Species-specific and individual differences in Nipah virus replication in porcine and human airway epithelial cells

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Highly pathogenic Nipah virus (NiV) causes symptomatic infections in pigs and humans. The severity of respiratory symptoms is much more pronounced in pigs than in humans, suggesting species-specific differences of NiV replication in porcine and human airways. Here, we present a comparative study on productive NiV replication in primary airway epithelial cell cultures of the two species. We reveal that NiV growth substantially differs in primary cells between pigs and humans, with a more rapid spread of infection in human airway epithelia. Increased replication, correlated with higher endogenous expression levels of the main NiV entry receptor ephrin-B2, not only significantly differed between airway cells of the two species but also varied between cells from different human donors. To our knowledge, our study provides the first experimental evidence of species-specific and individual differences in NiV receptor expression and replication kinetics in primary airway epithelial cells. It remains to be determined whether and how these differences contribute to the viral host range and pathogenicity.

INTRODUCTION

Nipah virus (NiV) is a highly pathogenic member of the genus Henipavirus within the family Paramyxoviridae (Wang et al., 2000). It encodes two surface glycoproteins that must act together during virus entry and cell–cell fusion processes. To fulfil this task, the receptor-binding glycoprotein G (NiV G) has to bind to its main host cell receptor, ephrin-B2 (Bonaparte et al., 2005; Negrete et al., 2005). In some neurological tissues, NiV G can additionally use ephrin-B3 as entry receptor, though the binding affinity was shown to be reduced compared to NiV G–ephrin-B2 binding (Negrete et al., 2006). After binding to ephrin, a conformational change in NiV G triggers NiV fusion protein (NiV F)–mediated virus–cell or cell–cell fusion events. As NiV F is synthesized as inactive precursor F0, NiV G/F-mediated fusion depends on functional F1 cleavage into F1 and F2 subunits by endosomal cathepsin L or B (Diederich et al., 2012; Pager et al., 2006).

Fruit bats in Southeast Asia have been identified as natural virus reservoir of zoonotic NiV (Clayton et al., 2013; Luby et al., 2009; Yob et al., 2001) that can cause clinical disease in a wide range of mammalian species. NiV was identified in 1998/1999 when it emerged for the first time in Malaysia and Singapore causing a major outbreak of severe respiratory disease in pigs. Upon close contact to infected pigs, the virus was transmitted to humans who developed severe encephalitis with mortality rates of 40% (Chua et al., 1999, 2000). In contrast to symptomatically infected pigs that all developed respiratory diseases, only 14–27% of NiV-infected human patients suffered from respiratory symptoms (Bellini et al., 2003; Hossain et al., 2008; Lo & Rota, 2008; Middleton et al., 2002). Also virus shedding via respiratory secretions was less frequently observed (Chua et al., 2001). In agreement with the distinct clinical representation, host-specific differences in lung pathology have been demonstrated histologically in fixed lung tissue samples from NiV-infected pigs and fatal human cases (Chua et al., 2000; Hooper et al., 2001; Maisner et al., 2009; Middleton et al., 2002; Tanimura et al., 2004; Weingartl et al., 2005; Wong et al., 2002). As porcine and human NiV isolates did not differ in their sequence (AbuBakar et al., 2004), species-specific host factors rather than genetic variabilities must be responsible for the differences in NiV replication in the respiratory tract of pigs and humans. On a molecular level, these host factors are not well understood, not least because direct comparative studies on NiV replication in airway epithelia of the two species have not been
performed so far. Therefore, and in contrast to studies comparing different henipaviruses (Hendra virus, NiVMalaysia and NiVBangladesh) in the same cell culture or in the same animal model that revealed several virus strain-dependent differences in replication, pathogenesis and cytokine profile (Baseler et al., 2015; DeBuysscher et al., 2013; Escaffle et al., 2013, 2016; Rockx et al., 2010, 2011), our study aimed to identify host factors varying between different species that influence replication of the same virus isolate. For this purpose, we analysed NiV growth and syncytia formation in freshly isolated porcine airway epithelial cells (PAEpC) and primary human airway epithelial cells (HAEpC). The host-specific differences in replication revealed in our study correlated with significant differences in endogenous NiV receptor expression levels that not only differed between porcine and human airway epithelial cells (species-specific differences), but also between cells of different human donors (individual variability).

