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# Humanized mice develop coronavirus respiratory disease

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Coronavirus infections historically were associated with mild upper respiratory tract diseases in infants, children, and adults. Human coronavirus (HCoV)-OC43 and HCoV-229E were associated with 15–30% of common colds in winter and occasionally linked with lower respiratory tract disease in populations with chronic underlying diseases. HCoV research was complicated by the lack of a reverse genetic system or animal model. These viruses propagated poorly, and the number of reagents was limited. However, coronaviruses are capable of rapid host switching and evolution in changing ecologies (1), suggesting that their diversity and role in human disease were underappreciated. The 21st century heralded the arrival of the more pathogenic coronaviruses, like severe acute respiratory syndrome (SARS)-CoV. Then, HCoV-NL63 was identified as an important cause of severe lower respiratory tract infections in children and adults, including a tentative linkage with Kawasaki disease (2), and HCoV-HKU1 was identified in adults with pneumonia (3–5), renewing interest in the replication mechanisms and pathogenesis of HCoV-OC43 and HCoV-229E. In this issue of PNAS, Lassnig *et al.* (6) describe a transgenic mouse model to study HCoV-229E replication and pathogenesis, laying the groundwork for developing transgenic mouse models for other HCoVs.

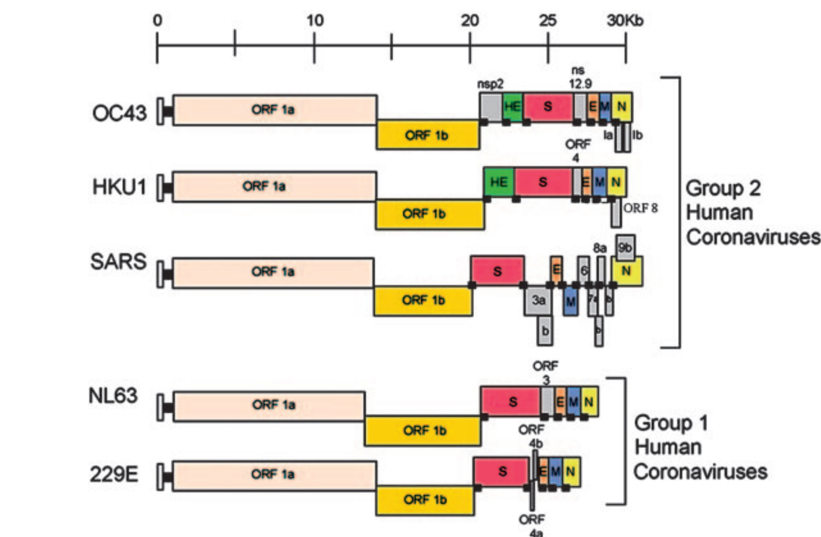


**Fig. 1.** Schematic of HCoV genomes. Schematic diagrams are shown for each HCoV. White rectangles and dark gray squares represent the leader transcription regulatory sequence and leader sequence, respectively. Color coding indicates hemagglutinin esterase (HE; green), spike (S; pink), envelope (E; orange), membrane (M; blue), nucleocapsid (N; yellow), accessory ORFs (gray).

## HCoVs

HCoVs include the group 1 (HCoV-229E and HCoV-NL63) and group 2 (HCoV-OC43 and HCoV-HKU1) serotypes (3, 4). The SARS-CoV classification is controversial, representing either the prototype group 4 strain or a distant relative of the group 2 viruses (5). They contain a linear single-stranded positive polarity RNA genome 27–32 kb in length with the 5′-most two-thirds of the genome encoding two large ORFs that function in replication (Fig. 1). Structural genes encode the spike (S) glycoprotein, a membrane (M) glycoprotein, the envelope (E) protein, and the nucleocapsid (N) protein. Structural ORFs are interspaced with accessory ORFs of unknown function, and all are expressed from subgenomic mRNAs that are arranged in the form of a nested set from the 3′ end of the genome (7). The S glycoprotein interacts with the receptor and is a critical determinant of host range, cross-species transmission, and pathogenesis (1). HCoV-OC43 and HKU1 also encode a hemagglutinin esterase whose function in replication is unknown. HCoVs use several host proteins for docking and entry, including aminopeptidase N (hAPN, HCV 229E), angiotensin-converting enzyme 2 (SARS-CoV), and neuraminic acid (HCoV-OC43) (8–10). Entry is a primary obstacle for initiating productive infection of cells *in vitro* and *in vivo*, although other sites of virus–host interaction likely contribute as well.

Fundamental barriers to HCoV research were the development of reverse genetic systems and robust animal models. Genetic obstacles were obliterated by Thiel *et al.* (11), who developed the first molecular clone for a HCoV, HCoV-229E, after inserting a full-length genome copy into a poxvirus vector to drive full-length infectious transcripts. Shortly thereafter, Yount *et al.* (12) isolated a molecular clone of the SARS-CoV by cloning the genome as six fragments that could be systematically assembled into a full-length cDNA *in vitro* and serve as the genomic template for the recovery of infectious transcripts (12). Using genetic approaches, the role of the accessory genes in coronavirus pathogenesis has been studied, foreign genes have been expressed, and coronavirus replicon particles have been developed as delivery vehicles for heterologous vaccines and therapeutics (13).

