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Short Communication

Replication-dependent downregulation of cellular angiotensin-converting enzyme 2 protein expression by human coronavirus NL63

Ronald Dijkman,[1]† Maarten F. Jebbink,[1] Martin Deijs,[1] Aleksandra Milewska,[1] Krzysztof Pyrc,[2] Elena Buelow,[1] Anna van der Bijl[1] and Lia van der Hoek[1]

Correspondence
Lia van der Hoek
c.m.vanderhoek@amc.uva.nl

[1]Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, The Netherlands

[2]Microbiology Department, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

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Like severe acute respiratory syndrome coronavirus (SARS-CoV), human coronavirus (HCoV)-NL63 employs angiotensin-converting enzyme 2 (ACE2) as a receptor for cellular entry. SARS-CoV infection causes robust downregulation of cellular ACE2 expression levels and it has been suggested that the SARS-CoV effect on ACE2 is involved in the severity of disease. We investigated whether cellular ACE2 downregulation occurs at optimal replication conditions of HCoV-NL63 infection. The expression of the homologue of ACE2, the ACE protein not used as a receptor by HCoV-NL63, was measured as a control. A specific decrease for ACE2 protein level was observed when HCoV-NL63 was cultured at 34 °C. Culturing the virus at the suboptimal temperature of 37 °C resulted in low replication of the virus and the effect on ACE2 expression was lost. We conclude that the decline of ACE2 expression is dependent on the efficiency of HCoV-NL63 replication, and that HCoV-NL63 and SARS-CoV both affect cellular ACE2 expression during infection.

Human coronaviruses (HCoVs) were previously associated with mild respiratory tract disease (common cold) that may cause more severe symptoms in young children, elderly or immune-compromised individuals (Bradburne *et al.*, 1967; Falsey *et al.*, 1997; Kraaijeveld *et al.*, 1980; Macnaughton *et al.*, 1981; Reed, 1984; van der Hoek, 2007). In 2002–2003 the appearance of the severe acute respiratory syndrome (SARS), caused by a formerly unknown coronavirus (SARS-CoV), changed the perspective on the impact on human health by HCoV infections (Fouchier *et al.*, 2003; Marra *et al.*, 2003; Perlman & Dandekar, 2005; Perlman & Netland, 2009; Rota *et al.*, 2003). Soon afterwards, HCoV-NL63 was identified in 2004 as a novel respiratory virus pathogen (van der Hoek *et al.*, 2004), a wide spread virus that has been associated with the childhood disease croup (van der Hoek, 2007; van der Hoek *et al.*, 2005).

Angiotensin-converting enzyme 2 (ACE2) has been identified as a homologue to the well known ACE (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000). Both metalloproteases are involved in the renin angiotensin system (RAS) by regulating the blood pressure and body fluid homeostasis (Donoghue *et al.*, 2000; Lambert *et al.*, 2010; Tipnis *et al.*, 2000). Alterations in the balanced RAS have been implicated in playing a crucial role in cardiovascular and renal disease, lung injury and liver fibrosis outcome (Lambert *et al.*, 2010). SARS-CoV uses ACE2 as a receptor to enter its target cell (Li *et al.*, 2003). The downregulation of ACE2 expression levels in the lungs upon SARS-CoV infection has been linked with the pathogenicity of the virus (Kuba *et al.*, 2005), reflected by the protective role of soluble ACE2 during experimental acid-induced lung injury (Imai *et al.*, 2005). Interestingly, HCoV-NL63 and SARS-CoV both utilize ACE2 protein as a receptor for cellular entry with overlapping binding regions, albeit the viruses differ in pathogenicity (Hofmann *et al.*, 2005; Li *et al.*, 2003, 2007; Smith *et al.*, 2006). Glowacka *et al.* (2010) reported for the first time differential aspects of SARS-CoV and HCoV-NL63 on ACE2 protein expression. Both viruses induce shedding of ACE2 with different efficiencies, although it is not a prerequisite for infectious entry (Glowacka *et al.*, 2010). Furthermore, it has been shown that the spike (S) protein

†Present address: Institute of Immunobiology, Kantonal Hospital St Gallen, St Gallen, Switzerland.

Supplementary figures are available with the online version of this paper.

