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TITLE PAGE

Gene silencing of endothelial von Willebrand factor reduces the susceptibility of human endothelial cells to SARS-CoV-2 infection

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Running title: vWF modulates SARS-CoV-2 infection via ACE2

Abbreviations: ACE2, angiotensin-converting enzyme 2; AngII, angiotensin II; COVID-19, coronavirus disease 2019; DAPI, 4′,6-diamidino-2-phenylindole; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; IFN, interferon; MOF, multiple organ failure; MOI, multiplicity of infection; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOX, NADPH oxidase; RIG-I, retinoic acid inducible gene I; ROS, reactive oxygen species; S, spike; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; siACE2, siRNA anti-ACE2; siNT, nontargeted siRNA; siRNA, short interference RNA; siVWF, siRNA anti-vWF; vWF, von Willebrand Factor.

Keywords: ACE2, endothelial cells, vWF, SARS-CoV-2, COVID-19

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Abstract

Mechanisms underlying vascular endothelial susceptibility to infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are not fully understood. Emerging evidence indicates that patients lacking von Willebrand factor (vWF), an endothelial hallmark, are less severely affected by SARS-CoV-2 infection, yet the precise role of endothelial vWF in modulating coronavirus entry into endothelial cells is unknown. In the present study, we demonstrated that effective gene silencing by short interfering RNA (siRNA) for vWF expression in resting human umbilical vein endothelial cells (HUVECs) significantly reduced by 56% the cellular levels of SARS-CoV-2 genomic RNA. Similar reduction of intracellular SARS-CoV-2 genomic RNA levels was observed in non-activated HUVECs treated with siRNA targeting angiotensin-converting enzyme 2 (ACE2), the cellular gateway to coronavirus. By integrating quantitative information from real-time PCR and high-resolution confocal imaging, we demonstrated that ACE2 gene expression and its plasma membrane localization in HUVECs were both markedly reduced after treatment with siRNA anti-vWF or anti-ACE2. Conversely, siRNA anti-ACE2 did not reduce endothelial vWF gene expression and protein levels. Finally, SARS-CoV-2 infection of viable HUVECs was enhanced by overexpression of vWF, which increased ACE2 levels. Of note, we found a similar increase in interferon-β mRNA levels following transfection with untargeted, anti-vWF or anti-ACE2 siRNA and pcDNA3.1-WT-VWF. We envision that siRNA targeting endothelial vWF will protect against productive endothelial infection by SARS-CoV-2 through downregulation of ACE2 expression and might serve as a novel tool to induce disease resistance by modulating the regulatory role of vWF on ACE2 expression.

Introduction

Despite being initially described as a respiratory virus, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), an enveloped positive-strand RNA betacoronavirus, revealed also endothelial cell tropism [1,2] contributing to the onset of severe coronavirus disease 2019 (COVID-19), characterized by endotheliitis, lung injury, cardiovascular and brain disease up to multiple organ failure (MOF) [3,4].

The direct SARS-CoV-2 infection of endothelial cells [5–8] may feed a sustained and self-perpetuating inflammatory loop that results in diffuse microangiopathy, hypercoagulability, and thromboembolic events, leading to a higher risk of mortality in COVID-19 rather than non-COVID-19 critically ill patients [1,9–12]. Although worldwide vaccination limited the spread of the virus, unraveling how to counteract endothelial infection by SARS-CoV-2 and its variants could provide new avenues for the development of complementary therapeutics to prevent/treat MOF in COVID-19 critically ill patients and postoperative venous thromboembolism in surgical patients with perioperative SARS-CoV-2 infection [13]

Initial evidence indicated that endothelial cells are resistant to direct SARS-CoV-2 infection due to low expression of angiotensin-converting enzyme 2 (ACE2) [14]. Yet, new studies have shown that the binding affinity between the receptor-binding domain of the viral spike (S) protein and the region surrounding the residue K353 of the ACE2 receptor located on the cell surface [15] is an important determinant of SARS-CoV-2 entry and replication rate also in endothelial cells [16,17]. Nonetheless, the SARS-CoV-2 entry route in endothelial cells remains controversial, and its understanding is of enormous clinical interest, even in light of recurrent viral variant reinfection.

Since the susceptibility of endothelial cells to viral infection is generally phenotype related, other endothelial proteins might influence the outcome of viral infection. Von Willebrand factor (vWF) is a multimeric glycoprotein expressed in all endothelial cells, stored in the Weibel Palade bodies as ultra-large vWF multimers, and secreted upon inflammation/injury [18]. Interestingly, high vWF levels have been associated with severe COVID-19 [19–22] and reduced vWF clearance occurs during the progression of the disease [23]. Moreover, oxygen therapy is highly required at admission in COVID-19 patients with higher levels of vWF [24,25]. Therefore, high vWF levels were suggested to powerfully predict mortality by several clinical studies [11,24,26,27]. However, it is not yet known whether disabling the expression of vWF at the endothelial level can halt the spread of SARS-CoV-2 from the mucosa to various organs, mitigating the severity of the disease.

It is noticeable that endothelial vWF is required for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) 2 activation-mediated oxidative stress [28], which is generally triggered through S protein:ACE2 interaction during endothelial infection by SARS-CoV-2 [29].

In our study, we tested the effects of short interfering RNA (siRNA)-mediated gene silencing of endothelial vWF in preventing SARS-CoV-2 infection of primary human umbilical vein endothelial cells (HUVECs), a well-established *in vitro* culture system for studying the molecular mechanisms underlying SARS-CoV-2 infection [30–32] and alterations in vWF expression [33]. Our results reveal a hitherto unsuspected role of

endothelial vWF downregulation in reducing ACE2 levels by preventing SARS-CoV-2 infection. These findings open new avenues for the development of vascular endothelium-specific siRNA-based drug targeting strategies for COVID-19.

Results

vWF downregulation limits SARS-CoV-2 infection of human endothelial cells

To evaluate the potential role of vWF levels on susceptibility to SARS-CoV-2 infection of human endothelial cells, the protein was downregulated in HUVEC cells by siRNA targeting vWF (siVWF) in HUVECs. Cell transfection with siVWF led to a significant reduction of vWF expression in HUVEC cells at a transcript level (-90%, $p<0.0001$), compared to primary endothelial cells treated with nontargeted siRNA (siNT) (**Fig. 1A**).

To confirm vWF downregulation at a protein level, we proceeded with vWF detection by fluorescent immunostaining and high-content analysis using the Operetta CLS™ confocal microscope platform. First, we assessed the transfection efficiency of HUVECs by using Atto550-labelled RNA as a control. As shown in **Figure 1B**, we automatically counted the number of Atto550-positive cells and calculated the average rate of transfection, which resulted in being ~30%. Then, cells were transfected with siRNAs, fixed, and stained for vWF and phalloidin 48h after transfection. In accord with the schematic representation of high-content confocal screening (**Fig.1C**), the analysis confirmed a significant 37% reduction of vWF production at a protein level detected as intensity per cell ($p<0.0001$) (**Fig. 1D-F**).

