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A nano-luciferase expressing human coronavirus OC43 for countermeasure development

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ABSTRACT

The genetic diversity of the coronavirus (CoV) family poses a significant challenge for drug discovery and development. Traditional antiviral drugs often target specific viral proteins from specific viruses which limits their use, especially against novel emerging viruses. Antivirals with broad-spectrum activity overcome this limitation by targeting highly conserved regions or catalytic domains within viral proteins that are essential for replication. For rapid identification of small molecules with broad antiviral activity, assays with viruses representing family-wide genetic diversity are needed. Viruses engineered to express a reporter gene (i.e. luminescence, fluorescence, etc.) can increase the efficiency, sensitivity or precision of drug screening over classical measures of replication like observation of cytopathic effect or measurement of infectious titers. We have previously developed reporter virus systems for multiple other endemic, pandemic, epidemic and enzootic CoV. Human CoV OC43 (HCoV-OC43) is a human endemic CoV that causes respiratory infection with age-related exacerbations of pathogenesis. Here, we describe the development of a novel recombinant HCoV-OC43 reporter virus that expresses nano-luciferase (HCoV-OC43 nLuc), and its potential application for screening of antivirals against CoV.

The genetic diversity of the coronavirus (CoV) family poses a significant challenge for drug discovery and development. Traditional antiviral drugs often target specific viral proteins from specific viruses which limits their use, especially against novel emerging viruses. Antivirals with broad-spectrum activity overcome this limitation by targeting highly conserved regions or catalytic domains within viral proteins that are essential for replication. For rapid identification of small molecules with broad antiviral activity, assays with viruses representing family-wide genetic diversity are needed. Viruses engineered to express a reporter gene (i.e. luminescence, fluorescence, etc.) can increase the efficiency, sensitivity or precision of drug screening over classical measures of replication like observation of cytopathic effect or measurement of infectious titers. We have previously developed reporter virus systems for multiple other endemic, pandemic, epidemic and enzootic CoV. Human CoV OC43 (HCoV-OC43) is a human endemic CoV that causes respiratory infection with age-related exacerbations of pathogenesis. Here, we describe the development of a novel recombinant HCoV-OC43 reporter virus that expresses nano-luciferase (HCoV-OC43 nLuc), and its potential application for screening of antivirals against CoV.

Prior to the COVID-19 pandemic, human CoVs caused approximately 10% of all upper and lower respiratory tract infections (Dijkman et al., 2012). Since December 2019, there have been almost 800 million cases of COVID-19 and almost 7 million deaths (https://covid19.who.int/). In the past twenty years, three known novel human CoVs (SARS-CoV, MERS-CoV, SARS-CoV-2) have emerged, thus demonstrating the emergence potential of the CoV family. HCoV-OC43 is closely related to Bovine CoV and is believed to have spilled over from cows into humans just over 200 years ago and has since become endemic in the human population (Vijgen et al., 2005). Although most children and healthy adults experience common cold-like symptoms upon HCoV-OC43 infection, disease can be severe in the elderly resulting in hospitalization, and although rare, infection can lead to death(Walsh et al., 2013). Currently there are no approved therapies or vaccines to treat or prevent HCoV-OC43 infections. Viruses similar to endemic, epidemic, and pandemic CoV circulate among wild and domestic animals and these

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enzootic viruses are likely to give rise to future emerging CoV. Because sequence data is limited for endemic CoV, it is not possible to appreciate the true extent of HCoV-OC43 genetic diversity. Additionally, as predicting the genetic identity of future emerging CoV is extremely complicated, broadly acting antivirals are needed to treat current and future emerging CoV infections. Currently, there are only two approved antivirals to treat COVID-19, remdesivir (Velkury) and nirmatrelvir/ritonavir (Paxlovid). To rapidly identify broadly active antiviral agents for the CoV family, assays for coronaviruses that are representative of family-wide genetic diversity are needed.