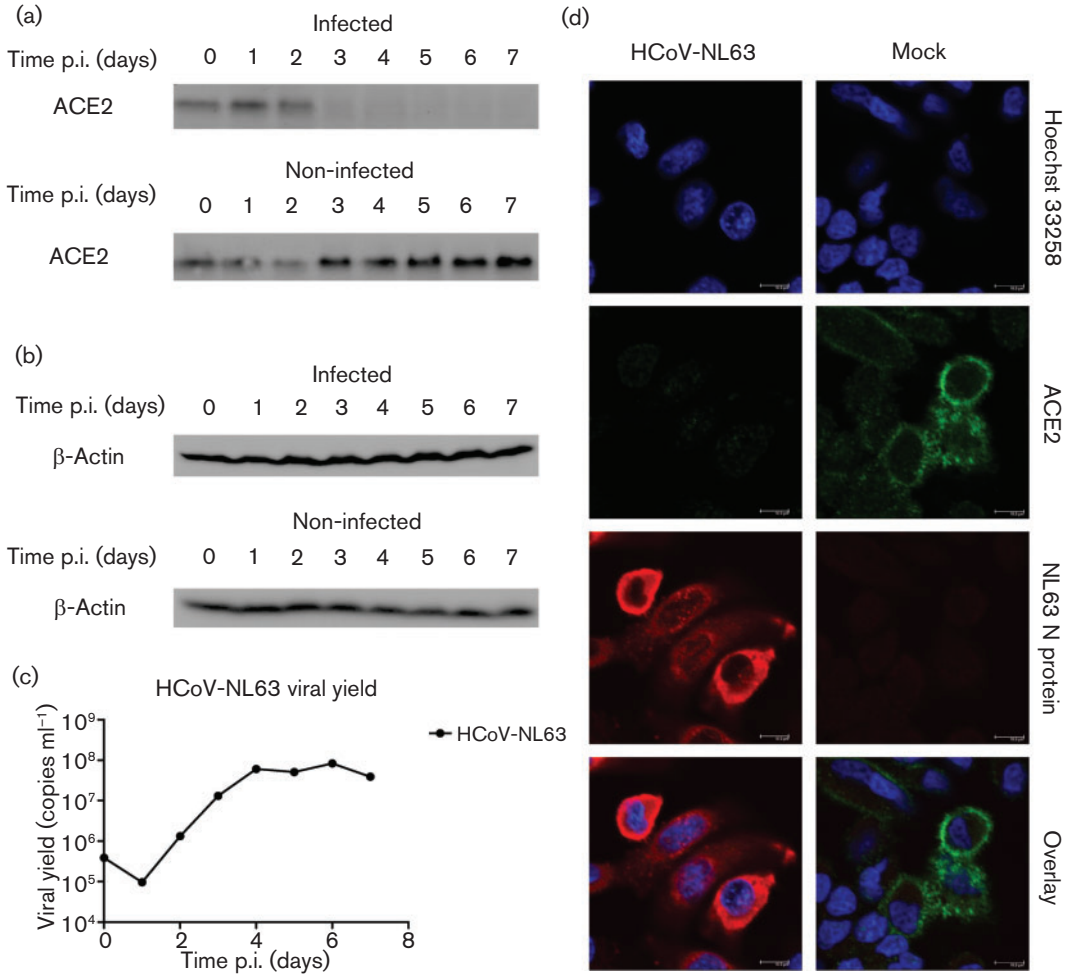

Fig. 1. Downregulation of ACE2 expression by HCoV-NL63. (a) Chemiluminescence detection of ACE2 protein expression in mock- or HCoV-NL63-infected LLC-MK2 cells cultured at 34 °C. (b) β-Actin protein expression in mock- or HCoV-NL63-infected cultures as loading control. (c) The HCoV-NL63 viral yield measured in the culture supernatant. (d) Confocal images of DNA (blue), ACE2 (green) and HCoV-NL63 N protein (red) in mock- and HCoV-NL63-infected LLC-MK2 cells 5 days p.i. The results from a representative experiment are shown.