RESULTS

NiV replication in porcine and human airway epithelial cells differs significantly

To compare NiV replication in primary airway epithelia of pigs and humans, PAEpC and HAEpC were infected at an m.o.i. of 0.5. To analyse infection kinetics, we monitored syncytia formation, quantified viral RNA in infected cell lysates by quantitative real-time PCR (qPCR) and determined virus titres in the supernatants at 24 and 48 h after infection. As demonstrated in Fig. 1a, syncytia formation in HAEpC was already visible 24 h after infection, while cell–cell fusion in PAEpC was only detected at 48 h post infection (p.i.). Quantification of fusion by counting cell nuclei within and outside of syncytia confirmed these prominent differences to be statistically significant (Fig. 1b). In agreement with the much higher numbers of infected HAEp cells at 24 h and 48 h p.i., cell-free virus titres (Fig. 1c) and cell-associated NiV RNA (Fig. 1d) were found to be drastically increased compared to titres produced in PAEpC. It has been shown recently that NiV infection only inefficiently induces interferon (IFN) production in human airway epithelial cultures (Escaffle et al., 2016). As IFN induction in porcine epithelial cells has not been studied to date, it could not be ruled out that infection of PAEpC was limited because of high IFN induction interfering with productive multi-cycle viral replication. To test this, we measured the expression of porcine and human IFN β. The finding that, in line with the lower numbers of initially infected cells, upregulation of IFN β was much less prominent in NiV-infected PAEpC (Fig. 1e) clearly indicated that limited NiV replication in PAEpC is unlikely due to IFN-dependent antiviral effects. It must rather be assumed that restricted infection of PAEpC cultures is the result of a less efficient virus entry and/or a limited spread of infection by cell–cell fusion or via cell-free infectious virus particles.

Using MDCK cells as a model epithelial cell line, we previously demonstrated that NiV fusion protein activation in polarized epithelial cells is mediated by endosomal cathepsin B (Diederich et al., 2012). When we repeated these experiments using PAEpC and HAEpC, we found similar cleavage rates and protease requirements (data not shown). We therefore ruled out the possibility that the observed host-specific differences in NiV replication were correlated to variations in the processing of the fusion protein.

Porcine and human ephrin-B2 do not differ in their capacity to mediate NiV fusion and cell entry

Amino acid sequences of ephrin-B2 from pigs and humans differ by 4%. Although both ephrins are known to support NiV infection (Bossart et al., 2008), there might be some qualitative differences in their ability to promote virus entry and fusion processes. To test this hypothesis, ephrin-B2-negative porcine aortic endothelial cells (PAoEC) (Erbar et al., 2008) were transfected with plasmids encoding porcine or human ephrin-B2 (pCAGGS-pEB2 or pCAGGS-hEB2). 16 h after transfection, cells were infected with NiV, and 24 h later infected cells were fixed and inactivated with 4% paraformaldehyde (PFA) for 48 h. After cell permeabilization, virus-positive syncytia were detected by immunofluorescence using a NiV-specific polyclonal antiserum and AF568-labelled secondary antibodies. As shown in Fig. 2a, untransfected PAoEC (control) did not show any cell–cell fusion while cells pre-transfected with either porcine or human ephrin-B2 (+pEB2 and +hEB2, respectively) showed large virus-positive syncytia of the same size. This finding was supported by a mixed fusion assay in which ephrin-B2-pre-transfected PAoEC were mixed with PAoEC transfected with NiV F, NiV G and eGFP (Fig. 2b). In cell–cell fusion in the mixed cultures monitored over 24 h did not show any obvious differences in numbers or sizes of syncytia formed with PAoEC expressing either human or porcine ephrin-B2. For quantitative analysis, we determined the total number of nuclei in syncytia and analysed the ephrin-B2 surface expression by flow cytometry, and did not find differences between the two cultures (Fig. 2c). We thus concluded that porcine and human ephrin-B2 do not differ significantly in their potency to function as NiV entry and fusion receptor.

