between contemporary human group 1b CoV and SADS-CoV S genes, it is not surprising that human sera efficiently neutralized hCoV NL63, but not SADS-CoV (Fig. 5A and B). Taking these data together, we find that the inability for PEDV vaccines and human polyclonal sera to protect against the SADS-CoV suggest that little, if any, cross-coronavirus herd immunity would exist to control the spread of this new pathogen, especially in swine. As previous studies have

Fig. 6. Potential receptors for SADS-CoV. Hu7.5 cells were treated with antibody against DPP4 or CD13 and then infected with SADS-CoV GFP. When compared to the untreated cultures, all cells and antibody treatment conditions did not block SADS-CoV entry or replication, suggesting that SADS-CoV is not using a known coronavirus entry receptor. Control DBT and DBT cells expressing hACE-2 were also infected and shown to be not permissive for SARS-CoV 2 GFP growth. All fluorescent images were taken at 10× magnification.
demonstrated that alphavirus VRP vaccines encoding the N and S proteins can provide protection against lethal SARS-CoV-1 and MERS-CoV infection and protect swine from PEDV infection (73), the SADS VRP N and S VRP vaccines developed herein may provide for similar opportunities for controlling SADS-CoV, and perhaps other group 1b swine coronavirus infections (74).

The coronavirus S glycoprotein is usually cleaved into distinct S1 and S2 polyproteins that include well-defined domains, including the S1 N-terminal domain (NTD) and C-terminal domain (CTD) (75). In some coronaviruses, the NTD contains folds that bind sugar residues, while the CTD encodes the receptor-binding domain, which engages various receptors, such as ACE-2, DPP4, CECAM1, and CD13 (75). SADS-CoV targeted a variety of host cell types from multiple species, including those characterized as of both human and nonhuman origin. The surprisingly broad host range of SADS-CoV does not appear to be driven by ACE-2, DPP4, or CD13 receptor usage, as antibodies targeting these receptors do not inhibit virus replication in human cells and overexpression of the human ACE-2 receptor also did not promote replication in mouse cells. As protease treatment enhances SADS-CoV host range, the data are also consistent with extensive literature that argues that protease availability is also a key regulator of coronavirus cross-species transmission (76–78). SADS-CoV tRFP replication in various CoV-neutralized human cells, including liver, stomach, intestinal, and rectal tumor cells (Fig. 34). However, a group 1 CoV camel coronavirus that was closely related to HCoV-229E also showed efficient replication in human tumor cell lines, but not in primary human lung airway cells, demonstrating the critical importance of evaluating zoonotic virus species specificity in primary cells (33). In contrast and in the absence of exogenous proteases, SADS-CoV replicated efficiently in primary human endothelial cells, primary lung fibroblasts cells, but growth in primary human airway and nasal epithelial cells. These cells play critical roles in maintaining lung architecture, compartmentalization, and airway function (79). Lung fibroblasts also play a central role in the homeostasis of the extracellular matrix and are effector cells during injury repair, while the microvascular endothelium regulates vascular homeostasis, as vascular leak can result in inefficient gas exchange in the lungs (80). In parallel, we showed efficient growth in primary human intestinal cells, including high virus titer after infection. Together, these data forge a compelling argument regarding the ability that the infected enter to SADS-CoV infection. Future studies must focus on identifying the entry and species-specificity mechanisms that regulate SADS-CoV cross-species transmission and pathogenesis, as well as the potential array of evolutionary pathways that could evolve to promote efficient replication in different human cells and tissues.