of SARS-CoV binds with a higher affinity to ACE2 as compared with the S protein of HCoV-NL63 (Glowacka *et al.*, 2010; Mathewson *et al.*, 2008). The downregulation of ACE2 protein expression on the cell surface was observed in context of SARS-CoV, but not HCoV-NL63 infection (Glowacka *et al.*, 2010). However, whether this difference was influenced by the higher replication efficiency of SARS-CoV under the conditions used was not investigated (Glowacka *et al.*, 2010). The levels of ACE2 protein expression in context of HCoV-NL63 replication were monitored in the African green monkey kidney derived cell line Vero-E6 at 37 °C (Glowacka *et al.*, 2010). However, the reference strain of HCoV-NL63 (Amsterdam-01) was isolated from LLC-MK2 cells, and best replication of this strain is observed in either this cell line or in Vero-B4 (Schildgen *et al.*, 2006; van der Hoek *et al.*, 2004). In order to keep HCoV-NL63 replication as efficient as possible we chose the most optimal culture condition for HCoV-NL63, so LLC-MK2 cells at 34 °C (van der Hoek *et al.*, 2004).

To monitor ACE2 protein expression levels during HCoV-NL63 replication we inoculated LLC-MK2 cells with HCoV-NL63 at an m.o.i. of 0.007 or used an equal volume of mock-supernatant as control. Cells were harvested by scraping 0, 1, 2, 3, 4, 5, 6 and 7 days post-inoculation (p.i.) in ice-cold PBS, supplemented with 5 mM EDTA/EGTA (Sigma). A constant number of viable cells (1×10^7 cells ml^{-1}) were transferred into Laemmli sample buffer, after which the whole-cell lysate was sheared and boiled. An aliquot of 10 µl was layered on a 15 % SDS-PAGE gel, and after separation the cellular ACE2 protein expression levels were analysed by Western blot. Detection of ACE2 protein was performed by using goat-derived anti-human ACE2

ectodomain antibody (0.2 μg ml^{-1}, AF933; R&D Systems) as primary, and rabbit-derived HRP-labelled anti-goat IgG (1 : 5000; Sigma) as secondary antibody. The β-actin protein control was measured after membranes were stripped and reprobed with mouse monoclonal anti-β-actin (0.4 μg ml^{-1}; Sigma) and goat-derived, HRP-labelled, anti-mouse IgG (0.4 μg ml^{-1}; Sigma) antibodies. The rhesus macaque ACE2 (805 aa, 85 kDa) appeared as a 120 kDa protein on Western blot, like the human glycosylated ACE2 protein (Jia *et al.*, 2009; Warner *et al.*, 2005). This showed that the anti-human ACE2 ectodomain antibody recognizes the rhesus macaque ACE2 protein.

The cellular ACE2 protein levels of HCoV-NL63-infected LLC-MK2 cells showed a clear signal reduction beyond 3 days p.i., which was not observed in the mock control (Fig. 1a). To investigate whether this alteration in ACE2 protein expression level correlated with the HCoV-NL63 viral yield we monitored the RNA yield in the supernatant during the same time frame using real-time PCR (van der Hoek *et al.*, 2010). We observed that the ACE2 signal decrease coincides with the exponential increase of HCoV-NL63 production that starts from 3 days p.i. and reaches plateau levels at 5 days p.i. (Fig. 1c). In accordance, the first signs of cytopathic effect (CPE) development were observed at 3 days p.i.

In addition to Western blot analysis, we used confocal microscopy to examine the ACE2 expression in HCoV-NL63-infected cells. LLC-MK2 cells were seeded upon coverslips and inoculated as described above. After 5 days p.i., the cultures were fixated and developed as described previously (Dijkman *et al.*, 2009). Coverslips were stained with serum from HCoV-NL63 nucleocapsid (N) protein immunized rabbits (1 : 400; Eurogentec) and goat polyclonal anti-human ACE2 ectodomain (2 μg ml^{-1}, AF993; R&D Systems), followed by incubation with donkey-derived, anti-rabbit IgG and anti-goat IgG [(H + L), 7.5 μg ml^{-1}; Jackson Immunoresearch], conjugated with Dylight 649 and Dylight 594, respectively. We observed that at 5 days p.i., the vast majority of cells stained positive for the NL63 N protein, indicating that they are infected by HCoV-NL63 (Fig. 1d). No ACE2 protein was detected on the surface of the infected cells, whereas ACE2 protein was clearly visible in the mock-infected culture. Analysis of ACE2 expression at 3 days p.i. indicated that ACE2 expression is only abolished in HCoV-NL63-infected cells and not in an uninfected adjacent cell (Fig. S1a, available in JGV Online). This indicates that the downregulation of ACE2 only occurs in virus-infected cells and, based on the antigen amount and localization, this most likely occurs during the early phase of viral replication (Fig. S2). Furthermore, we observed that not all LLC-MK2 cells express ACE2 on the surface, both at 3 and 5 days p.i. (Fig. S1a and b).

