The Nucleocapsid Protein of SARS-CoV-2 Abolished Pluripotency in Human Induced Pluripotent Stem Cells

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Abstract

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is raging across the world, leading to a mortality rate of 3.4%. As a potential vaccine and therapeutic target, the nucleocapsid protein of SARS-CoV-2 (nCoVN) functions in packaging the viral genome and viral self-assembly. To investigate the biological effect of nCoVN to human induced pluripotent stem cells (iPSC), genetically engineered iPSC overexpressing nCoVN (iPSC-nCoVN) were generated by lentiviral expression systems. Unexpectedly, the morphology and proliferation rate of iPSC were changed after nCoVN expressing for two weeks. The pluripotency markers SSEA4 and TRA-1-81 were not detectable in iPSC-nCoVN. Meanwhile, iPSC-nCoVN lost the ability for differentiation into cardiomyocytes when using a routine differentiation protocol. Our data suggested that nCoVN disrupted the pluripotent properties of iPSC and turned them into fibroblasts, which provided a new insight to the pathogenic mechanism of SARS-CoV-2.

Keywords:

SARS-CoV-2; nucleocapsid protein; human induced pluripotent stem cell; pluripotency; fibroblast

Introduction

Right now, the COVID-19 pandemic is sweeping the world, causing a huge crisis in public health and economics globally. According to the continuously updated data from World Health Organization, to date, more than 300,000 infected cases were confirmed, while more than 13,000 individuals died because of COVID-19 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was proved to be the pathogen of COVID-19, has 79% identity in genomes with severe acute respiratory syndrome coronavirus (SARS-CoV)[1]. 12 coding regions were predicted in SARS-CoV-2, including spike protein, nucleocapsid protein, envelope protein, and membrane protein[1-3]. The Cryo-EM structure of spike protein had been determined[4], and more and more evidences showed that the spike protein binds human ACE2 to entry into host cells[4,5], which indicated that SARS-CoV-2 might share similar pathogenic mechanisms with SARS-CoV. Because of the very limited knowledge of SARS-CoV-2, we sought to understand the biology of SARS-CoV-2 based on the

previous studies about SARS-CoV.

As one of most studied proteins in SARS-CoV, the nucleocapsid protein binds to viral RNA to package the genome in a ribonucleoprotein particle[6]. Unlike the spike protein with a certain mutation frequency, the sequence of nucleocapsid protein was more stable[7], which meant it was an ideal target for diagnostic tools[8-10] and antiviral therapy[11,12]. The pathogenic effects in host cells caused by the nucleocapsid protein were also studied. It was reported that the nucleocapsid protein inhibited type I interferon production after virion infected the host cells[13], which was considered as a possible mechanism of immune escape. The nucleocapsid protein inhibited cell cytokinesis and proliferation[14], and regulated several pathways, such as transforming growth factor-beta signaling[15], AP-1 signal transduction pathway[16], and NF-KappaB pathway[17]. Besides, the nucleocapsid protein was reported as an apoptosis inducer in COS-1 cells[18,19] and HPF cells[20].

As the nucleocapsid protein of SARS-CoV-2 (nCoVN) has 88.1% identity with the nucleocapsid protein of SARS-CoV[1], it is reasonable to speculate that they share a same pathogenic pathway in host cells. The original goal of this study is to determine the physiological malfunctions in human cardiomyocytes overexpressing nCoVN by using human induced pluripotent stem cells (iPSC) and direct differentiation protocols. Unexpectedly, the morphology of iPSC altered obviously when nCoVN had been expressed for 14 days. We turned to investigate whether nCoVN obstructed the pluripotency maintenance in iPSC. Here, we present a new deleterious effect of nCoVN to human pluripotent stem cells.