We then assessed how vWF downregulation affects the SARS-CoV-2 infection and replication in human endothelial cells (**Fig. 2**). For this purpose, we transfected HUVECs cells as described before and checked for SARS-CoV-2 genomes 24h after infection. As shown in Figure 2A, siVWF HUVECs cells showed markedly lower cellular levels of SARS-CoV-2 genomic RNA (-56%, $p=0.0058$), suggesting a strong reduction in viral infection of cultured cells (**Fig. 2A**). Of note, the magnitude of reduction was comparable to the SARS-CoV-2 genomic RNA levels detected when ACE2 itself, the cellular gateway to SARS-CoV-2, was reduced by siRNA anti-ACE2 (siACE2) (**Fig. 2A**). This anti-viral effect of siVWF and siACE2 was also quantified by measuring SARS-CoV-2 copies per HUVEC cell per day (**Fig.2C**). Similarly, viral replication was measured by quantifying SARS-CoV-2 release in the conditioned cell medium 48 hours after the entry of the virus as SARS-CoV-2 ($2^{-\Delta cT}$) (**Fig. 2B**) and SARS-CoV-2/ml (**Fig.2D**). The extent of reduction of SARS-CoV-2 RNA levels in the medium of siVWF cells was similar to the one of siACE2 cells (-24%, $p=0.034$).

vWF downregulation reduces the expression of ACE2 in human endothelial cells

To better define the relationship between attenuation of SARS-CoV-2 infection and endothelial vWF levels, we measured ACE2 receptor expression in HUVEC siVWFs. Of note, vWF silencing resulted in a downregulation of ACE2 mRNA on HUVEC cells (-55%, $p=0.0032$), similar to the one produced by treatment with siACE2

(**Fig. 3A**). A reduced ACE2 expression underlies the reduced susceptibility to SARS-CoV-2 infection of endothelial cells with lower vWF levels. Finally, cells with reduced ACE2 expression show vWF expression at a transcript and protein level similar to controls (siNT) (**Fig. 1A**).

vWF downregulation reduces ACE2 plasma membrane localization in human endothelial cells

To further elucidate the role of vWF levels in modulating ACE2 expression and its localization in the membrane of human endothelial cells, we performed a high-content confocal imaging screening on HUVEC cells transfected or not with siRNAs directed against ACE2 and vWF mRNAs. The experiment is schematically represented in **Figure 3B**. Briefly, cells were transfected or not with siRNAs, then fixed and stained for ACE2 and cell membrane 48h after transfection. As shown in **Figure 3C**, the analysis was performed by covering the whole surface of wells and counting several thousands of cells per treatment.

Then, we measured the number of ACE2-positive cells (**Fig.3C-D**) and the localization of ACE2 in the cell membrane (**Fig.3E-F**) in each experimental condition. As showed in **Figure 3D**, anti-vWF and anti-ACE2 siRNA similarly reduced the number of ACE2-positive HUVECs by about 40% ($p < 0.05$) compared with the siNT condition. Moreover, we observed a parallel significant decrease of ACE2 membrane intensity when HUVECs were transfected with anti-VWF siRNA, similarly to cells transfected with anti-ACE2 siRNA (**Fig. 3E**), as showed in **Figure3F**.

vWF overexpression enhances SARS-CoV-2 infection of human endothelial cells

The ~~complex~~ gene expression patterns in both knockdown cells suggested the existence of intricate interrelationships between vWF and ACE2. To better understand how the endothelial expression of the vWF factor is functionally related to ACE2 levels and viral infection, we performed gain-of-function experiments for vWF. Two days after transient transfection of HUVECs with pcDNA3.1-WT-vWF plasmid, we observed a substantial increase in vWF expression (130-fold increase, $p=7.4 \cdot 10^{-7}$) (**Fig. 4A**) and a significant upregulation of ACE2 receptor at a transcript level (24-fold increase, p=0.028) (**Fig. 4B**). Of note, vWF-overexpressing endothelial cells exhibited an increased susceptibility to SARS-CoV-2 infection demonstrated by measuring SARS-CoV-2 RNA levels at 24 hours after viral infection (18-fold increase, p=0.0009) (**Fig. 4C**). In order to better highlight the impact of vWF and ACE2 on SARS-CoV-2 entry into endothelial cells, we measured intracellular SARS-CoV-2 genome at 3h post-infection (**Fig. 4D**). We found that vWF-overexpressing endothelial cells exhibited a marked increase in the susceptibility to SARS-CoV-2 infection ($p<0.001$) (**Fig. 4E**). Interestingly, transfection with siRNA-ACE2 of HUVECs overexpressing vWF significantly reduced the intracellular amount of SARS-Cov-2 genome (**Fig.4D**). The impact of ACE2 silencing on limiting SARS-CoV-2 infection was similar in wild type and transgenic human endothelial cells ($p<0.001$).

IFN-β gene expression is increased in viable transfected human endothelial cells

An unwanted side effect of siRNA can be the induction of an innate antiviral response through induction of type I interferon (IFN)-alpha and/or -beta) [34]. As shown in **Figure 4E**, transfection with siNT reduced level of SARS-CoV-2 genome in HUVECs ($p<0.001$). Yet, the magnitude of antiviral state induced by siNT was significantly less than that of siACE2 without any significant harmful effect on viable endothelial cells (**Fig.5A**). As shown in **Figure 5B**, IFN-β □RNA levels in HUVECs following each transfection condition was similarly increased compared to untreated cells ($p<0.05$).

Discussion

In this study, we have demonstrated that endothelial vWF plays a hitherto unsuspected key role in modulating susceptibility to SARS-CoV-2 infection of human endothelial cells. Indeed, the SARS-CoV-2 entry and replication were safely hampered in vWF-knockdown HUVECs compared to wild-type cells without any significant harmful effect.

Our findings have a relevant clinical impact since SARS-CoV-2 infects mature vascular endothelial cells [15] leading to endothelial dysfunction and microvascular thrombosis [35], which exacerbate disease severity in COVID-19 patients. It is noteworthy that cardiovascular diseases [4,36], heart failure [37] and heart-brain axis dysfunction [3] may occur in patients infected by SARS-CoV-2. Therefore, we cannot exclude that the vascular endothelium ~~is~~ might be one of the main routes facilitating the spread of coronavirus in different tissues far from the primary site of infection [38,39] and causing multisystem disease.

Since endothelial proteins may play a key role in the endothelial tropism of SARS-CoV-2, we focused on the vWF, which is a hallmark of vascular endothelial cell phenotype. Indeed, critically ill patients with COVID-19 showed higher vWF levels [19–22] and direct SARS-CoV-2 infection has been observed in endothelial cells of non-survivors [7]. Yet, it is unclear how vWF may promote the endothelial spreading of SARS-CoV-2 regardless of its impact on hemostasis.

We have recently demonstrated that the steric hindrance on ACE2 is an effective strategy for the prevention of the entry of SARS-CoV2 variants into human cells [17]. Nevertheless, a previous study suggested that ACE2 may not be sufficient to sustain productive SARS-CoV-2 infection of endothelial cells [14] without the intervention of other hitherto unknown proteins. Interestingly, it has been shown recently that L-SIGN, a plasma membrane protein highly expressed on both human liver sinusoidal endothelial cells and lymph node lymphatic endothelial cells but not on blood endothelial cells, is a new receptor for SARS-CoV-2 infection [40]. In this regard, mapping the determinants of blood endothelial cells susceptibility to SARS-CoV-2 entry regardless of circulating factors is of paramount importance to understand the tropism of SARS-CoV-2 toward human endothelial cells from different vascular beds [16].

The severity of SARS-CoV-2 infections is generally attributed to altered redox balance [41]. We have previously demonstrated that vWF downregulation prevents oxidative stress-related endothelial dysfunction induced by angiotensin II (Ang II) [28], a peptide hormone activating NADPH oxidase resulting in a compensatory increase

in ACE2 that enhances SARS-CoV-2 infection [42]. Taken together, we hypothesized that downregulation of endothelial vWF expression, which blocks endothelial responsiveness of NOX4 and NOX2 to AngII (*28*), reduces ACE2 levels and thus, in turn, protects human endothelial cells from SARS-CoV-2 entry and subsequent genome replication.