The strong reduction of ACE2 protein expression observed in our experiments could have occurred due to the activation of ACE2 shedding from the cell surface after the binding of HCoV-NL63 S protein to the cellular receptor (Glowacka *et al.*, 2010). Therefore, we investigated the level of soluble ACE2 in culture supernatant by using an ACE2 enzyme activity assay, in which substrate degradation by soluble ACE2 ectodomain is measured (Vickers *et al.*, 2002). No ACE2 shedding could be detected by the assay within culture supernatant from HCoV-NL63-infected culture, nor control culture. In addition, stimulating ACE2 shedding with 10, 100 nM or 1 μM of phorbol 12-myristate 13-acetate, a known inducer for ACE2 shedding, did not show detectable ACE2 enzyme activity in the culture supernatant. Using serial dilutions of spiked recombinant human protein in complete culture medium revealed that our enzyme activity assay has a detection limit of 0.24 μg human ACE2 ml^{-1}, which is too high for the amounts of ACE2 in LLC-MK2 cells (data not shown). Therefore, we aimed to use Western blot analysis instead and increased the protein concentration by using serum-free medium (Optimem; Invitrogen), and after removal of cellular debris we concentrated the supernatant 50-fold, using centrifugal protein concentrators according to the manufacturer's instructions (Amicon Ultra, 30 kDa cut-off; Millipore). We harvested the supernatant at 0, 3 and 5 days p.i., and an aliquot of 10 μl concentrate was analysed with Western blot; however, we did not observe a difference in ACE2 shedding compared to the mock-infected cultures (Fig. S3a). The viral N protein could be detected in an increasing fashion in the HCoV-NL63-infected cultures using a specific NL63 mAb (Fig. S3b) (Sastre *et al.*, 2011), indicating that in our experimental setting ACE2 shedding could not be measured, most probably due to low number of cells that express ACE2 (Fig S1a and b).

Attempts to culture HCoV-NL63 on various human cell cultures did not lead to efficient HCoV-NL63 replication, or reduction of ACE2 expression (data not shown). A likely explanation is that the replication of the virus in human cells was too weak to have an effect on ACE2. To examine whether ACE2 downregulation is indeed influenced by replication efficiency, we propagated HCoV-NL63 in LLC-MK2 cells under optimal and suboptimal conditions, 34 and 37 °C respectively. Cells and supernatant were harvested at 0, 1, 2, 3, 4 and 5 days p.i. to monitor cellular ACE2 protein levels via Western blot analysis as described above, with the adjustment that ACE2 and control β-actin protein were measured via dual detection with donkey-derived, IRDye 800CW, anti-goat IgG (H + L) (0.2 μg ml^{-1}; Rockland) and donkey-derived, Dylight 649-labelled, anti-mouse IgG (H + L) (0.3 μg ml^{-1}; Jackson Immunoresearch) as secondary antibodies. Acquired images were analysed with the Odyssey application software package version 3.0 (Li-Cor). The ACE2 protein signal was normalized against the corresponding β-actin signal. Quantified ACE2 concentrations from different blots were corrected for between-session variation (Ruijter *et al.*, 2006). Comparison of the cellular ACE2 protein concentration during HCoV-NL63 infection, at 34 and 37 °C, was done with

