Detection of Synthetic Viral RNA Fragments in Human iPSC-Cardiomyocytes following Treatment with Precipitated Extracellular Vesicles from SARS-CoV-2 Coding-Sequence-Overexpressing Lung Epithelial Cells

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ABSTRACT

The novel coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has evolved into a worldwide pandemic. Early data suggest that the prevalence and severity of COVID-19 appear to be higher among patients with underlying cardiovascular risk factors. Despite the expression of angiotensin-converting enzyme 2 (ACE2), a functional receptor for SARS-CoV-2 infection, in cardiomyocytes, there has been no evidence of direct viral infection although the presence of inflammation and viral genome within the hearts of COVID-19 patients have been reported. Here we transduced A549 lung epithelial cells with lentivirus overexpressing selected genes of the SARS-CoV-2. We then isolated extracellular vesicles (EVs) from the supernatant of A549 cells and detected the presence of viral genome within the purified EVs. Importantly, we observed that human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were able to actively uptake these EVs and viral genes were subsequently detected in the cardiac cells. Accordingly, uptake of EVs containing viral genes led to an upregulation of inflammation-related genes in hiPSC-CMs. Thus, our findings indicate that SARS-CoV-2 RNA-containing EVs represent an indirect route of viral RNA entry into cardiomyocytes although future work is required to determine if the RNA is functional.

INTRODUCTION

Since the initial outbreak in China, coronavirus disease 2019 (COVID-19) which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has evolved into a global pandemic. While COVID-19 affects both healthy individuals as well as those with comorbid conditions such as cardiovascular diseases, the severity and risk of adverse outcomes of COVID-19 are especially pronounced in the latter[1]. Patients with COVID-19 have also been reported to start developing cardiovascular complications which were previously absent. However, it remains unclear whether exacerbated cardiac injury seen in COVID-19 patients results directly from viral SARS-CoV-2 infection of the myocardium or indirectly from the complications of COVID-19. Cardiomyocytes express angiotensin-converting enzyme 2 (ACE2), the SARS-CoV-2 binding site[2]. However, there is no evidence of direct viral infection of cardiomyocytes to date, although the presence of myocardial inflammation and viral particles among the interstitial cells of the myocardium have been reported[3] and viral RNA was also detected in some COVID-19 patients' hearts[4]. The majority of cells in the body are known to release lipid bilayer membrane vesicles, also known as extracellular vesicles (EVs), that are capable of transferring various genetic materials such as RNAs to other recipient cells[5]. Therefore, in the present work, we hypothesized that rather than direct viral infection of cardiomyocytes, SARS-CoV-2-infected cells such as airway epithelial cells secrete EVs carrying viral genetic material that are incorporated into cardiomyocytes and establish a critical indirect route of SARS-CoV-2 transmission.

RESULTS AND DISCUSSION

To test whether the viral genome of SARS-CoV-2 can be transmitted via EVs into cardiomyocytes without the need for direct infection, we transduced lung A549 epithelial cells with lentivirus encoding selected SARS-CoV-2 proteins[6] (**Figure 1A**). A549 cells were chosen as a model cell type since COVID-19 appears to mainly infect respiratory tract cells in patients. Viral genes encoding for two non-structural proteins (*Nsp1* and *Nsp12*) and two structural proteins (envelope *E* and nucleocapsid *N*) were used for this proof-of-principle study. We opted not to include the spike (*S*) protein which is required for receptor binding and viral entry to demonstrate the absence of components required for direct viral entry does not preclude EV-mediated transfer of viral genome into recipient cardiomyocytes. The use of lentivirus overexpressing viral subunits also allows us to distinguish EV-mediated SARS-CoV-2 RNA transfer from canonical virus infection since EV preparations inevitably contain infectious virions due to the overlap in size.