## Animal Models of Human Disease

The receptor for HCoV-229E, hAPN, also termed CD13, is a 150-kDa membrane-bound exopeptidase, which is constitutively expressed and forms dimers on the surface of a wide variety of cells (10). APN functions in digestion, angiogenesis, and synaptic activity and cleaves peptides bound to MHC molecules of antigen-presenting cells. Transfection of nonpermissive cells with hAPN is sufficient to confer susceptibility to HCoV-229E infection (10). For the group 1 coronaviruses, early attempts at developing transgenic mice expressing the hAPN receptor failed to produce a susceptible model, suggesting that other cofactors might be essential for *in vivo* replication (14). This outcome is not unique to HCoV-229E; similar findings were reported in transgenic mice expressing the poliovirus and measles virus receptors (15). Although the fundamental basis for this dichotomy remains undetermined, modifications that enhanced virus growth in these models included the generation of double-transgenic mice that

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also contained defects in innate immune responses, virus strain variation, or changes in the route of inoculation.

Lassnig *et al.* (6) resolved issues with the hAPN transgenic mice by using a comprehensive APN regulatory element, which allowed for near equivalent levels of hAPN expression in the appropriate organs with that seen by endogenous APN levels. Unfortunately, although the primary embryo fibroblasts (PEFs) from the transgenic mice were susceptible to infection by HCoV-229E, the animals were not productively infected with virus. To increase HCoV-229E replication, double-transgenic mice were obtained by crossing the hAPN homozygous males to immunocompromised Stat1$^{-/-}$ females, which have impaired innate immune responses. PEF cultures from the double-transgenic mice were robust hosts for HCoV-229E replication, but, again, virus failed to infect the hAPN$^{+/+}$Stat1$^{-/-}$ mice. Like other RNA viruses that have narrow host ranges, coronaviruses evolve quickly after blind serial passage either *in vitro* or *in vivo* and can rapidly be adapted to new host species (1). Refusing to be deterred, Lassnig *et al.* passaged WT HCoV-229E four times in PEF cultures from hAPN$^{+/+}$Stat$^{-/-}$ mice, selecting a variant HCoV-229E-37 that replicated more efficiently in this genetic background and in cultures derived from hAPN$^{+/+}$Stat$^{+/+}$ mice. Consistent with the role of the S glycoprotein gene in influencing host range and pathogenesis, two amino acid changes ($T_{278}$I and $N_{814}$A) were noted in S1. Although these mutations flank the principal hAPN receptor binding domain located between amino acids 407 and 547 in the HCoV-229E S (16), their functional significance is unknown. These mutations may mitigate functions associated with entry or release or encode residues that interact with murine coreceptor molecules critical for *in vivo* infection. Previous studies have indicated that other group I coronavirus S glycoproteins might interact with sialic acid (17), and dendritic cell-specific intracellular adhesion molecule 3 grabbing nonintegrin and CD209L may function as coreceptors for SARS-CoV docking and entry into particular cell types (18). Similar *in vitro* passages have enhanced rhinovirus replication in murine cells harboring the human intercellular adhesion molecule-1 receptor (19).

Importantly, HCoV-229E-37 replicated efficiently in hAPN$^{+/+}$Stat$^{-/-}$ mice but not in hAPN$^{-/-}$ or Stat$^{+/+}$ control animals after inoculation through oral, intranasal, intragastric, and i.p. routes simultaneously or via the nasal route only. Virus was detected in large amounts in the lungs and the gut after multiple route challenge and in the lungs after intranasal challenge. Clinically, animals showed a mild weight loss, slight temperature increases, and hemorrhagic areas in the lungs and small intestine. Animals inoculated via the intranasal route also displayed histopathology in the lung consistent with active virus replication.

## Future Directions

Lassnig *et al.*'s article (6) provides a paradigm for the generation of transgenic animals that are susceptible to HCoVs. SARS-CoV replicates efficiently in BALB/c mice without significant clinical disease or pathology, limiting the usefulness of this model for viral pathogenesis studies and the identification of virulence determinants (20). The development of human angiotensin-converting enzyme 2 transgenic animals may well serve as a more robust model for SARS-CoV. Importantly, hAPN humanized mice allow studies into HCoV-229E pathogenesis, tropism, replication, and spread in an immunocompromised host. hAPN is highly polymorphic in human populations, and the availability of this animal model allows investigation into whether allelic variation in the receptor influences HCoV-229E replication and pathogenesis *in vivo*, as has been so elegantly described for other RNA viruses like noroviruses (21, 22). The molecular basis for HCoV-229E adaptation represents another rich avenue of investigation. Although the S glycoprotein may be critical, other genetic changes could equally contribute to *in vivo* replication, adaptation, and pathogenesis. Because virus entry and spread are oftentimes restricted by innate host defense mechanisms, it is likely that one or more mutations may evolve that restrict activation of antiviral host genes in Stat$^{-/-}$ mice. The molecular clone for HCoV-229E provides the necessary tool for the identification and functional analysis of genetic elements responsible for *in vivo* adaptation and pathogenesis.

Murine models for HCoV-229E and SARS-CoV infection provide a means to study viral vector tropism and the efficacy and biosafety of HCoV-based vaccine vectors. The coronaviruses' unique genome organization and replication strategy allow simultaneous regulated expression of multiple foreign genes from transcription regulatory sequences encoded at the 3′ end of the genome. Coronavirus vectors can be targeted to different species, tissues, and mucosal compartments allowing for directed gene expression. It is likely that several kilobases of foreign genetic material can be stably incorporated and expressed in coronavirus genomes. HCoV vectors can be designed to safely target payloads to specific tissues or organs (13). The achievement by Lassnig *et al.* (6) will encourage further studies into human coronavirus replication and pathogenesis *in vivo* while simultaneously propelling the development of second-generation animal models for applying coronavirus-based vaccines and therapeutics in the treatment of human diseases.


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