In agreement with our own studies and those of others [28,43], we found that an effective siRNA-based downregulation of vWF gene expression (−90% vWF mRNA transcript) with transfection efficiency just over 30%, consistent with reduced cellular protein localization, does not affect cell viability. Although primary endothelial cells are notoriously difficult to transfect, the efficacy of gene silencing of our siRNAs with Lipofectamine is in line with previous studies [44, 45]. Our findings further demonstrate that vWF is a protein that is well conserved across species and in different vascular beds since the same anti-vWF siRNA is effective in porcine and human, arterial, and venous endothelial cells respectively.

In the present study, we have shown that wild-type HUVECs can be efficiently infected with SARS-CoV-2 and sustain viral replication *in vitro*. Our findings confirm previous studies [15], even if some experimental evidence excluded that HUVECs can productively be infected by SARS-CoV-2 due to low ACE2 expression [16,43]. Despite these controversial results, we performed additional experiments to assess the reliability of our *in vitro* model of SARS-CoV-2 infection and found that ACE2 silencing in HUVECs significantly reduces coronavirus entry and replication. Finally, the finding that the magnitude of viral load in cell lysates and supernatants is similar in both ACE2- and vWF-knockdown HUVECs caught our attention. Moreover, SARS-CoV-2 genome is similarly reduced per day in cells transfected with siVWF or siACE2 providing a first snap-shot to quantitatively describe the potential impact of vWF on the kinetics of virus infection in HUVEC cell culture. Our data suggest, for the first time, vWF as a new upstream modulator of endothelial susceptibility to SARS-CoV-2, yet molecular mechanisms underlying potential interdependence between vWF and ACE2 in viral infection requires further investigation.

In this regard, we have measured ACE2 expression in HUVECs in different experimental conditions. The relative ACE2 gene expression is similarly reduced in either ACE2- or vWF-knockdown cells compared to wild-type HUVECs, as confirmed by its membrane localization. On the other hand, ACE2 silencing does not significantly alter vWF gene and protein expression in viable cells. This finding suggests that vWF may specifically influence the expression of other genes coding for mediators that regulate ACE2 expression, and not vice versa. Although which genetic loci are associated with congenital reduction of VWF levels in humans is still under investigation [46], our evidence-driven hypothesis is supported by our previous study showing that vWF downregulation reduces endothelial nitric oxide synthase (eNOS) protein expression without impairing nitric oxide (NO) availability in viable endothelial cells not exposed to AngII [28]. Since NO is a known antioxidant, we exclude that lower ACE2 levels in vWF-knockdown HUVECs are mainly caused by inefficient scavenging of reactive oxygen species. The question, therefore, remains whether changes in endothelial ACE2-dependent susceptibility to SARS-CoV-2 infection result from the direct change of vWF expression levels.

To further confirm what ~~was~~ we observed, we also performed a gain-of-function experiment in which vWF overexpression increased ACE2 mRNA levels leading to higher productive SARS-CoV-2 infection of transgenic HUVECs, compared to wild type cells. Interestingly, gene silencing of ACE2 in viable HUVECs overexpressing vWF counteracts their susceptibility to SARS-CoV-2 entry at 3h after infection. Our findings further strength the key role of ACE2 in mediating vWF-dependent susceptibility of human endothelial cells to SARS-Cov2 infection. Our data well support clinical evidence showing that conditions that upregulate vWF expression, such as aging [47], diabetes [48], obesity [49], nonalcoholic fatty liver disease [50] and cancer [51], offer a favorable environment to SARS-CoV-2 infection [52].

Finally, we assessed whether the antiviral effect of anti-vWF siRNA is mediated by the activation of the innate immunity response in HUVECs. Indeed, retinoic acid inducible gene I (RIG-I) senses viral RNAs, siRNAs and DNA triggering innate antiviral responses through induction of type I interferons (IFN-α and/or -β) [34]. Among different mediators, we focused on IFN-β since SARS-CoV-2 is more sensitive to IFN-β than IFN-α [53,54]. Of note, the level of endothelial IFN-β gene expression is similar under each transfection condition and it may represent a potential off-target antiviral effect of siRNA. In fact, siNT induces IFN-β leading to anti-viral response as previously demonstrated by others [55]. However, the magnitude of the antiviral effect of siNT is significantly lower than that induced by siACE2 transfection and co-transfection in the presence of similar levels of IFN-β gene expression. Instead, it is plausible that the raise IFN-β gene expression in transfected HUVECs than wild type cell may contribute to reduce the average number of viable cells, although not significantly different from untreated condition. It is already known that IFN-β reduces HUVECs proliferation without increasing apoptosis [56]. Taken together, our data suggests that endothelial IFN-β gene expression has not a prominent role in vWF-dependent anti-SARS-CoV-2 effects, which is mainly related to reduction of ACE2 expression.

Moreover, we cannot ignore that binding of viral spike protein to ACE2, which is no longer available to cleave Ang II, increases intracellular reactive oxygen species (ROS) production [31] that enhances endothelial vWF levels [28,57]. Therefore, our data suggest the use of siRNA-based vWF gene silencing might halt the sustained and self-perpetuating loop of endothelial susceptibility to SARS-CoV-2 infection due to enhanced ACE2 expression. In this manner, reduction of endothelial vWF levels could also not predispose to re-infection by SARS-CoV-2 variants and disease severity in high-risk patients.

Limitations of the study

We performed most experiments on HUVECs isolated from a single donor to assess, for the first time, the positive correlation between vWF and ACE2 levels in the susceptibility of human endothelial cells to SARS-CoV-2 infection. Our experimental design aimed to exclude all potential confounding factors related to immortalized cell lines and cell source. Further experiments on cells isolated from different endothelia and patients of different sexes and ages are needed to further strength our first experimental evidence. Although HUVECs more faithfully represents human endothelial cell behavior as compared to other cell lines [58] and are

a well-established *in vitro* model to investigate endothelial tropism of SARS-CoV-2 [30–32], clinically relevant *in vivo* studies are mandatory to confirm our findings. However, our results well support previous clinical evidence demonstrating that patients with vWF disease are less severely affected by SARS-CoV-2 [59] and reduction of vWF levels by plasma exchange attenuates the inflammatory state in patients with COVID-19 pneumonia [60]. Further investigations are required to define the molecular mechanisms underlying the regulation of vWF-ACE2 axis and its impact on SARS-CoV-2 viral kinetics. For this purpose, other approaches (such as CRISPR Cas9 mediated gene editing) should complement siRNA-mediated vWF gene silencing to further prove robustness of its impact on SARS-CoV2 replication.

In conclusion, by using an *in vitro* approach, we demonstrate that siRNA-based vWF gene silencing increases tolerance against productive SARS-CoV-2 infection of healthy human endothelial cells via reduction of ACE2 expression and plasma membrane localization without interfering with cell viability. Conversely, upregulation of endothelial vWF increases endothelial susceptibility to SARS-CoV-2 infection due to rising of ACE2 expression. Our study, for the first time, reveals the existence of a vWF-ACE2 axis that regulates endothelial SARS-CoV-2 infection.

Taken together, our results reveal the efficacy of siRNA-based approach to break new grounds towards endothelial protection to prevent the outcome worsening in COVID19 patients and late cardiovascular sequelae after recovery from the disease.