PAEpC and HAEpC differ in their endogenous ephrin-B2 expression levels

Though there are no species-specific functional differences between porcine and human ephrin-B2, endogenous receptor expression levels in primary airway epithelial cell cultures may differ, thereby influencing NiV infection efficiencies. Supporting this idea, qPCR analysis revealed much lower ephrin-B2 mRNA levels in PAEpC (Fig. 3a). In line with this, surface expression analysis of ephrin-B2 determined by flow cytometry revealed significantly lower receptor densities in PAEpC compared to HAEpC (Fig. 3b). To determine whether lower receptor levels contribute to
**Fig. 1.** NiV infection of primary airway epithelial cells of pigs and humans. (a) Syncytia formation in PAEpC and HAEpC. Cells were infected with NiV at an m.o.i. of 0.5. At 24 and 48 h p.i., NiV-induced syncytia formation was documented in live cells by phase-contrast microscopy at ×100 magnification. (b) Quantification of cell–cell fusion. Cells in and outside of syncytia in five randomly chosen microscopic fields were counted and averaged. The percentages of cells in syncytia (>5 nuclei) are given. Mean values and standard deviations (error bars) are shown. (c) Virus titration in cell supernatants. Cell-free viruses in supernatants were quantified by the limited dilution method at 24 and 48 h p.i. Titres are expressed as 50% tissue culture infectious doses (TCID<sub>50</sub> ml<sup>-1</sup>) (n=3). Error bars indicate the standard deviation. (d) qPCR of cell-associated NiV N RNA. Total RNA was isolated from cell lysates and reverse transcribed using random hexamer primers. cDNA was then analysed by quantitative real-time PCR (Applied Biosystems/SYBR Green) using specific primers for NiV N. Ct values were normalized to the internal control (α-tubulin), and are shown as 2<sup>-ΔΔCt</sup>. Error bars indicate the standard deviation of three replicate experiments. (e) IFN induction. At 24 and 48 h p.i., cellular RNA was reverse transcribed using random hexamer primers. qPCR was performed with specific primers for porcine and human IFN β. The fold-change over mock is shown (as 2<sup>-ΔΔCt</sup>). Bars indicate the standard deviation. Asterisks indicate statistically significant differences (unpaired t-test; **, P<0.01; ***, P<0.001).
the limited NiV infection of PAEpC, primary porcine airway cultures were pre-transfected with pCAGGS-pEB2, or pCAGGS-eGFP as a control. 16 h after transfection, cells were infected with NiV at a high m.o.i., and virus infection was analysed at 24 h p.i.. As depicted in Fig. 4a, cell–cell fusion was significantly enhanced in PAEpC expressing plasmid-encoded ephrin-B2 (+pEB2). Also, cell-associated viral RNA (Fig. 4b) and virus titres in the cell supernatants (Fig. 4c) were increased accordingly. Due to the low transfection efficiency in primary cells, we did not find infection rates similar to that in human airway cells by transfecting ephrin-B2 into PAEpC. We therefore cannot rule out the possibility that there are additional host factors restricting NiV replication in porcine airway epithelia. However, the finding that ephrin-B2 over-expression led to a more productive NiV infection of PAEpC supported the conclusion that limited endogenous receptor expression restricts the permissiveness of primary PAEpC to NiV infection.