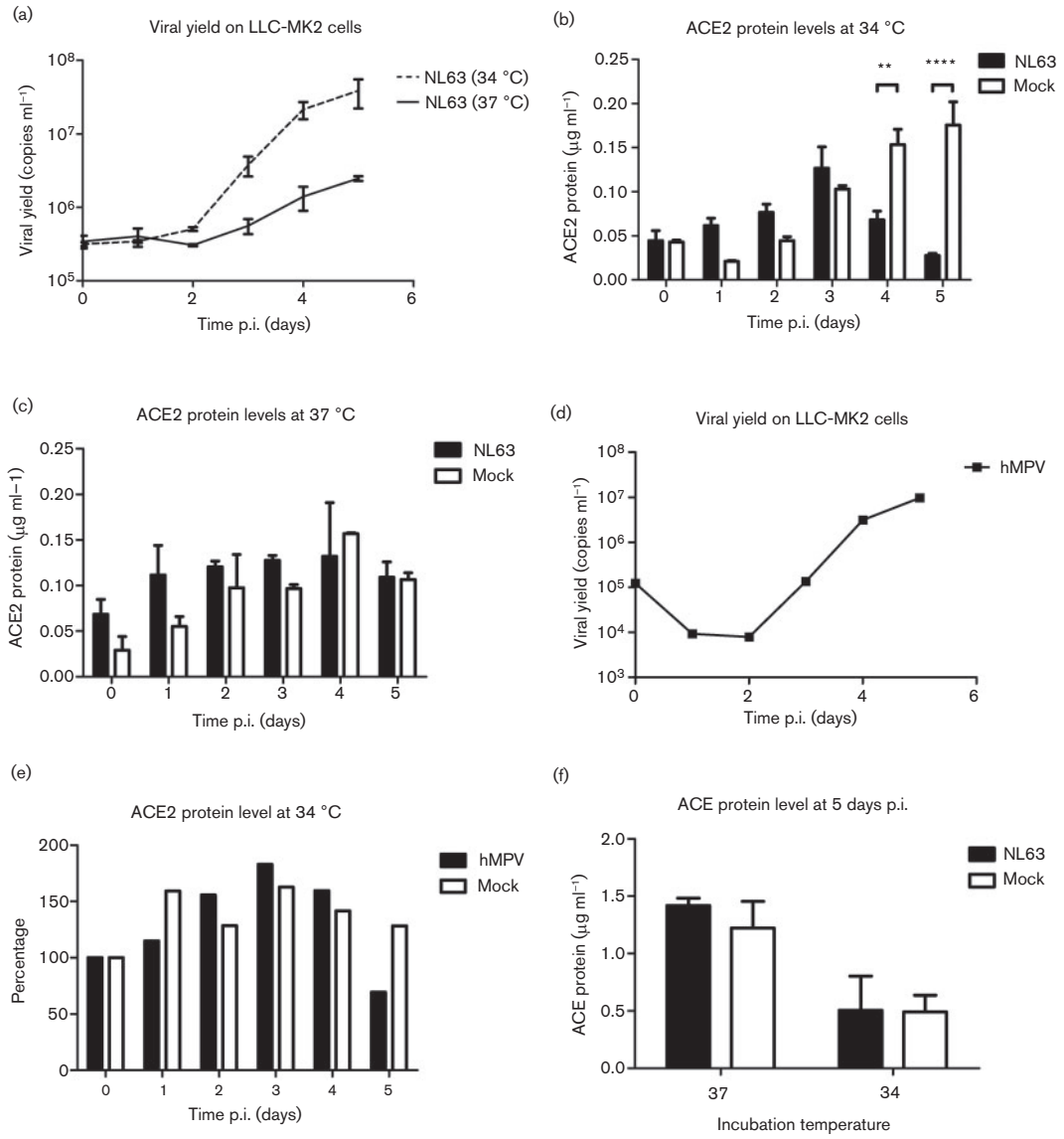

Fig. 2. ACE2 down modulation is dependent on HCoV-NL63 replication efficiency. (a) The HCoV-NL63 viral yield monitored at 34 and 37 °C. (b) The ACE2 concentration at 34 °C in mock- and HCoV-NL63-infected cultures (**=P<0.01, ****=P<0.0001). (c) The ACE2 concentration at 37 °C in mock- and HCoV-NL63-infected cultures. (d) Human metapneumovirus (hMPV) yield monitored at 34 °C. (e) The percentage of ACE2 expression in mock- and hMPV-infected cultures (f) ACE concentration at 34 and 37 °C in mock- and HCoV-NL63-infected cultures. The results are shown as the mean of two independent experiments with SEM, except for panel (d) and (e) where the results from a representative experiment are shown.

two-way ANOVA, using the Bonferroni multiple comparison test with a threshold for significance of P-value <0.05, in the Prism software version 5 (Graphpad).

At 34 and 37 °C, a CPE was visible at day 4, yet the effect was notably weaker at 37 °C. The total HCoV-NL63 viral yield at 37 °C increased only 1-log over time, whereas a 2-log increase was detected at 34 °C (Fig. 2a). In accordance, the amount of NL63 N protein within the cell lysate was lower under suboptimal conditions compared with optimal conditions (data not shown). Therefore, the lower viral yield detected in the supernatant was not due to reduced budding that may lead to the accumulation of virions within the cells. It was evaluated whether the difference in replication could be due to variation of the initial level of cellular ACE2 protein expression at these temperatures, yet no difference in initial ACE2 expression at both temperatures was noted (data not shown). Significant decrease of cellular ACE2 protein was detected 4 days p.i. (P=<0.01) in HCoV-NL63-infected cultures incubated at 34 °C (Fig.