Materials and Methods

Cell culture

Human Umbilical Vein Endothelial cells (HUVECs, single female Caucasian donor) were purchased from Lonza (Basel, CH). Cells were cultured in complete Endothelial Cell Growth Medium™-2 (EGM-2) (C-22111; PromoCell, Heidelberg, Germany) supplemented with antibiotic-antimycotic solution (100 units/mL penicillin, 100 ug/mL streptomycin and 250 ng/mL amphotericin B) (A5955; Sigma-Aldrich, St.Luis, MO, USA) under standard culture conditions (5% CO2, 37 °C). For the transfection experiments, cells were maintained in an appropriate EGM-2 medium with the addition of all supplements excluding heparin and without addition of the antibiotic-antimycotic compounds, hereby referred to as complete antibiotic-free heparin-free EGM-2 medium. HUVECs at early passages (up to 5) were grown to 75-90% confluence and used in the in vitro studies.

siRNA-mediated gene silencing

The task of specific gene knockdown *in vitro* was achieved through the use of short interfering RNAs (siRNA) specifically targeting human vWF (siVWF) and human ACE2 (siACE2) (ON-TARGETplus SMARTpool

siRNA, Dharmacon™, Horizon Discovery, Cambridge, UK). As a control, cells were transfected with a non-targeting scrambled siRNA (ON-TARGETplus Non Targeting Pool, Dharmacon™, Horizon Discovery, Cambridge, UK). Briefly, HUVEC cells were seeded at a 15,000 cells/cm^2 density in complete antibiotic-free and heparin-free medium to reach 80-90% confluence in 24 hours. The day after, cells were transfected with 25 nM of siRNA using DharmaFECT™-1 transfection reagent (Horizon Discovery, Cambridge, UK) following the manufacturer's guidelines, in complete antibiotic-free and heparin-free medium, and transfection was maintained for 48 hours. Quantitative RT-PCR for the specific target genes verified transfection efficiency.

Endothelial vWF upregulation

To induce expression of exogenous human vWF in HUVEC cells, pcDNA3.1-WT-VWF plasmid was purchased from Addgene (Watertown, MA, USA) and purified following the supplier's indications with PureYield™ Plasmid Midiprep System (Promega, Madison, WI, USA). HUVEC cells were seeded in 6–well plates (growth area ~10 cm^2) at a density of 15,000 cells/cm^2 in complete growth medium to reach 90% confluence in 24 hours. The day after, transient transfection of pcDNA3.1-WT-VWF was performed using Lipofectamine™ 2000 reagent (Thermo Fisher Scientific, Carlsbad, CA, USA), following the manufacturers' guidelines. Briefly, cell monolayer was washed with PBS and 2 mL complete antibiotic-free and heparin-free medium was added to each well. Cells were supplemented with 200 µL of liposome-DNA complex in Opti-MEM™ medium (Thermo Fisher Scientific, Carlsbad, CA, USA) (containing 1 µg pcDNA3.1-WT-VWF and 5 µL Lipofectamine™ 2000) for 5 hours, then medium was replaced with fresh antibiotic-free and heparin-free medium and maintained for further 43 hours to achieve a 2-day transient transfection. Mock transfection was performed as a control. Transfection efficiency was confirmed by quantitative RT-PCR for the specific target genes.

In order to induce the over-expression of vWF and at the same time down-regulate the expression of ACE2, the HUVEC cells were seeded in 6-well plates (growth area ≈10 cm2) at a density of 75.000 cells/cm2. After 24 hours, the cells were co-transfected with ACE2 siRNA and pcDNA3.1-WT-VWF plasmid using Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). Untreated cells, mock, cells transfected with pcDNA3.1-WT-VWF or siRNA anti-ACE2 transfected alone were included as controls. Briefly, 1 mL of Opti-MEM™ medium (Thermo Fisher Scientific, Carlsbad, CA, USA) containing 2.5 µg of pcDNA3.1-WT-VWF, 10 pmol of siRNA anti-ACE2 (ON-TARGETplus SMARTpool siRNA, Dharmacon™, Horizon Discovery, Cambridge, UK), 3.75 µL of Lipofectamine™ 3000 Reagent and 5 µL of P3000TM Enhancer Reagent was added. Then, Opti-MEM™ medium was removed and a new antibiotic-free and heparin-free medium was added after 6h. Cells were ready for SARS-CoV-2 infection.

Endothelial Infection by SARS-CoV-2

At the end of the 48 hours transfection, cell medium was removed and cells were infected with 1 multiplicity of infection (MOI) of clinical strains of SARS-CoV-2/Human/ITA/PAVIA10734/2020 for 1 hour at 37°C, obtained

from the U.O. of Virology, AOUP, Pisa, Italy. Our strain was isolated from a 74 years-old symptomatic male patient isolated in February 2020 that has been fully sequenced (sequence available on GISAID, accession no. EPI_ISL_568579, and GenBank®, accession no. M527178.1). Cells were washed three times in ice-cold PBS to remove residual virions and maintained in antibiotic-free heparin-free medium to allow replication of the virus in the infected cells. Cells and supernatants were collected 24 or 48 hours after infection to investigate viral RNA in the medium. After this time, in fact, the cells were ready for RNA isolation, quantitative RT-PCR (RT-qPCR) and data analysis. In additional experiments following new experimental protocol (**Fig. 4D**), at 1h after SARS-CoV-2 infection HUVECs were washed three times in ice-cold PBS to remove the residual virions and maintained in the antibiotic-free and heparin-free medium for 3 hours at 37 °C. After this time, the cells were ready for RNA isolation, RT-qPCR and data analysis. All the procedures were performed in a BSL3 facility.

RNA extraction and RT-qPCR

In order to evaluate the modulation of ACE2 and vWF expression in HUVEC cells, total RNA was isolated from a cell monolayer using Aurum Total RNA isolation Kit (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. Reverse transcription of equal RNA amounts was achieved using PrimeScript™ RT Reagent Kit (RR037; Takara, Kusatsu, JP), following manufacturer's guidelines. To perform quantitative RT-PCR, 1 µL of cDNA was amplified on a 20 µL total reaction volume using TB Green Premix Ex Taq (Tli RNase H Plus) reagent (RR420; Takara, Kusatsu, JP) and 500 nM primers targeting human ACE2 (NM_021804; FW: 5'-GGGATCAGAGATCGGAAGAAGAAA-3'; RV: 5'-AGGAGGTCTGAACATCATCAGTG-3') [61], human vWF (NM_000552.5; FW: 5'-AGCCTCTCCGTGTATCTTGG-3'; RV: 5'-CATCGATCCTGGCCACAAAG-3') or human β-actin (NM_001101.5; FW: 5'-AACTGGAACGGTGAAGGTGACAG-3'; RV: 5'-AGAAGTGGGGTGGCTTTTAGG-3'). The reaction was carried out on a standard Rotor-Gene Q real-time PCR cycler (Qiagen, Venlo, NL). In keeping with manufacturers' guidelines, the following thermocycling conditions were employed: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s). Relative gene expression was calculated by Delta-Delta Ct method [62]; β-actin was employed as the housekeeping gene to normalize mRNA levels of target genes in accord to previous studies [63,64].

In order to evaluate extent of SARS-CoV-2 infection, total viral RNA was isolated from infected HUVECs using QIAzol Lysis Reagent according to the manufacturer's instructions (QIAGEN, Hilden, Germany). In order to evaluate magnitude of SARS-CoV-2 replication, viral genomes were extracted from the supernatants 48 hours post-infection using Takara MiniBEST Viral 643 RNA/DNA (Takara Bio, Kyoto, Japan) according to manufacturer's instructions. Then, 200 ng of RNA isolated from cultured cells or 5 µL of RNA isolated from supernatants were amplified by One Step PrimeScriptTM III RT-qPCR Mix kit (Takara Bio, Kyoto, Japan) using the following primers: SARS-CoV-2 RdRp forward 5' -TCACCTATTTTAGCATGGCCTCT-3', reverse 5'-CGTAGTGCAACAGGACTAAGC-3', probe 5'-/5(6)-FAM/TGC TTGTGCCCATGCTGC-3'; β-actin

forward 5'-AAGGAGAAGCTGTGCTACGTC-3', reverse 5'-AGACAGCACTGTGTTGGCGTA-3', probe 5'-/5(6)-FAM/TGGCCACGGCTGCTTCCA.