Both quantitative and traditional RT-PCR on total RNA extracted from A549 cells 48 hours after lentivirus transduction confirmed the successful overexpression of viral RNAs encoding for

Nsp1, Nsp12, E and N respectively compared to control blank vector (**Figure 1B**). To investigate whether A549 cells secrete EVs, the supernatant of A549 cells grown in culture medium supplemented with exosome-depleted FBS for 48 hours was collected for EV purification. Immunoblotting of EV preparations confirmed the enrichment of the EV markers CD63 and CD81 (**Figure 1C**). In addition, there was no significant difference between control and viral proteins overexpressing A549 cells in terms of EVs production with an average of 100 µg of EVs being secreted over a period of 48 hours by 1 x 10^7 cells (data not shown). Due to the temporary closure of the university's core facilities, we were unable to perform electron microscopy (EM) to further verify the identity of our isolated EVs, but it should be noted we have previously used the same method to isolate EVs from other cell types and verified the purity of isolated EVs by EM[7].

We next asked whether the RNAs encoding for SARS-CoV-2 are packaged into purified EVs of A549 cells. qRT-PCR revealed the presence of mRNA in purified EVs for each of the four tested SARS-CoV-2 proteins (**Figure 1D**). Treatment of EV preparation with RNase A prior to RNA isolation did not reduce the mRNA levels and thus further confirmed their encapsulation within EVs and consequently protection from nuclease digestion (data not shown). To study if human cardiomyocytes are able to uptake EVs, we labeled EVs with a fluorescent dye ExoGlow, and incubated them (100 µg) with human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CMs, 1 x 10^6 cells). Following 6 hours of 37°C incubation and washout of unbound EVs, the presence of labeled EVs was observed in treated hiPSC-CMs, confirming the successful uptake of EVs by recipient cells (**Figure 1E**). Moreover, after exposure of hiPSC-CMs to A549 EVs for 24 hours, we detected a significant presence of all four tested viral RNAs compared to hiPSC-CMs treated with control EVs (**Figure 1F**). Functionally, A549 control EVs resulted in an induction of pro-inflammatory genes in hiPSC-CMs, in accordance with previous reports of these EVs of cancer origin being immunomodulatory[8]. This induction of pro-inflammatory genes was further elevated by EVs containing Nsp1 suggesting the transferred viral genes are capable of eliciting cardiac inflammation (**Figure 1G**).

Overall, our results collectively demonstrated that lung epithelial cells amenable to SARS-CoV-2 infection can secrete EVs containing viral components that can be taken up by cardiomyocytes resulting in an indirect route of entry of viral RNA into cardiac cells. This may potentially lead to cardiac dysfunction via increased inflammation although further work is needed to clarify the mode of action of these viral genes.

METHODS

Cell Culture

HEK293T cells were purchased from TakaraBio and cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Thermo Fisher, 11995-065) supplemented with 10% serum. A549 cells were purchased from ATCC and cultured in Ham's F-12K medium (Thermo Fisher, 21127022). Human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CMs) were differentiated using a chemically defined monolayer differentiation protocol as previously described[9]. Briefly, iPSCs at 90% confluence were incubated with differentiation basal medium comprising RPMI 1640 medium (Thermo Fisher, 11875095) and B27 supplement minus insulin (Thermo Fisher, A1895601). CHIR99021 (8 μM) was added to the differentiation basal medium. On day 2, medium was removed and replaced with differentiation basal medium minus CHIR99021. On day 3, the Wnt antagonist, IWR-1, was added to the medium. After 48 hours, medium was removed and replaced with differentiation basal medium without any inhibitors. On day 7, the cells were incubated with complete CM medium consisting of RPMI 1640 medium and B27 supplement plus insulin (Thermo Fisher, 17504044). The medium was changed every 2 days. Monolayers of hiPSC-CMs were cultured for 30 days and subsequently dissociated for experimental use using TrypLE Express (Life Technologies). For measurement of inflammatory genes, hiPSC-CMs were exposed to either TNF-α (50 ng/mL) or EVs (control vs Nsp1) for 6 hours prior to RNA extraction. All procedures conformed to the UIC institutional review board-approved protocol.