Ephrin-B2 levels of airway epithelial cells vary between different human donors

While symptomatic respiratory disease was consistently observed in NiV-infected pigs, the frequency and severity of respiratory symptoms varied significantly in human NiV infections. We therefore wanted to determine whether there is variability in ephrin-B2 expression in airway epithelial cells of different individuals that could affect the susceptibility to NiV. For this purpose, we analysed ephrin-B2 mRNA levels in PAEpC of five different pigs and in HAEpC of ten different human donors. While PAEpC cultures of the five pigs did not show major differences in their low content of ephrin-B2 mRNA, the ephrin-B2 mRNA levels in HAEpC cultures from ten different human donors showed a high variability (Fig. 5). To evaluate whether such variations in receptor mRNA levels have any biological significance, HAEpC cultures from two human donors with different ephrin-B2 expression profiles were infected. As shown in Fig. 6a, cells from donor 073 expressed higher receptor amounts on the cell surface compared to HAEpC cultures from donor 072 (MFI 22.2 and MFI 14.98, respectively). When we infected these cultures with NiV, we found increased syncytia formation in HAEpC from donor 073 (Fig. 6b). The increased susceptibility to NiV of cells from donor 073 with higher endogenous ephrin-B2 levels was also reflected by the production of 100-fold higher virus titres in infected cell supernatants (Fig. 6c). These findings support the idea that not only species-specific differences but also individual variations in ephrin-B2 expression can influence the kinetics of NiV infection in airway epithelial cells.
showed a more rapid spread via cell-cell fusion compared to PAEpC. We found that more efficient infection of human cells could neither be explained by reduced type I interferon upregulation in HAEpC nor by improved receptor functions of human ephrin-B2. Rather, distinctions in NiV replication kinetics correlated with differences in endogenous NiV receptor expression that varied between the two species and between different human donors.

Ephrin-B2, as natural ligand of Eph receptors, has important functions in embryonic patterning, axon guidance, blood vessel remodelling and lymphangiogenesis (Kullander & Klein, 2002). Therefore, ephrin-B2 is widely expressed and highly conserved in all mammalian species. In agreement with an earlier study that demonstrated the principal capacity of ephrin-B2 from different species to function as NiV entry receptor (Bossart et al., 2008), we did not see functional differences between cells transfected with porcine and human ephrin-B2. However, we found significant differences in the intrinsic receptor expression levels in primary airway epithelial cultures. The generally low amount of surface receptors in PAEpC correlated with an overall limited NiV replication. Experiments on ephrin-B2 over-expression in PAEpC (Fig. 4) and comparative studies of human donor cells expressing different receptor amounts (Fig. 6) support our hypothesis that endogenous ephrin-B2 expression levels critically influence the extent and the kinetics of NiV replication in primary airway epithelial cells.

During the initial NiV outbreak in pigs in Malaysia, the virus was efficiently transmitted from pig to pig via direct contact (Mohd Nor et al., 2000). Our finding of rather low expression levels of NiV entry receptors in porcine airway cells suggests that productive primary infection of these cells does not represent a very efficient entry pathway that would allow the virus to overcome the epithelial barrier and initiate systemic infection. It thus will be interesting to determine whether NiV can establish primary infection by hijacking immune cells in the respiratory tract, a highly efficient entry pathway known to be used by other paramyxoviruses causing systemic infections, such as measles virus or measles virus.

**DISCUSSION**

This study presents a systematic comparison of productive NiV replication in primary porcine versus human airway epithelial cells. Although both were permissive to NiV infection, infected HAEpC produced higher viral titres and showed a more rapid spread via cell–cell fusion compared to PAEpC. We found that more efficient infection of human cells could neither be explained by reduced type I interferon upregulation in HAEpC nor by improved receptor functions of human ephrin-B2. Rather, distinctions in NiV replication kinetics correlated with differences in endogenous NiV receptor expression that varied between the two species and between different human donors.

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canine distemper virus (de Swart et al., 2007; Lemon et al., 2011; Rudd et al., 2006).