Reaction was performed on a CFX Connect real-time PCR cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following thermocycling conditions: 52°C for 5 min, 95°C for 10 s, 44 cycles of 95°C for 5 s and 62 °C for 30 s, as previously reported [65,66]. The absolute copy number of viral loads was also determined using a serial diluted recombinant plasmid (ranging from 2.0×10^6 to 2.0×10^3 viral copies number) containing the target fragment of real-time PCR. Absolute viral quantification was expressed in terms of number of SARS-CoV-2 genome copies per HUVEC cell per day and as SARS-CoV-2 Log copies per mL of supernatant. .

In order to evaluate the pattern of IFN-β mRNA expression in plasmid and siRNA transfected HUVEC cells after 48h post-transfection, total RNA extracted from cells using QIAzol Lysis Reagent according to the manufacturer's instructions (QIAGEN, Hilden, Germany) was used for a quantitative RT-PCR using commercially one-step QuantiNova SYBR Green RT-PCR kit (Qiagen Inc., Valencia, California, USA). β -Actin gene was chosen for normalization. The IFN-β primer set was as follows: forward 5′-CTTGGATTCCTACAAAGAAGCAGC-3′ and backward 5′- TCCTCCTTCTGGAACTGCTGCA-3′, while the β-actin primer set was previously described. The reaction was carried out in a 20 µl PCR mixture containing 1x of QuantiNova SYBR Green RT-PCR buffer, primers (0.5 µM each) and 5 µl of extracted RNA (10-100 ng). The standardized thermal program consisted of 50.0 ◦C for 10 minutes, 95 ◦C for 2 minutes, followed by 40 cycles of 5 seconds at 95.0 ◦C, and 60 ◦C for 10 seconds. Melting curves were produced using fluorescence intensity plotted against temperature, increasing from 65.0 to 95.0 ◦C at 0.5 ◦C/s. Each sample was amplified in triplicate, and negative PCR controls without RNA templates were included in each round of tests. Bio-Rad CFX Maestro 1.1 software (Bio-Rad, Hercules, CA, USA) was used to analyze the data. The differences in IFN- β expression compared to the untreated control were represented as the fold change in gene expression using the $2^{-\Delta\Delta CT}$ method [62]. β-actin was used to normalize the mRNA fraction in cell lysates in accord to previous studies [63,64].

Analysis of endothelial vWF and ACE2 expression by immunofluorescent staining

Cultured HUVEC (2×10^4/well) (**Figure 1B**) were preliminary tested for mycoplasma contamination as described [67] and then transfected with siRNA against ACE2 (ON-TARGETplus SMARTpool siRNA, Dharmacon™, Horizon Discovery, Cambridge, UK), and irrelevant target (Non Targeting, NT) (ON-TARGETplus Non Targeting Pool, Dharmacon™, Horizon Discovery, Cambridge, UK), and vWF (ON-TARGETplus SMARTpool siRNA, Dharmacon™, Horizon Discovery, Cambridge, UK) using RNAiMax (Thermo Fisher Scientific, Carlsbad, CA, USA) following manufacturer's instructions. Track-RNA-Atto550 (IDT, Coralville, USA) was used as internal transfection control. Transfected cells were cultured in Cell-carrier Ultra plates (Perkin-Elmer, UK) overnight. Media was then replaced with fresh medium. Cells were then fixed

and stained 48 hours after transfection: nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Carlsbad, CA, USA), vWF with anti-vWF antibody (#65707; Cell Signaling Technology, Danvers, MA; 1:150) and secondary antibody (Alexa Fluor™ 647, Thermo Fisher Scientific, Carlsbad, CA, USA), ACE2 with anti-ACE2 antibody (ab15348, Abcam, Cambridge, UK; 1:200) and secondary antibody (Alexa Fluor™ 488, Thermo Fisher Scientific, Carlsbad, CA, USA), F-actin with Alexa Fluor™ 488 Phalloidin (A12379, Thermo Fisher Scientific, Carlsbad, CA, USA) and cell membrane with CellMask Deep Red (Thermo Fisher Scientific, Carlsbad, CA, USA). Non-specific isotype control antibody (IgG Isotype Control #31235, Thermo Fisher Scientific, Carlsbad, CA, USA) was used as a negative control in anti-vWF (1:150) or ACE2 (1:200) staining applications following the manufacturers' guidelines.

Images were acquired with Operetta CLS confocal fluorescent microscope (Perkin-Elmer, Seer Green, UK). High-content confocal screening assessed living cells by counting the number of nuclei in the very same fields stained for vWF and ACE2, using the Find Nuclei (DAPI) building block to evaluate nuclear morphology and level of DAPI staining [68].

The vWF expression was analyzed by quantifying both vWF signal intensity per cell and the number and the intensity of vWF positive (vWF+) spots per cell. The analysis was performed using Harmony software (Perkin-Elmer, Seer Green, UK) on over 165 fields/well, taking images with 63× water objective in confocal, analyzing an average of ~5000 cells per well, using the following building blocks: find nuclei (DAPI) > find cytoplasm (Phalloidin-488 > Calculate Intensity Property (vWF – 647) > Find spots (vWF – 647) > calculate spot region intensity (vWF – 647).

For ACE2 staining, the analysis was performed using Harmony software (Perkin-Elmer, Seer Green, UK) on over 165 fields/well, taking images with 63× objective and analyzing an average of ~ 2000 cells using the following building blocks: find nuclei (DAPI) > find cytoplasm (CellMask 647) > find region – membrane (CellMask 647) > find intensity properties (ACE2 -568) > find population (ACE+/ACE-) as previously described [65].

Statistical analysis

Data are presented as mean ± SEM. Graphical representation and statistical analysis were performed using Prism GraphPad software. Normality of sample distribution was evaluated by Kolmogorov-Smirnov test, while homoscedasticity was evaluated by F-test of equality of variances (two groups) or Brown-Forsythe test (more than two groups). Independent samples Student's T test (with or without Welch's correction, depending on homoscedasticity) or Mann Whitney test were performed to assess statistical differences between two independent groups according to their distribution. One-way ANOVA with Tukey's *post hoc* correction was applied when more than two groups were compared. All statistical tests were performed as two-sided, statistical significance was considered for P values <0.05.

Authors' contributions

VL conceived the study. ADC and GF performed most of the experiments. FS, PGS and RF accomplished some of the *in vitro* experiments. ADC and GF performed the statistical analysis. ML, MP and VL secured funding. ML and VL supervised the studies. ADC, FS, GF, ML, MP, PGS and VL critically revised the draft. GF and VL wrote the manuscript. All authors read and approved the submitted version.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, VL, upon reasonable request.

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FIGURES

Fig. 1 Efficient VWF knock-down in HUVEC cells. A. HUVEC cells were transfected with vWF-targeting siRNA (siVWF) or ACE2-targeting siRNA (siACE2) for 48 hours as described in the methods. Non targeting scrambled siRNA was employed as a control (siNT); vWF mRNA expression was measured after transfection via RT-qPCR. B-F. Protein expression of vWF after siRNA transfection was evaluated via immunofluorescent staining followed by high-content confocal screening by Operetta CLS High-Content Analysis System. (B) Transfection efficiency of HUVEC cells was assessed via transfection with Atto550-labeled RNA, the statistical analysis on the average rate of Atto550-labeled RNA transfection in HUVEC cells is presented in the graph. (C) Schematic representation of high-content confocal screening: cells were transfected with the following siRNAs: not targeting (siNT), anti-ACE2 (siACE2) and anti-vWF (siVWF) as described in the experimental procedure. At the end of transfection, cells were stained for nuclei (DAPI), cytoskeletal F-actin (Alexa Fluor™ 488 Phalloidin) and vWF (Alexa Fluor™ 647, secondary antibody). (D) Overview of a representative acquisition; (E) 63x confocal images of every experimental condition including IgG isotype control (#31235). (F) Analysis of vWF signal intensity and positive (vWF+) spots out of total events (number of nuclei) (A.U., arbitrary unit). Data information: In (A,B,F) data are presented as mean ± SEM. Statistical analyses were performed using One-way ANOVA, followed by post hoc Tukey's test, N≥3, α=0.5, * $p<0.05$, **$p<0.01$,**** $p<0.0001$, ns = not significant