2b), whereas no significant difference between cellular ACE2 protein levels was detected in cultures incubated at 37 °C (Fig. 2c). These results suggest that downregulation of cellular ACE2 is related to the fold increase in viral yield of HCoV-NL63.

The m.o.i. value that is used for inoculation is of influence to the number of infected cells, and the measurability of the effect on infected cells. A difference in m.o.i. value might explain the discrepancy between our results and those from Glowacka and colleagues. In our experiment, we used an m.o.i. of 0.007 for HCoV-NL63, whereas Glowacka and colleagues used an m.o.i. of 0.0001. An m.o.i. of 0.0001 will, in the case of a 2-log increase of HCoV-NL63, result in 1–10 % of the cells infected, whereas an m.o.i. of 0.007 will result in infection of the majority of cells. As shown above, a majority of infected cells is required to measure the decrease in cellular ACE2 expression during HCoV-NL63 infection.

We observed the first signs of CPE development at 3 days p.i., which coincides with the decrease in ACE2 expression. To determine whether the effect on ACE2 expression is HCoV-NL63-specific or an unspecific result of the CPE the virus is inducing, we used a control virus that replicates in LLC-MK2 cells with comparable efficiency as HCoV-NL63: human metapneumovirus (hMPV) (kindly provided by Oliver Schildgen, Institute for Pathology, Kliniken der Stadt Köln gGmbH, Private University of Witten/Herdecke, Cologne, Germany). The same m.o.i. value of hMPV was used as described for HCoV-NL63. The level of viral yield and cellular ACE2 expression was monitored for 5 days at 34 °C. The exponential increase of 2-log in hMPV viral yield is comparable to that of HCoV-NL63 (Fig. 2a and d), as well as the development of CPE characterized by focal rounding without syncytia formation. However, the effect of infection on the ACE2 levels was different from the HCoV-NL63 effect. Until day 4, ACE2 levels are equal to the mock infection, so also during exponential rise in virus titres. Only at day 5 an intermediate decrease in ACE2 expression is observed compared with day 4, which is probably caused by the CPE development in combination with amino acid starvation (Fig. 2e). Unlike HCoV-NL63, hMPV cultures are maintained in serum-free medium supplemented with 0.025 % trypsin. Surprisingly, the presence of trypsin did not affect the cellular ACE2 protein level.

A final control to determine whether the ACE2 expression decrease is HCoV-NL63 replication-specific was provided by monitoring the levels of ACE during infection. ACE is the homologue of ACE2 that has an opposite biological role. The levels of ACE protein were determined by Western blot analysis within cell lysates of HCoV-NL63-infected cultures, as described above using goat-derived anti-human ACE ectodomain antibody (2 μg ml^{-1}, AF929; R&D Systems). The concentration of ACE protein at 37 °C was higher compared with the concentration at 34 °C; however, both remained unaffected during HCoV-NL63 infection (Fig. 2f). Thus, HCoV-NL63 infection specifically affects expression of its receptor ACE2. This phenomenon, decreased expression of a viral receptor during infection, has also been described for Epstein–Barr virus, hepatitis B, influenza C, measles and human immunodeficiency virus 1 (Aiken *et al.*, 1994; Breiner *et al.*, 2001; Marschall *et al.*, 1997; Naniche *et al.*, 1993; Tanner *et al.*, 1987). Future studies focusing on HCoV-NL63 cell entry should clarify which viral and cellular proteins are employed for the downregulation of ACE2.

In summary, we investigated whether the cellular level of the ACE2 protein, the receptor of HCoV-NL63, is affected by HCoV-NL63 infection. A robust decline in ACE2 expression was observed 3 days p.i., while the levels of cellular ACE, which is not used as a receptor, remained constant. The decrease in ACE2 is dependent on HCoV-NL63 replication efficiency.

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References

Aiken, C., Konner, J., Landau, N. R., Lenburg, M. E. & Trono, D. (1994). Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* **76**, 853–864.