Multi-partite reverse genetic systems for CoV were first engineered in the year 2000 (Yount et al., 2000). Using this well-established strategy, we cloned cDNA fragments for HCoV-OC43 VR-1558 each with unique flanking junctions created by the class II restriction enzyme Esp3I into the pCR-TOPO vector backbone to facilitate the directional assembly of genome length cDNA with a 5′ T7 promoter site. We inserted the nano-luciferase gene (GenBank: KF811457.1) in place of accessory gene NS2 nucleotides 21,523 – 22,220 (Fig. 1). Previous studies have demonstrated NS2 to stably tolerate reporter gene insertions, and that these reporter viruses exhibited no apparent growth defects in vitro (Shen et al., 2016). To generate the recombinant HCoV-OC43 nLuc, in vitro transcribed genomic RNA was electroporated into the highly electrocompetent BHK cells and then co-cultured with Vero E6 TMPRSS2 cells which support efficient infection and replication of HCoV-OC43 (Hirose et al., 2021). After a two-day incubation at 37 °C, the P0 stock of the HCoV-OC43 nLuc virus was harvested, nano-luciferase expression was confirmed, and this P0 stock was expanded and passaged four times on Vero E6 TMPRSS2 cells to generate a P4 stock. As the P4 titer was relatively low (10^4 PFU/mL), we sought to identify a cell line with increased permissivity to increase replication and infectious titer and provide a more suitable cell line for antiviral assays. We also tested HRT-18 cells, a line commonly used to grow HCoV-OC43. However, HRT-18 cells did not support adequate replication of HCoV-OC43 nLuc. NS2 is a known antagonist of the host's innate immune response (Goldstein et al., 2017). The growth defect of HCoV-OC43 nLuc in HRT-18 cells could therefore be due to an impaired ability of the reporter virus to antagonize host innate immune response pathways and requires further investigation.

Interferon-induced transmembrane 3 (IFITM3) protein is an interferon stimulated gene whose expression is typically induced by virus infection. IFITM3 is primarily located at the plasma membrane and the membranes of endosomes and lysosomes where it can restrict the entry of a broad range of enveloped viruses, including influenza A virus, HIV-1, Zika virus, and SARS-CoV-2 by inhibiting the fusion of the viral envelope with cellular membranes, preventing entry into the host cell (Drouin et al., 2021; Feeley et al., 2011; Prelli Bozzo et al., 2021; Savidis et al., 2016). In addition, IFITM3 influences endosomal acidification, a process required for efficient viral fusion and replication. Paradoxically, emerging research has shed light on a potentially pro-viral role of IFITM3 in certain viral infections, including HCoV-OC43. Recent studies have suggested that IFITM3 may facilitate HCoV-OC43 entry into host cells by enhancing fusion and promoting more efficient viral entry (Zhao et al., 2014). To see if IFITM3 expression would increase the replication efficiency of HCoV-OC43 nLuc virus, we generated a Huh7.5 cell line stably expressing human IFITM3 with an N-terminal FLAG tag (Huh7.5 IFITM3) or an empty vector (EV) control. Parental Huh7.5 cells were obtained by MTA from Apath LLC in 2015. We confirmed FLAG-IFITM3 expression via Western blot and upon infection with HCoV-OC43, noted an increase in nucleocapsid protein suggesting expression of FLAG-IFITM3 increases viral replication (Fig. 2A). We then passaged our HCoV-OC43 nLuc an additional time in the Huh7.5 IFITM3 cells at an MOI of 0.01 at 37 °C for 48 h and generated a working stock (10^5 PFU/mL) that was utilized for all subsequent analysis/characterization.

To determine the optimal growth conditions for HCoV-OC43 nLuc virus, we performed a growth curve in Huh7.5 IFITM3 cells and EV cells. We performed this analysis at two different temperatures: 32 °C and 37 °C to mimic the lower and upper respiratory tracts, respectively. Infection supernatants were collected at 24 hour intervals and titered using the focus forming assay (FFA) on Huh7.5 IFITM3 cells (Fig. 2C) as described previously (Carbaugh et al., 2020). HCoV-OC43 nLuc growth was the highest over time in the Huh7.5 IFITM3 cell line at 37 °C (Fig. 2C). Overall, the virus grew better in Huh7.5 IFITM3 cells regardless of temperature, and the virus grew to higher titers and exhibited faster replication kinetics at 37 °C as compared to 32 °C in both cell lines (Fig. 2C). The differences in virus titer were greatest at the earliest timepoint of 24 h (Fig. 2C), suggesting that the growth advantage of HCoV-OC43 nLuc in Huh7.5 IFITM3 cells at 37 °C is a result of greater initial infectivity. These data are concordant with previous studies showing that IFITM3 specifically enhances the entry steps (viral/host cell membrane fusion) of the HCoV-OC43 replication cycle (Zhao et al., 2014). The growth of HCoV-OC43 nLuc began to slow-down and stabilize around 72 h for all conditions (Fig. 2C). This is potentially a consequence of cell death resulting from viral replication and the decline in the viability of the cells over time due to over-confluency and extended incubation in minimal media (OPTI-MEM, 1X Pen/strep). Overall, we found that using the Huh7.5 IFITM3 cell line and a 37 °C incubation temperature was optimal for the growth of HCoV-OC43 nLuc in infection assays. Collectively, our results are largely concordant with previous studies and demonstrate a clear pro-viral role for IFITM3 during HCoV-OC43 infection.