Fig. 2 VWF knock-down reduced SARS-COV-2 infection in HUVEC cells. Human ACE2 and VWF knock down were achieved on HUVEC cells by transfection with specific siRNAs (siACE2; siVWF) for 48 hours. Untreated cells and cells transfected with non targeting scrambled siRNA (siNT) were employed as a control (siNT). After transfection, cells were infected with SARS-CoV-2 for one hour as described in the Material and Methods. Total RNA isolated was collected from cell lysates 24h after infection and the expression of SARS-CoV-2 within the cells evaluated via One Step RT-qPCR (A) and SARS-CoV-2 copies/HUVEC/day (C) using specific primers. Total RNA was isolated from conditioned medium 48 hours after infection and the amount of SARS-COV-2 released in the medium evaluated using the same One Step RT-qPCR approach (B) and SARS-CoV-2 Log copies/mL (D). Data information: Data are presented as mean ± SEM. Statistical differences between groups were evaluated by One-way ANOVA followed by post hoc Tukey's test. N≥3, α=0.5, ** $p<0.01$, ***$p<0.001$, ns = not significant

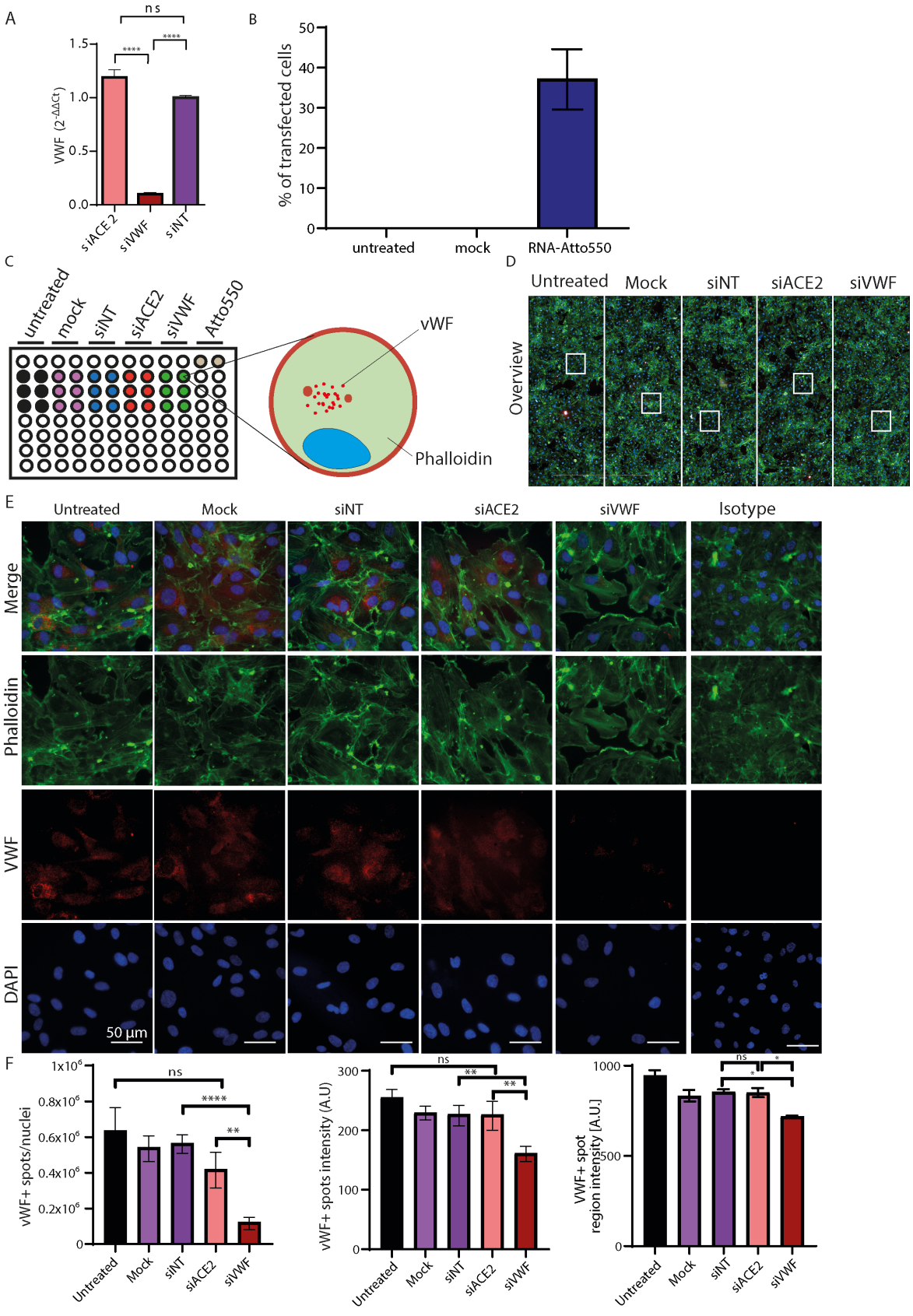
Fig. 3 VWF knock-down determines ACE2 downregulation in HUVEC cells. Human ACE2 and vWF downregulation was performed on HUVEC cells by transfection with specific siRNAs (siACE2; siVWF) for 48 hours. Untreated cells and cells transfected with non targeting scrambled siRNA (siNT) were employed as a

control (siNT). A. After transfection, ACE2 mRNA expression was measured in the cells via RT-qPCR as described in the experimental procedure and expressed as normalized on β-actin housekeeping gene (Ct_{ACE2} - $Ct_{β\text{-}actin}$; -Δ□□). B-F. Protein expression of ACE2 after siRNA transfection was evaluated via immunofluorescent staining followed by high-content confocal screening by Operetta CLS High-Content Analysis System. (B) Schematic representation of high-content confocal screening. HUVECs were transfected with the following siRNAs: not targeting (siNT), anti-ACE2 (siACE2) and anti-vWF (siVWF) as described in the experimental procedure. (C) At the end of transfection, cells were stained for nuclei (DAPI), cytoskeletal F-actin (Alexa Fluor™ 488 Phalloidin) and ACE2 (Alexa Fluor™ 488, secondary antibody), including IgG isotype control (#31235). 63x confocal images of every experimental condition. (D) Statistical analysis on ACE2+ cells out of total events (number of nuclei). (E) Statistical analysis of ACE2 intensity localized in the cell membrane. (F) At the end of transfection, cells were stained for nuclei (DAPI), membrane (CellMask Deep Red) and ACE2 (Alexa Fluor™ 488, secondary antibody), including IgG isotype control (#31235). Data information: In (A,D-E) data are presented as mean ± SEM. Statistical analyses were performed using One-way ANOVA, followed by post hoc Tukey's test, N≥3, α=0.5, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns = not significant