Bradburne, A. F., Bynoe, M. L. & Tyrrell, D. A. (1967). Effects of a "new" human respiratory virus in volunteers. *BMJ* **3**, 767–769.

Breiner, K. M., Urban, S., Glass, B. & Schaller, H. (2001). Envelope protein-mediated down-regulation of hepatitis B virus receptor in infected hepatocytes. *J Virol* **75**, 143–150.

Dijkman, R., Mulder, H. L., Rumping, L., Kraaijvanger, I., Deijs, M., Jebbink, M. F., Verschoor, E. J. & van der Hoek, L. (2009). Seroconversion to HCoV-NL63 in rhesus macaques. *Viruses* **1**, 647–656.

Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K. & other authors (2000). A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* **87**, E1–E9.

Falsey, A. R., McCann, R. M., Hall, W. J., Criddle, M. M., Formica, M. A., Wycoff, D. & Kolassa, J. E. (1997). The "common cold" in frail older persons: impact of rhinovirus and coronavirus in a senior daycare center. *J Am Geriatr Soc* **45**, 706–711.

Fouchier, R. A., Kuiken, T., Schutten, M., van Amerongen, G., van Doornum, G. J., van den Hoogen, B. G., Peiris, M., Lim, W., Stöhr, K. & Osterhaus, A. D. (2003). Aetiology: Koch's postulates fulfilled for SARS virus. *Nature* **423**, 240.

Glowacka, I., Bertram, S., Herzog, P., Pfefferle, S., Steffen, I., Muench, M. O., Simmons, G., Hofmann, H., Kuri, T. & other authors (2010). Differential downregulation of ACE2 by the spike proteins of

severe acute respiratory syndrome coronavirus and human coronavirus NL63. *J Virol* **84**, 1198–1205.

Hofmann, H., Pyrc, K., van der Hoek, L., Geier, M., Berkhout, B. & Pöhlmann, S. (2005). Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc Natl Acad Sci U S A* **102**, 7988–7993.

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., Yang, P., Sarao, R., Wada, T. & other authors (2005). Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* **436**, 112–116.

Jia, H. P., Look, D. C., Tan, P., Shi, L., Hickey, M., Gakhar, L., Chappell, M. C., Wohlford-Lenane, C. & McCray, P. B., Jr (2009). Ectodomain shedding of angiotensin converting enzyme 2 in human airway epithelia. *Am J Physiol Lung Cell Mol Physiol* **297**, L84–L96.

Kraaijeveld, C. A., Reed, S. E. & Macnaughton, M. R. (1980). Enzyme-linked immunosorbent assay for detection of antibody in volunteers experimentally infected with human coronavirus strain 229 E. *J Clin Microbiol* **12**, 493–497.

Kuba, K., Imai, Y., Rao, S., Gao, H., Guo, F., Guan, B., Huan, Y., Yang, P., Zhang, Y. & other authors (2005). A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med* **11**, 875–879.

Lambert, D. W., Clarke, N. E. & Turner, A. J. (2010). Not just angiotensinases: new roles for the angiotensin-converting enzymes. *Cell Mol Life Sci* **67**, 89–98.

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., Somasundaran, M., Sullivan, J. L., Luzuriaga, K. & other authors (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450–454.

Li, W., Sui, J., Huang, I. C., Kuhn, J. H., Radoshitzky, S. R., Marasco, W. A., Choe, H. & Farzan, M. (2007). The S proteins of human coronavirus NL63 and severe acute respiratory syndrome coronavirus bind overlapping regions of ACE2. *Virology* **367**, 367–374.

Macnaughton, M. R., Hasony, H. J., Madge, M. H. & Reed, S. E. (1981). Antibody to virus components in volunteers experimentally infected with human coronavirus 229E group viruses. *Infect Immun* **31**, 845–849.

Marra, M. A., Jones, S. J., Astell, C. R., Holt, R. A., Brooks-Wilson, A., Butterfield, Y. S., Khattra, J., Asano, J. K., Barber, S. A. & other authors (2003). The genome sequence of the SARS-associated coronavirus. *Science* **300**, 1399–1404.

Marschall, M., Meier-Ewert, H., Herrler, G., Zimmer, G. & Maassab, H. F. (1997). The cell receptor level is reduced during persistent infection with influenza C virus. *Arch Virol* **142**, 1155–1164.