While respiratory disease was regularly observed in pigs during the NiV outbreak in Malaysia, respiratory symptoms were generally less frequent and less severe in human infections (Lo & Rota, 2008; Mohd Nor et al., 2000). We therefore did not expect to observe a more efficient NiV replication in our human tracheal/bronchial airway cultures. This apparent inconsistency with the clinical data could be explained by limitations of our in vitro model, in which macrophages and other cells of the innate immune system are lacking. Furthermore, Escaffre and colleagues provided conclusive evidence that NiV-infected small airway epithelia rather than infected tracheal/bronchial epithelial cultures produced high levels of pro-inflammatory cytokines and chemokines (Escaffre et al., 2016). This, together with an earlier histopathological study on lung tissue samples from a NiV-infected fatal human case (Wong et al., 2002), indicates that virus-induced inflammation processes in small-airway epithelia may be more critical than productive replication in the upper airway epithelia for disease severity and respiratory symptoms during NiV infection.

Fig. 5. Variations in ephrin-B2 expression in primary airway epithelial cells from different porcine and human donors. To compare endogenous ephrin-B2 expression in different individuals, total RNA was isolated from PAEpC and HAEPc cultures prepared from cells of five pigs and ten human donors. Ephrin-B2-specific qPCR was performed as described in the legend to Fig. 3.

Fig. 6. NiV infection of HAEPc from two human donors with different ephrin-B2 expression profiles. (a) Ephrin-B2 surface expression in HAEPc from two different donors. HAEPc cultures of donors 072 and 073 were detached and fixed with PFA before they were immunostained with soluble EphB4/Fc and a FITC-labelled secondary antibody. Flow cytometry was carried out with a Guava easyCyte Flow Cytometer. MFI, mean fluorescence intensity. (b, c) Spread and release of NiV at 16 h p.i. HAEPc cultures of donors 072 and 073 were infected with NiV at an m.o.i. of 0.5. (b) Cell–cell fusion was documented 16 h.p.i. by phase-contrast microscopy at ×100 magnification. White lines have been added to indicate syncytia. (c) Virus production was measured by titrating viruses in the supernatants (n=3). Asterisks indicate statistically significant differences (unpaired t-test; **, P<0.01).
A recent study described differences in the susceptibility to influenza virus H1N1pdm infections of primary alveolar epithelial cells from different human donors (Travanty et al., 2015). Although the authors had not yet identified the molecular basis for the observed variations, this study is consistent with our finding of individual differences in the permissiveness to NiV infection of primary human airway cells from different donors. Our data suggest that these differences are determined, at least in part, by variation in NiV receptor levels on the cells among individual donors. It remains unclear why such variability in receptor expression and sensitivity to infection was not seen in airway cells of individual pigs. A potential explanation could be the much higher variations between humans in terms of age, genetic background and health status. This idea clearly needs to be evaluated further since, to our knowledge, endogenous ephrin-B2 expression levels have never been analysed in human airway epithelial cells in vivo. The study of Hafner et al. (2004) described high ephrin-B2 levels in lung homogenates from healthy human donors, in agreement with the high ephrin-B2 levels we found in HAEpC. However, that study did not analyse the cell type (fibroblasts, endothelial, epithelial or immune cells) in which ephrin-B2 was abundantly expressed, and whether there were variations among different donors. Though it remains to be elucidated whether the individual differences found in primary airway epithelial cell cultures also occur in human airway epithelial cells in vivo, it is tempting to speculate that individual variations in NiV receptor expression contributed to the susceptibility to NiV infection and/or to the variability in respiratory symptoms in human NiV infections observed in the Malaysian outbreak in 1998.

Compared to infections with the NiV Malaysia strain, human NiV infections with the Bangladesh strain were much more regularly associated with clinical respiratory disease (Hossain et al., 2008). The recent finding by Escatire et al. (2016) that NiV_Bangladesh replicates more efficiently in differentiated human airway cultures supports the view that the observed differences in respiratory disease are linked to intrinsic differences between the two NiV strains. In view of the more consistent respiratory symptoms, it will be interesting to determine whether replication of NiV_Bangladesh in airway epithelia from different human donors varies to a similar extents = as the infection kinetics of NiV_Malaysia.