Fig. 4 VWF overexpression promotes ACE2 upregulation and SARS-CoV-2 infection in HUVEC cells. Human VWF overexpression was achieved on HUVEC cells by transfection with pcDNA3.1-WT-VWF as described in the experimental procedures. Mock transfection was performed as a control. A-B. Total RNA was collected from cells 48 hours after transfection and expression of vWF (A) and ACE2 (B) was evaluated via RT-qPCR. C. Transfected cells were infected with SARS-CoV-2 (1 M.O.I) for one hour as described in the Material and Methods. Total RNA was collected from cells 24h after infection and the expression of SARS-CoV-2 within the cells evaluated via One Step RT-qPCR. All procedures were performed as described in the Material and methods on 5 (Mock), or 6 (pcDNA3.1-WT-VWF, pcDNA3.1-VWF) independent replica/group. D. Schematic representation of additional infection protocol to evaluate SARS-CoV-2 entry: at 1h after SARS-CoV-2 infection (1 M.O.I.) HUVECs were washed three times in ice-cold PBS to remove the residual virions and maintained in the antibiotic-free and heparin-free medium for 3 hours at 37 °C. Then, cells were ready for RNA isolation, RT-qPCR and data analysis. All procedures were performed as described in the Material and methods on 3 independent replica/group. Data information: In (A-C) data are presented as mean ± SEM. Statistical differences between groups were evaluated by T-test (with Welch's correction, in case of unequal variances) or Mann Whitney test depending on data distribution. In E, data are presented as mean ± SEM. Statistical differences between groups were evaluated by one-way Anova followed by post hoc Tukey's test. N=3, α=0.5, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns = not significant

Fig.5 IFN-β gene expression is increased in viable transfected human endothelial cells. (A) HUVEC cells were treated with transfection reagent (Mock), transfected with the following siRNAs (not targeting, siNT; anti-

ACE2, siACE2; anti-vWF, siVWF;), with plasmid pcDNA3.1-WT-VWF (pcDNA3.1-VWF) or co-transfected with pcDNA3.1-WT-VWF and siACE2 (pcDNA3.1-VWF+siACE2) as described in the experimental procedure. At the end of tranfection, nuclei were stained with DAPI and high-content confocal screening assessed the effect of the different siRNAs on the number of living cells, by counting the number of nuclei using the find nuclei (DAPI) building block. Untreated cells were used as additional control.(B) Quantification of endothelial IFN-β mRNA expression in abovementioned experimental condition by RT-qPCR was performed at 48h after each treatment. Untreated cells were used as additional control. Data are presented as mean ± SEM. Statistical differences between groups were evaluated by One-way ANOVA followed by post hoc Tukey's test . N≥3, α=0.5, *p<0.05, ns = not significant



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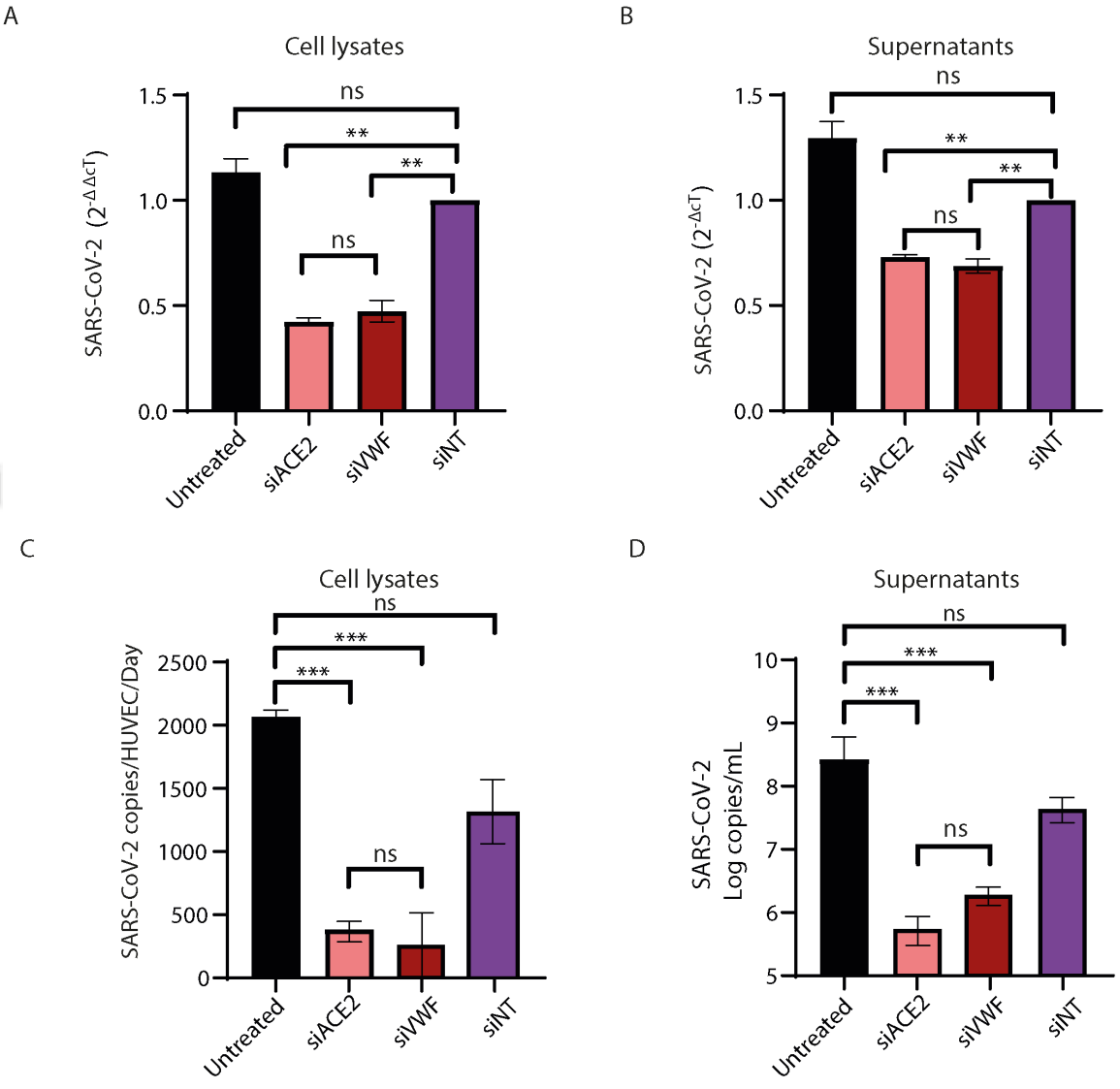