Production of lentivirus expressing SARS-CoV-2 subunits

Plasmids encoding for codon-optimized Nsp1, Nsp12, E and N of SARS-CoV-2 with a 2xStrep tag at the C-terminus were kindly provided by Dr. Nevan Krogan[6]. Empty vector or respective overexpression plasmids were packaged into virus using HEK293T cells as the packaging cell line in 10 cm dishes. Target DNA, helper plasmids VSVG and PAX2 were transfected at 9, 3 and 9 μg respectively using Lipofectamine 2000 (Thermo Fisher, 11668027). Infectious supernatant was collected at 48 and 72 hours after transfection and filtered to remove cell debris. Supernatant was then concentrated using Lenti-X Concentrator (TakaraBio, 631232) according to the manufacturer's protocol. A549 cells were then transduced with either control or overexpression lentivirus with polybrene (8 μg/ml) overnight, and fresh medium supplemented with exosome-depleted FBS (Thermo Fisher, A2720803) was added and incubated for 48 hours before being collected for EV isolation.

RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Direct-Zol RNA Miniprep Kit (Zymo Research, R2051). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher, 4374966) and qRT-PCR was performed using the PowerUp SYBR Green Master Mix (Thermo Fisher, A25742) on a QuantStudio 7 Flex real-time PCR detector (Thermo Fisher). Relative mRNA levels were normalized to those of GAPDH mRNA in each reaction and undetermined raw Ct values were set to 40 for analysis purposes. Primers sequences are shown in **Table 1**. Three to four replicates per group were used for qRT-PCR.

Protein extraction and western blot analysis

EV samples were prepared in 1x RIPA buffer (Sigma, R0278) supplemented with protease and phosphatase inhibitor (Thermo Fisher, 78440). Samples were subjected to electrophoresis on 4-12% NuPAGE Bis–Tris gels (Thermo Fisher, NP0335BOX) and proteins were transferred to nitrocellulose membranes using wet-based transfer system (Bio-Rad). Membranes were incubated overnight with the indicated primary antibodies, followed by incubation for 1 hour with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling; 7074 and 7076). Signals were detected by chemiluminescence. Primary antibodies used include CD63 (System Biosciences, EXOAB-CD63A-1) and CD81 (Thermo Fisher, 10630D).

Isolation of extracellular vesicles

Supernatant of A549 cells was collected at 48 hours after transduction with lentivirus for isolation of EVs. Briefly, supernatant was first centrifuged at 300 x g for 5 minutes, followed by 1500 x g for 10 minutes, filtered (0.2 μM) and concentrated using Ultracel-100K (Millipore). EVs in concentrate were then isolated using Total Exosome Isolation Reagent (Thermo Fisher, 4478359) according to the manufacturer's instructions overnight at 4°C, followed by centrifugation at 12 000 x g at 4°C for 1 hour. Isolated EVs were immunoblotted for EV markers CD63 and CD81.

Uptake of EVs by hiPSC-CMs

Purified EVs were labeled using the ExoGlowTM-Protein EV Labeling Kit (System Bio, EXOGP400A-1). Briefly, 150 μg of EVs were resuspended in 500 μl of PBS and incubated with 1 μl of ExoGlow dye with shaking at 37°C for 20 minutes. Labeled EVs were then precipitated overnight as described above and added to hiPSC-CMs for 6 hours at 37ºC. hiPSC-CMs were

then fixed and stained with cardiac troponin T (Abcam, AB45932) and imaged by confocal microscopy to visualize uptake of labeled EVs.

Statistics

The values presented are the means ± standard deviation from at least three samples. Statistical differences were determined by a 2-tailed, unpaired Student t-test or two-way ANOVA with Tukey's multiple comparison test as appropriate. A value of $P<0.05$ was considered significant.