In addition to taking the infection supernatant from the 0, 24, 48, and 72 hour timepoints of the growth curve to determine infectious virus titers using the FFA, we also monitored for nano-luciferase activity to evaluate whether it was a good correlate for infectious virus production. Like the FFA data, nano-luciferase activity was highest over time during the infection of Huh7.5 IFITM3 cells at 37 °C (Fig. 2D). Additionally, nano-luciferase production peaked around 72 h like the FFA titers (Fig. 2D). A slight difference between the FFA and nano-luciferase data was that the nano-luciferase production over time clustered more based

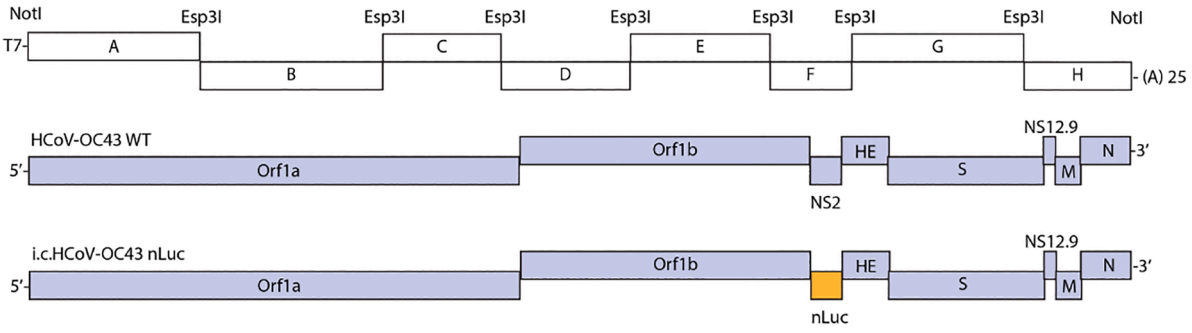

Fig. 1. Schematic design of HCoV-OC43 nLuc infectious cDNA clone. The full-length HCoV-OC43 genome was divided into eight contiguous cDNAs flanked by the type II restriction enzyme Esp3I to facilitate the directed assembly of full-length cDNA as template for in vitro transcription and subsequent electroporation.