Mathewson, A. C., Bishop, A., Yao, Y., Kemp, F., Ren, J., Chen, H., Xu, X., Berkhout, B., van der Hoek, L. & Jones, I. M. (2008). Interaction of severe acute respiratory syndrome-coronavirus and NL63 coronavirus spike proteins with angiotensin converting enzyme-2. *J Gen Virol* **89**, 2741–2745.

Naniche, D., Wild, T. F., Rabourdin-Combe, C. & Gerlier, D. (1993). Measles virus haemagglutinin induces down-regulation of gp57/67, a molecule involved in virus binding. *J Gen Virol* **74**, 1073–1079.

Perlman, S. & Dandekar, A. A. (2005). Immunopathogenesis of coronavirus infections: implications for SARS. *Nat Rev Immunol* **5**, 917–927.

Perlman, S. & Netland, J. (2009). Coronaviruses post-SARS: update on replication and pathogenesis. *Nat Rev Microbiol* **7**, 439–450.

Reed, S. E. (1984). The behaviour of recent isolates of human respiratory coronavirus in vitro and in volunteers: evidence of heterogeneity among 229E-related strains. *J Med Virol* **13**, 179–192.

Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A., Campagnoli, R., Icenogle, J. P., Peñaranda, S., Bankamp, B., Maher, K. & other authors (2003). Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* **300**, 1394–1399.

Ruijter, J. M., Thygesen, H. H., Schoneveld, O. J., Das, A. T., Berkhout, B. & Lamers, W. H. (2006). Factor correction as a tool to eliminate between-session variation in replicate experiments: application to molecular biology and retrovirology. *Retrovirology* **3**, 2.

Sastre, P., Dijkman, R., Camuñas, A., Ruiz, T., Jebbink, M. F., van der Hoek, L., Vela, C. & Rueda, P. (2011). Differentiation between human coronaviruses NL63 and 229E using a novel double-antibody sandwich enzyme-linked immunosorbent assay based on specific monoclonal antibodies. *Clin Vaccine Immunol* **18**, 113–118.

Schildgen, O., Jebbink, M. F., de Vries, M., Pyrc, K., Dijkman, R., Simon, A., Müller, A., Kupfer, B. & van der Hoek, L. (2006). Identification of cell lines permissive for human coronavirus NL63. *J Virol Methods* **138**, 207–210.

Smith, M. K., Tusell, S., Travanty, E. A., Berkhout, B., van der Hoek, L. & Holmes, K. V. (2006). Human angiotensin-converting enzyme 2 (ACE2) is a receptor for human respiratory coronavirus NL63. *Adv Exp Med Biol* **581**, 285–288.

Tanner, J., Weis, J., Fearon, D., Whang, Y. & Kieff, E. (1987). Epstein–Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* **50**, 203–213.

Tipnis, S. R., Hooper, N. M., Hyde, R., Karran, E., Christie, G. & Turner, A. J. (2000). A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* **275**, 33238–33243.

van der Hoek, L. (2007). Human coronaviruses: what do they cause? *Antivir Ther* **12** (4 Pt B), 651–658.

van der Hoek, L., Pyrc, K., Jebbink, M. F., Vermeulen-Oost, W., Berkhout, R. J., Wolthers, K. C., Wertheim-van Dillen, P. M., Kaandorp, J., Spaargaren, J. & Berkhout, B. (2004). Identification of a new human coronavirus. *Nat Med* **10**, 368–373.

van der Hoek, L., Sure, K., Ihorst, G., Stang, A., Pyrc, K., Jebbink, M. F., Petersen, G., Forster, J., Berkhout, B. & Uberla, K. (2005). Croup is associated with the novel coronavirus NL63. *PLoS Med* **2**, e240.

van der Hoek, L., Ihorst, G., Sure, K., Vabret, A., Dijkman, R., de Vries, M., Forster, J., Berkhout, B. & Uberla, K. (2010). Burden of disease due to human coronavirus NL63 infections and periodicity of infection. *J Clin Virol* **48**, 104–108.

Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J., Godbout, K., Parsons, T., Baronas, E. & other authors (2002). Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* **277**, 14838–14843.

Warner, F. J., Lew, R. A., Smith, A. I., Lambert, D. W., Hooper, N. M. & Turner, A. J. (2005). Angiotensin-converting enzyme 2 (ACE2), but not ACE, is preferentially localized to the apical surface of polarized kidney cells. *J Biol Chem* **280**, 39353–39362.