Table 1. List of primers used in this study

Gene	Primer (Forward)	Primer (Reverse)
Nsp1	TTATGGAGCCGACCTCAAATC	GAGTAACGCCACTGCTATGT
Nsp12	GACGACGCTGTAGTATGCTTTA	TCAGTCTCGGTCCAACATTTC
E	GTATTTCTCCTCGTCACACTGG	CACCCTGCTGTAAACGTAGAA
N	CGGGACATGGCTCACTTATAC	GGTGGGTGGAAACGTCTTAT
Gapdh	GGTGTGAACCATGAGAAGTATGA	GAGTCCTTCCACGATACCAAAG
Il1β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
Il6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
Mcp1	AGAATCACCAGCAGCAAGTGTCC	TCCTGAACCCACTTCTGCTTGG

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FIGURE LEGEND

Figure 1. Transmission of SARS-CoV-2 genome into human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) through EVs. A, Schematic depiction of study design. Nsp1 indicates non-structural protein 1; Nsp12, non-structural protein 12; E, envelope protein; N, nucleocapsid protein. **B**, Expression of SARS-Cov-2 genes in A549 lung epithelial cells. A549 cells were infected with indicated lentiviral particles for 48 hours and mRNA levels were measured by qRT-PCR (left) (n=3, mean± S.D) and semiquantitative PCR (right). *P<0.05; **P<0.01; ***P<0.001 versus pLVX-Blank (Student's t-test). **C**, Immunoblotting of EV markers demonstrating enrichment in the EV fraction compared to supernatant. **D**, SARS-CoV-2 genetic materials (Nsp1, Nsp12, E and N) were detected in EVs secreted from A549 lung epithelial cells. EVs were purified from A549 cell culture media and mRNA levels were measured by qRT-PCR (n = 3, mean ± S.D.). *P<0.05; ***P<0.001 versus pLVX-Blank (Student's t-test). **E**, Uptake of ExoGlow-labeled EVs (pseudocolor red) by hiPSC-CMs stained with cardiac troponin T (green) were visualized by confocal imaging. Scale bar = 10 μM. **F**, qRT-PCR was performed to detect the presence of viral genes in hiPSC-CMs following EV uptake. mRNA levels were measured by qRT-PCR (n = 3, mean ± S.D.). ***P<0.001 versus pLVX-Blank (Student's t-test). **G**, Expression of inflammatory genes in hiPSC-CMs. hiPSC-CMs were treated with EVs released by A549 cells transduced with pLVX-Blank or pLVX-Nsp1 lentiviral particles for 6 hours and mRNA levels were measured by qRT-PCR (n = 3, mean ± S.D.). Tumor necrosis factor-α (TNF-α, 50 ng/ml) was used as a positive

control. **P<0.01; ***P<0.001; n.s., not significant (Two-way ANOVA followed by Tukey's multiple comparisons test). Interleukin 1β: Il1β; interleukin 6 (Il6); monocyte chemoattractant protein 1 (Mcp1).