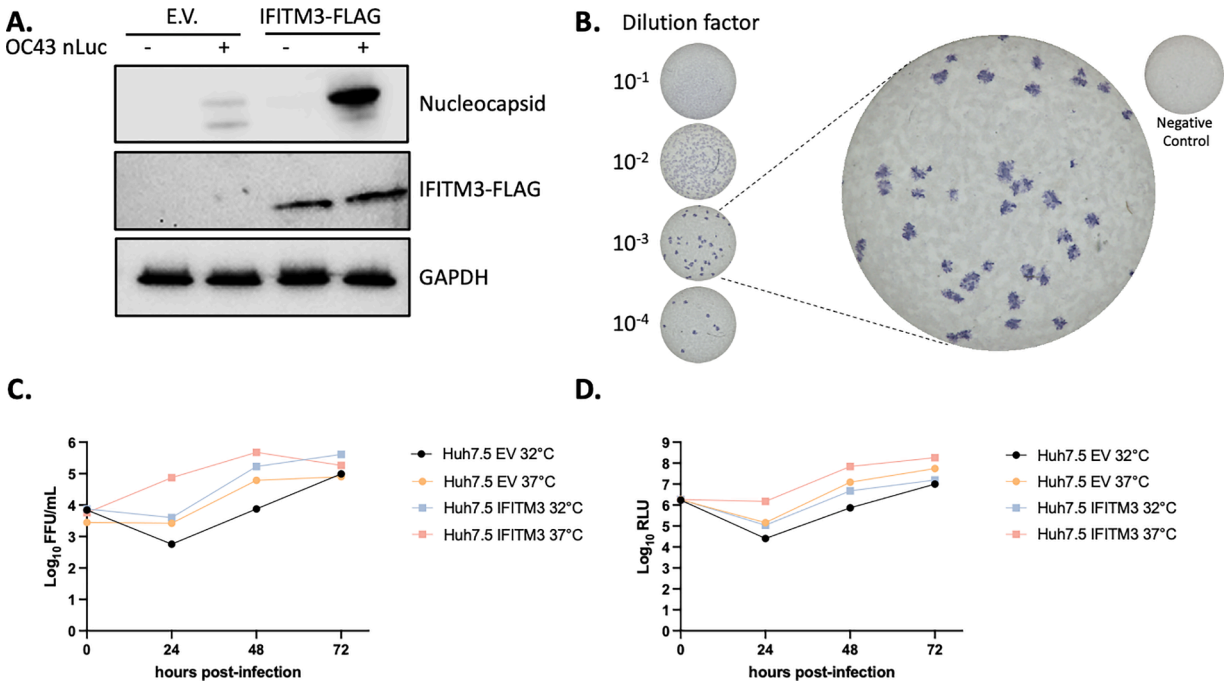

Fig. 2. HCoV-OC43 nLuc replication kinetics in Huh7.5 IFITM3 over-expressing cells. **A.** Western blot analysis of HCoV-OC43 replication in Huh7.5 cells expressing FLAG-tagged IFITM3. HCoV-OC43 nucleocapsid was detected by mouse anti-coronavirus primary antibody (Millipore Sigma: MAB9012). FLAG-tagged human IFITM3 was detected by anti-FLAG M2 antibody (CST: 14793A). GAPDH was detected by anti-GAPDH (CST: 2118S). Following primary antibody incubation, blots were incubated with either anti-mouse (SeraCare: 5220–0341) or anti-rabbit (CST: 7074S) HRP secondary antibodies. **B.** Representative example of HCoV-OC43 nLuc foci observed upon infection of Huh7.5 IFITM3 cells with serially diluted virus sample after 48hpi at 37 °C. For foci visualization, cells were fixed with 2.0% PFA for 1 hour at room temperature, stained with mouse anti-coronavirus primary antibody (Millipore Sigma: MAB9012) followed by goat anti-mouse HRP secondary antibody (SeraCare: 5220–0341), and then incubated with KPL TrueBlue Substrate (SeraCare: 5510–0030). Cells were washed 3 times with PBS between antibody incubations, and antibodies were diluted in blocking buffer (PBS, 0.1% BSA, 0.1% saponin). **C.** Growth of infectious HCoV-OC43 nLuc over time in Huh7.5 EV or Huh7.5 IFITM3 cells at 32 °C or 37 °C. Huh7.5 EV or Huh7.5 IFITM3 cells were infected with HCoV-OC43 nLuc at an MOI of 0.01 at 32 °C or 37 °C, and virus supernatants were titered by focus-forming assay (FFA) at 0, 24, 48, & 72hpi. **D.** Growth of HCoV-OC43 nLuc as determined by nano luciferase luminescence over time in Huh7.5 EV or Huh7.5 IFITM3 cells at 32 °C or 37 °C. Huh7.5 EV or Huh7.5 IFITM3 cells were infected with HCoV-OC43 nLuc at an MOI of 0.01 at 32 °C or 37 °C, and nano luciferase activity in the virus supernatants was measured at 0, 24, 48, & 72 h.p.i. using the Promega NanoGlo Assay. Data is from two independent, biological replicates with two or three technical replicates each. Focus forming units (FFU). Relative light units (RLU).

on temperature than cell line with both cell lines exhibiting higher nano-luciferase production during HCoV-OC43 nLuc infection at 37 °C compared to 32 °C (Fig. 2D). A potential explanation could be that the kinetics of biochemical processes such as translation increase with temperature, thereby increasing baseline levels nano-luciferase protein production at 37 °C independent of viral replication. Overall, for infection assays, nano-luciferase production appears to be a viable correlate of the production of infectious virus.