The emergence of the group 2b SARS-CoV-2 in Wuhan, China SADS-CoV demonstrated the predictive utility of using primary human lung cells, receptor interaction, and antigenic studies to predict the preepidemic potential of novel zoonotic viruses, and then use this recombinantly derived collection of viruses to identify readily available drugs for compassionate use (6–8, 66, 68). SADS-CoV also has a broad host range and replicates efficiently in primary human lung and intestinal cells. Due to the variability in infection efficiency seen between various donors of human primary cells, it is likely that in the case of a spillover, we would see a range of SADS-CoV severity in human patients. Swine are known amplifying hosts for several human pathogens, providing an infrastructure for the possibility of future emergence events. To date, there is no evidence of virus replication in humans (10). However, the ability of SADS-CoV to replicate in primary human primary cells indicates the potential for spillover of SADS-CoV into humans. With the 2019 reemergence of this virus in the swine population in China (81), continued surveillance of swine is critical. Additionally, individuals in the swine industry should be regularly evaluated for evidence of infection in order to reduce the potential of outbreaks. Consistent with the phylogenetic distance in the S glycoproteins (Fig. L4), little, if any, significant levels of cross-neutralizing human or swine herd immunity appear to exist between the contemporary alphacoronavirus tested and the SADS-CoV. While recognizing an unexplored potential for T cell contributions and given this collection of phenotypes, we suggest the need for continued One Health surveillance (82, 83), screening of swine workers in outbreak settings, BSL3 containment and that the continued rigorous and testing and testing of candidate vaccines. These drugs should be prioritized to protect the health of human populations as well as economically important domesticated livestock.

Materials and Methods

Virus and Cells. The recombinant SADS-CoV and its RFP-expressing derivative (SADS-CoV tRFP) were propagated in Vero CCL-81 and LLC-PK1 cells. All cells were maintained prior to infection in complete DMEM media supplemented with 10% serum, nonessential amino acids, sodium pyruvate, and 1% antibiotic. To generate virus stocks, cells were washed twice with PBS and cultured in serum-free DMEM media supplemented with 8% tryptose phosphate broth and 2.5 to 10 μg/mL trypsin and grown at 37 °C in a humidified CO2 incubator. Samples were then titrated by plaque assay on Vero CCL-81 cells. To enhance viral growth and spread, 10 μg/mL trypsin was added to the overlay for plaque assay. Growth curves were performed in Huh7.5 and LLC-PK1 cells, and supernatants were titrated on Vero CCL-81 cells as previously described (84, 85). Huh7.5 cells did not require additional trypsin for viral growth and spread, although it is likely that the inoculum used to perform the growth curves contained minimal levels of residual trypsin. For growth curves of SADS-CoV tRFP, fluorescent foci were counted to determine titer. Primary human airway epithelial cells, lung fibroblast, and lung microvascular endothelial cells prepared as described previously (86) were purchased from the Marsico Lung Institute, Tissue Procurement and Cell Culture Core, University of North Carolina at Chapel Hill, and used to evaluate SADS-CoV growth using previously described methods (84, 85). The cells obtained from human lungs were approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board (protocol #03-1396). Two-dimensional primary human intestinal cells were derived from human ileal crypts that form a continuous polarized epithelium with proliferative and nonproliferative zones (87). All primary cells (lung and intestinal) were infected with SADS-CoV tRFP diluted at 1:10 in PBS. Incubated cultures were incubated at 37 °C in a humidified CO2 incubator for 2 h before washing with PBS. Viral titers were determined using apical washes of airway ALI cultures, or apical media of intestinal cultures at appropriate time points. All recombinant SADS-CoVs were maintained in a BSL3 laboratory to enhance biosafety and all waste material was disinfected and autoclaved prior to removal from the facility. All personnel were equipped with fully protective Tyvek suits,double gloves, and a powered air-purifying respirator, as previously described by our group (8).