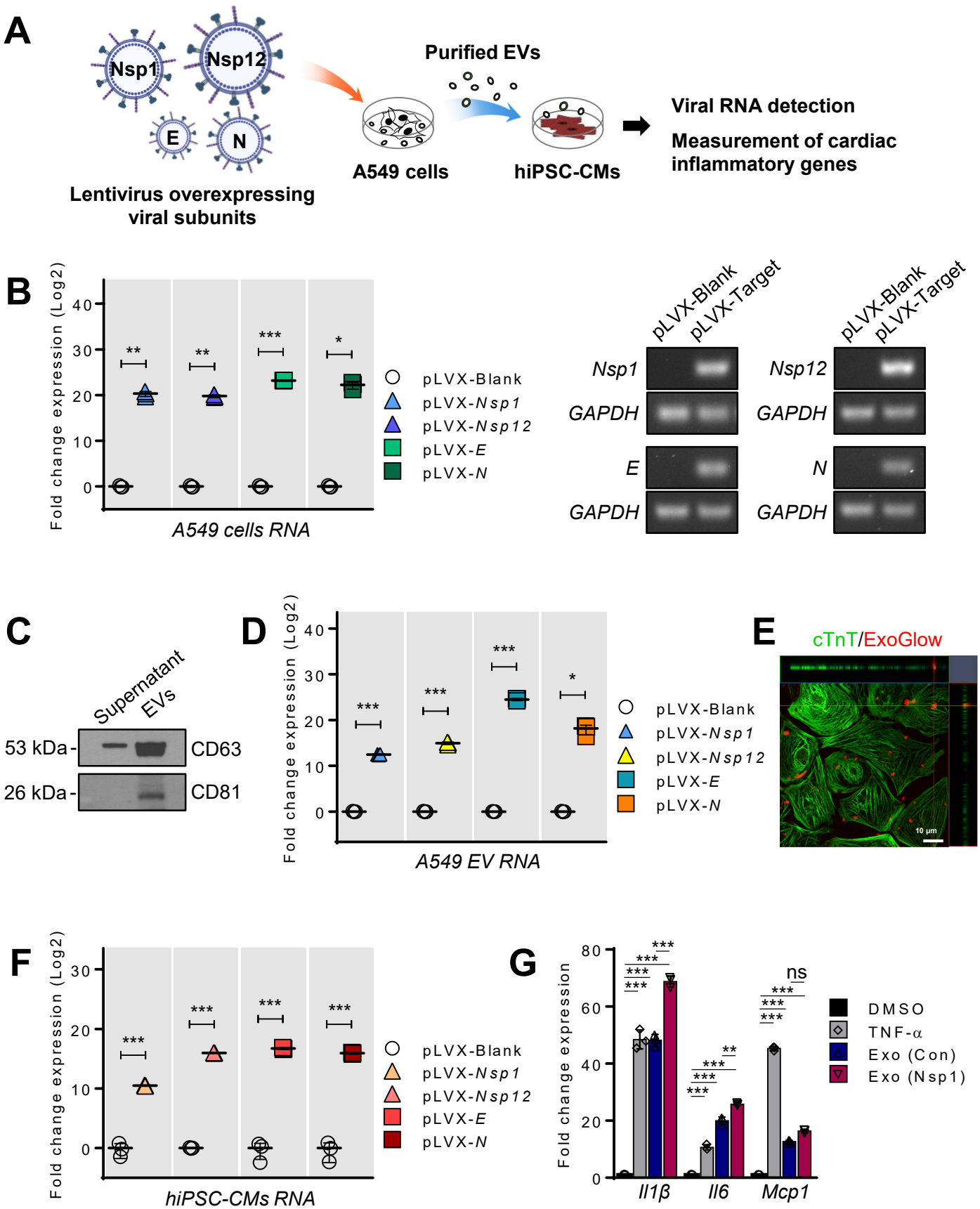
A
Nsp1
Nsp12
E
N
Lentivirus overexpressing viral subunits
Purified EVs
A549 cells
hiPSC-CMs
Viral RNA detection
Measurement of cardiac inflammatory genes
B
Fold change expression (Log2)
A549 cells RNA
pLVX-Blank
pLVX-Nsp1
pLVX-Nsp12
pLVX-E
pLVX-N
pLVX-Blank
pLVX-Target
Nsp1
GAPDH
Nsp12
E
N
C
Supernatant
EVs
53 kDa
26 kDa
CD63
CD81
D
A549 EV RNA
E
cTnT/ExoGlow
10 µm
F
hiPSC-CMs RNA
G
Fold change expression
Il1β
Il6
Mcp1
ns
DMSO
TNF-α
Exo (Con)
Exo (Nsp1)

Figure 1