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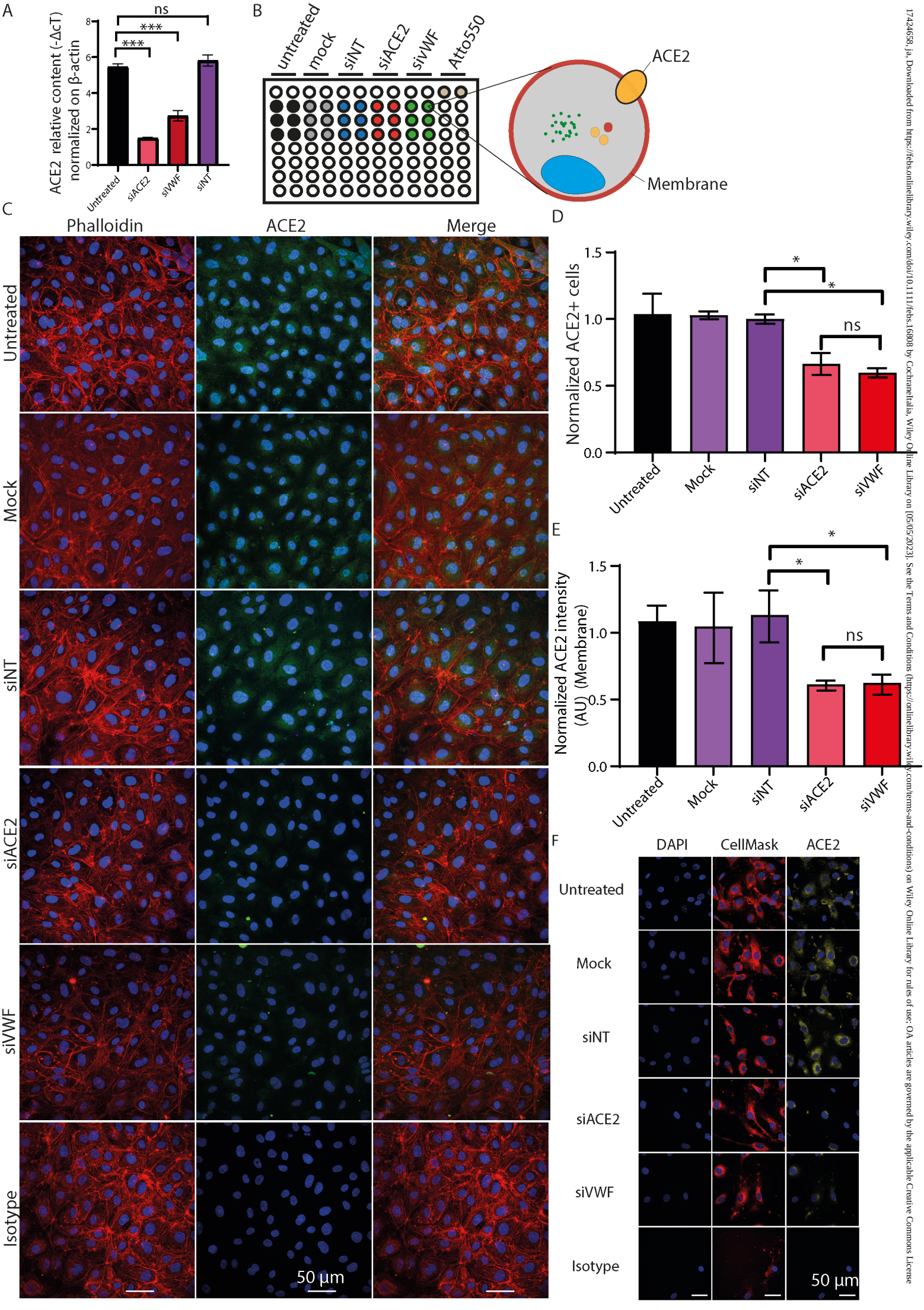

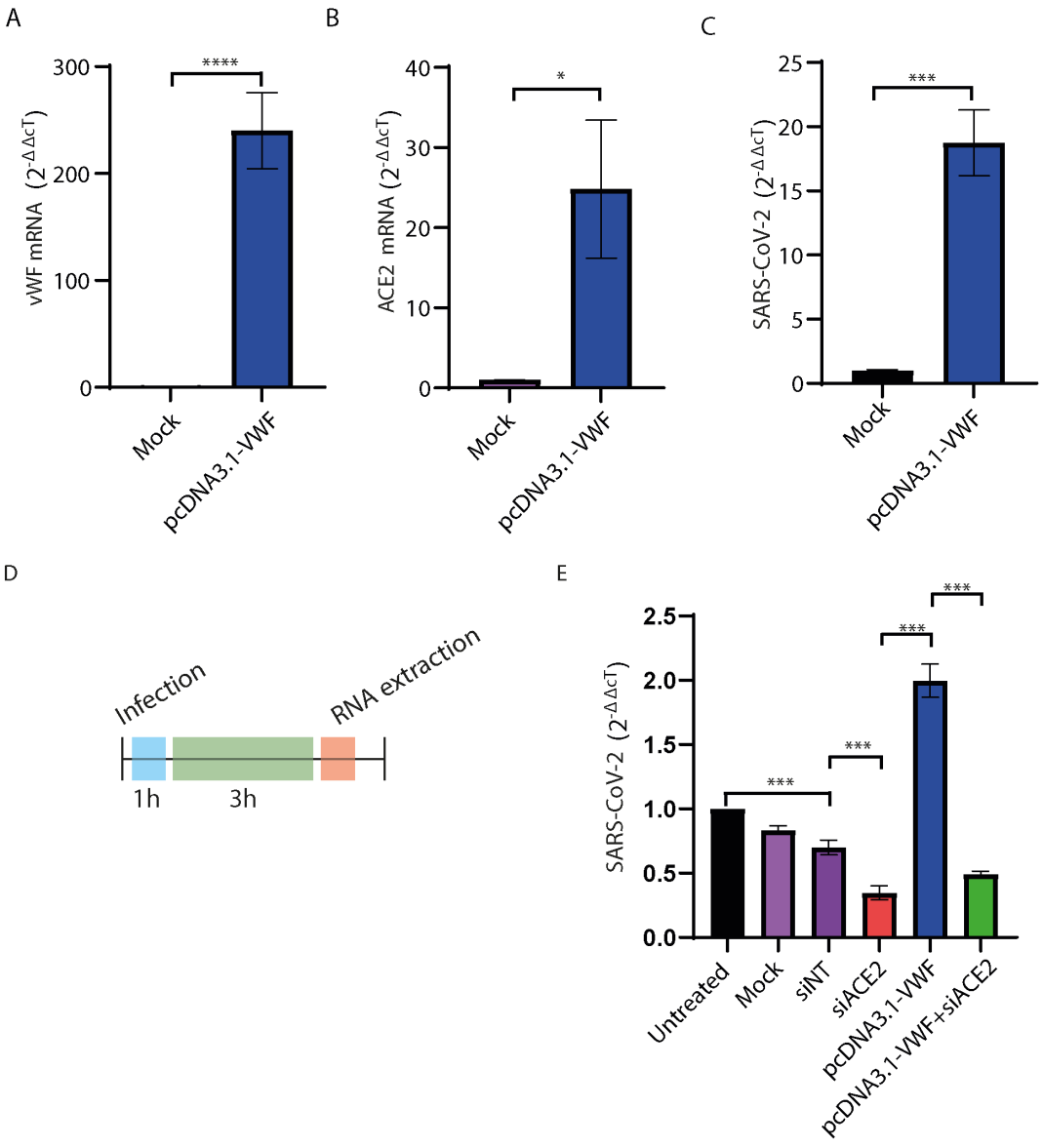

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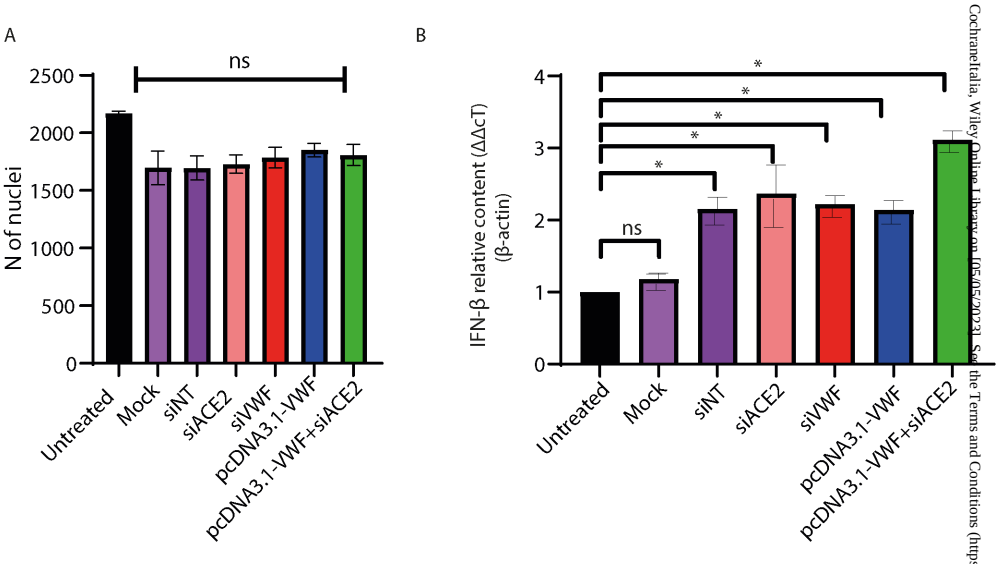

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