In summary, this study demonstrates that NiV receptor expression levels differ in primary airway epithelial cells isolated from porcine and human lungs, thereby influencing NiV replication in terms of cell–cell fusion and virus release. The finding that infection not only differed in cells from the two species, but also varied in human epithelial cells from different donors, should be considered for the design and interpretation of NiV replication studies in primary airway cultures.

METHODS

Cell culture. Porcine airway epithelial cells (PAEpC) were isolated from fresh porcine lung tissue obtained from two local slaughterhouses, as described by Goris et al. (2009). Briefly, bronchi were prepared by removing the connective tissue and were then incubated in Dulbecco’s modified minimal essential medium (DMEM) containing 0.05 % DTT for 24 h to remove the mucus, followed by treatment with Protease 14 (0.1 %, diluted in DMEM) for 24 h. Epithelial cells were scraped off from the bronchi and pelleted by centrifugation for 10 min at 230 g. Cells obtained from one pig lung were resuspended in 5 ml airway epithelial cell growth medium (AEGM, PromoCell) and seeded on culture flasks (Costar, Corning) coated with collagen type I from rat tail (30 µg µl−1 in 0.02 M acetic acid; ThermoFisher). Cells were cultivated at 37 °C and 5 % CO2 until they had reached 80 % confluence, and were then frozen and stored in liquid nitrogen. Human airway epithelial cells (HAEpC) from 10 different human donors were purchased from three different companies (Provitro, cat. no. EP408B; Lonza, cat. no. CC-2540).

For infection studies, PAEpC and HAEpC were seeded on collagen-coated 6-well plates and cultivated with AEGM supplemented with 100 U ml−1 penicillin, 0.1 mg ml−1 streptomycin, 50 µg ml−1 kanamycin and 2.5 µg ml−1 amphotericin B (Fungizone; Gibco). Ephrin-B2-negative porcine aortic endothelial cells (PAoEC) (Erbar et al., 2008) were cultivated in DMEM-F12 (Gibco) containing 5 % foetal calf serum (FCS), 4 mM l-glutamine, 100 U ml−1 penicillin and 0.1 mg ml−1 streptomycin. Vero76 cells were maintained in DMEM (Gibco) containing 10 % FCS, glutamine, penicillin and streptomycin.

Virus infections. All experiments with live NiV were performed under biosafety level 4 (BSL-4) conditions at the Institute of Virology, Philippus University Marburg. The NiV_Malaysia strain used in this study was described previously (Diederich et al., 2012; Dietzel et al., 2015; Moll et al., 2004).

For infection studies, confluent PAEpC and HAEpC were infected with NiV at a multiplicity of infection (m.o.i.) of 0.5 or 5. After virus adsorption at 37 °C for 60 min, cells were washed and incubated at 37 °C with DMEM containing 2 % FCS. To quantify cell–cell fusion in the infected cultures, cells within and outside of syncytia were counted and averaged in five randomly chosen microscopic fields (% cells in fusion). After 24 and 48 h, cells in replicate cultures were lysed using RLT-Buffer (Qiagen) containing 1 % β-mercaptoethanol (ME), followed by RNA isolation according to the manufacturer’s instructions (RNeasy Kit; Qiagen). Virus concentrations in the culture supernatants were determined by titration in Vero76 cells and expressed as 50 % tissue culture infective doses per ml (TCID50 ml−1) as described previously (Diederich et al., 2008).

cDNA synthesis and quantitative real-time PCR (qPCR). Total RNA was extracted from PAEpC and HAEpC using the Qiagen RNeasy Kit according to the manufacturer’s protocol. Purified RNA (100 ng) was reverse transcribed using random hexamer primers and the ReverTag & Maisner (2015) and are available on request. Amplification was carried out using a StepOne Real-Time PCR System (Applied Biosystems), and 100 nM forward and reverse primers specific for NiV N or the respective porcine or human gene were added [primer sequences are listed in Frei et al. (2009) as described previously (Diederich et al., 2008).
experiments. The statistical significance of differences was analysed by unpaired t-test using GraphPad software.