Results

ACE2 was expressed in various of stem cells

As *ACE2* is the major receptor of SARS-CoV-2 on the cell membrane[4,5], we first examined whether *ACE2* were expressed in the stem cells. Thanks to the gene expression data collection in Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), it is convenient to analyze the *ACE2* expression profiles in sorts of stem cells,. Figure 1A exhibited the *ACE2* expression values in different stem cells from different projects, including human embryonic stem cells[21], iPSC[22], human epithelial stem cells[22], human adipose stem cells[23], human hematopoietic stem cells[24], and human mesenchymal stem cells[25]. The expression values of a housekeeping gene *GAPDH*

were simultaneously collected as controls. *ACE2* was expressed in each kind of stem cells, though the expression values were relatively low compared with *GAPDH*. The reverse transcription-PCR (RT-RCR) results showed that *ACE2* was expressed in iPSC, iPSC-derived cardiomyocytes (iPSC-CM) and human coronary artery endothelial cells (HCAEC), suggesting these cells were the potential targets of SARS-CoV-2 (Figure 1B).

Overexpression of nCoVN drove iPSC to fibroblasts

To study whether physiological activities in iPSC were disturbed by nCoVN, a human induced pluripotent stem cell line (iPSC-nCoVN) in which the expression of nCoVN could be modulated by a Tet-On system was generated by a lentiviral expression system. In this system, nCoVN cDNA sequence was conjugated to puromycin resistance gene through a T2A peptide encoding sequence, and the transcription was relied on the induction of tetracycline or doxycycline (Dox). After puromycin selection, iPSC-nCoVN were divided into two groups: one was induced by Dox for nCoVN expression (Dox+), the other was added with DMSO as a control set (DMSO+). The transcriptional level of nCoVN was detected by Real-time PCR in iPSC, Dox+ and DMSO+ groups. nCoVN expression increased about 267-fold in Dox+ group (Figure 2A). The proliferation rate was compared between iPSC and Dox+ groups by using a Cell Counting Kit-8. The absorbance at 450 nm (A450) was measured at 24 hours, 42 hours, 48 hours, 60 hours and 72 hours after cell seeding. After three days of cell seeding, iPSC showed a higher proliferation rate than Dox+ group, indicating that nCoVN might hamper the growth and division of iPSC (Figure 2B). This observation was consistent with the previous finding about the nucleocapsid protein of SARS-CoV[14]. We continued to induce nCoVN expression in iPSC. The morphology of some sub-clones started to change after a 7-day induction; most of the cells exhibited distinct shapes from wild-type iPSC after a 14-day induction; after induction for 28 days, a typical fibroblast-like morphology was appeared (Figure 2C). The antibodies against fibroblast markers alpha-smooth muscle actin (α-SMA) and S100A4 were used to verify the cell type of these fibroblast-like cells. The results from immunofluorescence assays confirmed that Dox+ cells had turned to fibroblasts (Figure 2D).

Overexpression of nCoVN disabled the pluripotent properties of iPSC

Next, we examined the pluripotency markers in iPSC and iPSC-nCoVN. The pluripotency markers SSEA4 and TRA-1-81, which were expressed on the membranes of human embryonic stem cells and iPSC, were widely applied in identification of pluripotent stem cells[26,27]. The images

obtained from immunofluorescence assays clearly illustrated that iPSC-nCoVN completely lost the expression of SSEA4 and TRA-1-81, namely, iPSC-nCoVN lost the pluripotency in the presence of nCoVN (Figure 3). To further test the pluripotency in iPSC and iPSC-nCoVN, we directly differentiated these cells to cardiomyocytes by using a routine protocol and under same conditions. As expected, the differentiation efficiency could reach 60% in iPSC; however, on differentiation day 12, only a very small portion of cells were expressed cardiac Troponin T, accompanied by most of cells death (Figure 4). This differentiation assay provided solid evidence that the pluripotency maintenance of iPSC-nCoVN was disrupted by nCoVN.

Discussion

According to the current knowledge about the life cycle of SARS-CoV, the nucleocapsid protein was translated by the host cell translation protein synthesis machinery[28,29], and was localized mainly in the cytoplasm[30]. The primary function of nucleocapsid protein was to package the viral genome into nucleocapsids to protect the genomic RNA[28]. During the formation of nucleocapsids, numerous nucleocapsid proteins bound to the viral RNA and started oligomerization. The viral reproductive strategies would synthesize nucleocapsid proteins as many as possible to meet the requirements of viral assembly, which meant the nucleocapsid proteins were overproduced. The findings that redundant nucleocapsid proteins interfered with the normal physiology of host cells were reported[13-15,18-20]. In this study, we first presented that nCovN abolished pluripotency, reduced the proliferation rate, but did not cause apoptosis in human induced pluripotent stem cells. Long-term expression of nCovN drove iPSC to fibroblasts in spite of using the stem cell culture conditions. It was reported that the nucleocapsid protein of SARS-CoV facilitated TGF-β-induced PAI-1 expression to promote lung fibrosis[15], which was also the possible pathway that nCovN turned iPSC to fibroblasts.