To efficiently screen compounds for antiviral breadth against the CoV family, assays for viruses representing family-wide genetic diversity are needed. To this end, we sought to establish an antiviral assay using our newly developed HCoV-OC43 nLuc virus and Huh7.5 IFITM3 cell line. We infected 2.5×10^4 cells in a black-bottom 96-well plate using 800 FFU HCoV-OC43 nLuc in the presence of a dose response of two antiviral drugs with proven activity against the CoV family, remdesivir and molnupiravir, in order to determine the concentration at which replication was inhibited by 50% (IC_{50}) (Fig. 3) Here we show dose dependent inhibition of HCoV-OC43 replication for both antiviral drugs

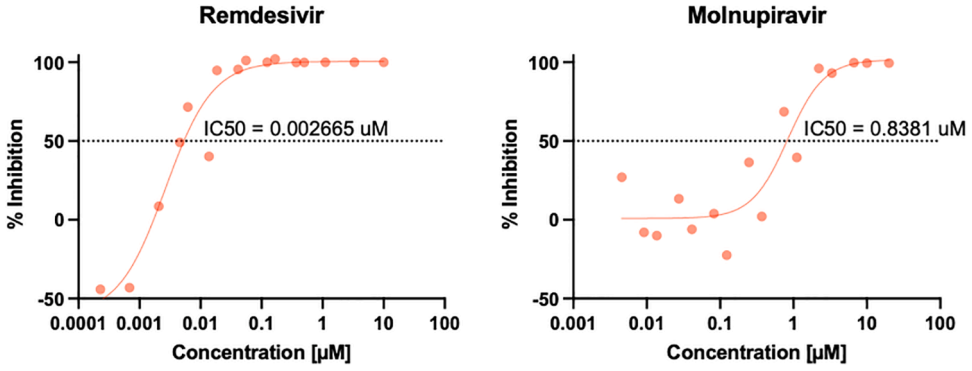

Fig. 3. HCoV-OC43 nLuc antiviral assay and in vitro potency of remdesivir and molnupiravir. Huh7.5 expressing IFITM3 cells were seeded in black bottom 96 well plates (2.5×10^4 cells per well). Antiviral activity is based on RLU of HCoV-OC43 nLuc replication in the presence of decreasing concentration of antivirals. Subsequent quantification of nano-luciferase activity was measured as described in Fig. 2D. Data is from two independent, biological replicates with two technical replicates each.

with potent submicromolar IC_{50} values (remdesivir IC_{50} = 0.002 μM, molnpiravir IC_{50} = 0.83 μM). The potency of molnupiravir against HCoV-OC43 was previously reported to be in a similar in range (0.57 μM) in human airway organoid cultures (Li et al., 2022). Interestingly, we had previously reported a higher IC_{50} (0.1 μM) for remdesivir in Huh7 cells infected with HCoV-OC43 using an antigen stain-based focus forming assay (Brown et al., 2019). This discrepancy is likely driven by the increased sensitivity of our assay using recombinant HCoV-OC43 nLuc coupled with our Huh7 IFITM3 cells.

Multiple broadly active antivirals are needed to treat current and future emerging CoV infections. We created a reverse genetic system for HCoV-OC43 and a recombinant reporter virus expressing nano-luciferase. This virus had suboptimal replicative fitness in Huh7 cells leading to the creation of Huh7 cells expressing the pro-HCoV-OC43 ISG, IFITM3. Together, these new reagents facilitated the rapid screening of antiviral drugs. These virologic and cell biologic tools for HCoV-OC43 complement the existing reverse genetic systems we have for endemic, pandemic and enzootic CoV which together facilitate the rigorous testing of antiviral breadth against the CoV family.

Author statement

All authors contributed equally to this work.

CRediT authorship contribution statement

Meghan V. Diefenbacher: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Thomas J. Baric:** Investigation. **David R. Martinez:** Conceptualization. **Ralph S. Baric:** Conceptualization, Funding acquisition. **Nicholas J. Catanzaro:** Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Timothy P. Sheahan:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

T.P.S. has received funding from ViiV Healthcare and GlaxoSmithKline and has collaboratins with Ridgeback Biosciences and Gilead. R.S.B. is a member of the advisory board of VaxArt and Inviyd, and has collaborations with Takeda, Pfizer, Moderna, Ridgeback Biosciences, Gilead, and Eli Lily.

Data availability

Data will be made available on request.

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