Systematic Assembly of a Full-Length SADS-CoV cDNA. The SADS-CoV clone was designed using the previously published sequence (10). Six contiguous cDNAs (A–F) flanked by unique restriction endonuclease sites (BsmBI/SapI/ BglI) were purchased from BioBasic (Fig. 1). Breakpoints were designed to maximize the stability of cloned fragments in bacteria as previously described by our group (21–24). The fragments were digested, separated through 0.8% agarose gels, visualized, excised, and purified using a QIAquick Gel Extraction Kit. The SADS-CoV cDNAs were ligated overnight at 4 °C, phenol/chloroform extracted, and precipitated. Full-length T7 RNA transcripts were generated in vitro as described by the manufacturer with modifications (21). RNA transcripts (wild-type or tRFP) were added to a Vero CCL-81 cell culture at 37 °C in a humidified CO2 incubator. Samples were then titered by plaque assay on Vero CCL-81 cells. To enhance viral growth and spread, 10 μg/mL trypsin was added to the overlay for plaque assay. Growth curves were performed in Huh7.5 and LLC-PK1 cells, and supernatants were titrated on Vero CCL-81 cells as previously described (84, 85). Huh7.5 cells did not require additional trypsin for viral growth and spread, although it is likely that the inoculum used to perform the growth curves contained minimal levels of residual trypsin. For growth curves of SADS-CoV tRFP, fluorescent foci were counted to determine titer. Primary human airway epithelial cells, lung fibroblast, and lung microvascular endothelial cells prepared as described previously (86) were purchased from the Marsico Lung Institute, Tissue Procurement and Cell Culture Core, University of North Carolina at Chapel Hill, and used to evaluate SADS-CoV growth using previously described methods (84, 85). The cells obtained from human lungs were approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board (protocol #03-1396). Two-dimensional primary human intestinal cells were derived from human ileal crypts that form a continuous polarized epithelium with proliferative and nonproliferative zones (87). All primary cells (lung and intestinal) were infected with SADS-CoV tRFP diluted at 1:10 in PBS. Incubated cultures were incubated at 37 °C in a humidified CO2 incubator for 2 h before washing with PBS. Viral titers were determined using apical washes of airway ALI cultures, or apical media of intestinal cultures at appropriate time points. All recombinant SADS-CoVs were maintained in a BSL3 laboratory to enhance biosafety and all waste material was disinfected and autoclaved prior to removal from the facility. All personnel were equipped with fully protective Tyvek suits, double gloves, and a powered air-purifying respirator, as previously described by our group (8).

Recombinant SADS-CoV tRFP Expression Construct. To generate a SADS-CoV tRFP recombinant virus, the nonstructural ORF, N55a, was replaced with tRFP. A total of three linker amino acids and ligation into the SADS F fragment. One PCR amplicon was generated using primers SADS TFRP #1 (5′-gcttctggtgctgctgctcgg-3′) and SADS TFRP #2 rev (5′-nnnnnncttttctggctgcttgac ctccccattc-3′). A second PCR amplicon was generated using primers SADS
Western Blot Analysis. To produce protein lysates for analysis by Western blot, infected cells were washed with 1× PBS and lysed in buffer containing 20 mM Tris HCl (pH 7.6), 150 mM NaCl, 0.5% deoxycholine, 1% nonidet P-40, 0.1% SDS. After initial lysis and removal of nuclei, supernatants were added to an area of fluorescence.

Data Availability. The sequences of recombinant SADS-CoV viruses have been deposited in GenBank under accession nos. MT039232 for rSADS-CoV wild-type (90) and MT039232 for rSADS-CoV tRFP (91). All other study data are included in the main text and SI Appendix.

ACKNOWLEDGMENTS. Provision of primary human lung cells by Dr. Randall was supported by grants from the Cystic Fibrosis Foundation (BOUCHE190) and NIH (DK062988). We thank Dr. Camille Ehre for providing the primary human intestinal cells for this study. This project was supported by the North Carolina Policy Collaboratory at the University of North Carolina at Chapel Hill with funding from the North Carolina Coronavirus Relief Fund established and appropriated by the North Carolina General Assembly. This work was also supported by NIH Grants AI089728, AI142759, AI132178, and AI151797 (to R.S.B.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Edwards et al.


31. G. Jiang et al., Comprehensive comparison of molecular portraits between cells lines and patients in breast cancer. *MGC Genomics* 17 (suppl. 7), 525 (2016).


34. Y. J. Hou et al., SARS-CoV-2 reverse genetics reveals a variable infection gradient in the respiratory tract. *Cell* 172, 849–446.e14 (2020).


42. M. L. Agostini et al., coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exonuclease. *mBio* 9, e00188 (2018).


89. S. Agnihothram et al., A mouse model for betacoronavirus subgroup 2c using a bat coronavirus strain HKU5 variant. mBio 5, e00047-14 (2014).