**Plasmid-driven ephrin-B2 expression.** To clone the porcine ephrin-B2 gene, total RNA was isolated from PAEC pC with the RNeasy Kit (Qiagen) and reverse transcribed into cDNA using random hexamer primers. The ephrin-B2 gene was amplified using specific primers described by Bossart et al. (2008), and the cDNA was cloned into a pCAGGS vector (Niwa et al., 1991) using the restriction enzymes NotI and NheI. Cloning of human ephrin-B2 has been described previously (Thiel et al., 2008). Expression of cloned ephrin-B2 genes was assayed by transfecting 7.5 μg pCAGGS-pEB2 or pCAGGS-hEB2 into ephrin-B2-negative PAEC using Lipofectamine 2000 (Life Technologies). 16 h after transfection, surface-expressed ephrin-B2 was immunostained with soluble EphB4/Fc as described previously (Lamp et al., 2013).

**Flow cytometric analysis of ephrin-B2 surface expression.** To control surface expression of ephrin-B2, FACSscan analysis was performed with either ephrin-B2-transfected PAEC (in parallel with the fusion assay), or with primary PAEC pC HAE pC. Transfected PAEC were detached with accutase (Life Technologies), while PAEC and HAE pC were detached with detachment solution (Passagekit 4; ProVitro). Single-cell suspensions were then fixed with 0.5 % PFA for 15 min. After washing with FACS buffer (PBS containing 1 % bovine serum albumin), cell suspensions were incubated overnight at 4 °C with 50 μg ml⁻¹ recombinant mouse EphB4/Fc, a soluble receptor for ephrin-B2 fused to the Fc region of human IgG (R&D Systems) known to detect ephrin-B2 of all species. EphB4/Fc was then detected by FITC-conjugated goat-anti-human IgG (Dianova). Analysis was carried out using the Guava easyCyte Flow Cytometer (Merck Millipore). The ephrin-B2 cell surface expression (CSE) was calculated by multiplying the percentage of ephrin-B2-positive cells with the mean fluorescence intensity (MFI) of this cell population.

**Immunostaining of NIV-infected cells.** PAEC were transfected with either porcine or human ephrin-B2 and 16 h after transfection, cells were infected with NIV at an m.o.i. of 0.5. At 24 h p.i., the cells were transferred to 4 % PFA and incubated for 48 h for fixation and virus inactivation. After permeabilization with methanol/acetone (1:1, v/v), virus-positive cells were stained using a NIV-specific monoclonal guinea pig antiserum as described previously (Diederich et al., 2012). For quantification, the total number of nuclei in syncytia on the complete coverslip was determined.

**Mixed fusion assay.** Ephrin-B2-negative PAEC were grown to 80 % confluence in 6-well plates (Greiner Bio One). One well was transfected with either pCAGGS-pEB2 or pCAGGS-hEB2. A second culture was transfected with a mixture of plasmids pCAGGS-NIV F, pCAGGS-NIV G and pCAGGS-eGFP at a ratio of 3:3:2. After 16 h, the cells were detached using accutase, and 1×10⁵ cells from each of the two cultures were mixed and seeded on a coverslip to allow cell–cell fusion. After incubation for 24 h at 37 °C, the mixed cultures were fixed with 4 % PFA. Nuclei were counterstained with DAPI. Samples were embedded in Mowiol and analysed with an Axiosvert 200 fluorescent microscope (Zeiss). To quantify cell–cell fusion in the sample, the total number of nuclei in syncytia was determined.

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