In addition, how nCovN breaks the pluripotency maintenance of iPSC is still a riddle. The pluripotency maintenance in stem cells requires delicate regulations to maintain the balance of pluripotency gene expression in a complicated network. Since nCovN is able to bind RNAs, it is possible that nCovN suppresses the key pluripotency gene's translation through occupying the particular sites of RNAs. Although the mechanism is unknown, the toxic effects of nCovN are clear, which reminds us that SARS-CoV-2 could impair the reproductive system and hematopoietic system.

More functional experiments are ongoing and we will provide detailed data to illustrate the deleterious mechanism of nCovN in iPSC later.

Materials and Methods

Cell culture and differentiation assay

Human induced pluripotent stem cells (iPSC) DYR0100 (The American Type Culture Collection, ATCC) were plated on Matrigel matrix (hESC-Qualified, LDEV-Free, Corning, 354277)-coated plates, and then were cultured in DMEM/F-12 medium (Gibco, 11320033) supplemented with STEMUP® ES/iPS cell culture medium supplement (Nissan Chemical Corporation). STEMUP Medium was changed every two days. IPSC were passaged every three to four days or when the cell culture was 80-90% confluent. During passages, iPSC were rinsed with 1× DPBS (Gibco, 14040133) for one time then were treated with 0.5mM EDTA (Invitrogen, 15575020) in 1× DPBS (Gibco, 14190144) for 10 mins at room temperature. The split ratio was 1:3-1:6. The detailed differentiation protocol was described in the previous published reports[31,32]. Briefly, iPSC were treated with small molecule CHIR99021 (Tocris, 4423, final concentration 10 μM) in the RPMI-BSA medium [RPMI 1640 Medium (HyClone, SH30027.01) supplemented with 213 μg/ml AA2P (l-ascorbic acid 2-phosphate magnesium) (Sigma, A8960) and 0.1% bovine serum albumin (BSA) (Sigma, A1470)] for 24 hours, then were incubated with RPMI-BSA medium for 48 hours. On differentiation day 4, cells were treated with the small molecule IWP2 (Tocris, 3533, final concentration 5 μM) in RPMI-BSA medium. After 48 hours, medium was changed to RPMI-BSA medium. Then, RPMI 1640 Medium supplemented with 3% KnockOut Serum Replacement (Gibco, 10828-028) was used to culture the cardiomyocytes in the following experiments. For nCoVN expression induction, doxycycline hyclate (Sigma, D9891) was supplemented in the cardiomyocyte culture medium at a final concentration of 2 μg/mL.

Generation of iPSC-nCoVN

The cDNA of nCoVN (Sangon Biotech) and puromycin resistance gene were sub-cloned into the plasmid pCW-Cas9-Blast (Addgene, 83481) to replace Cas9 and Blast cDNA, respectively. Lentivirus preparation using a third generation lentivirus packaging system were referred to the previous report[33]. We followed and modified the protocol from Zhang lab to detect MOI of the lentivirus and perform transduction[34]. After 24 hours of transduction, medium was changed to

fresh STEMUP medium supplemented with doxycycline hyclate (Sigma, D9891) for induction. Three days later, puromycin (InvivoGen, ant-pr-1, final concentration 2 μg/mL) was added into the STEMUP medium supplemented with doxycycline hyclate. After 2-3 days' selection, which resulting in a transduction efficiency of ~30%, cells were dissociated and re-plated in new 6-well plates for future culture.

Reverse transcription-PCR and Quantitative Real-time PCR

Total RNA was extracted using the UNlQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech, B511321-0100) prior to the treatment with DNase I (Sangon Biotech, B618252) for 30 minutes. mRNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, 1708841). Quantitative Real-time PCR was performed using a PikoReal Real-Time PCR System (Thermo Fisher) with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, 1725271). The primers for Reverse transcription-PCR and Quantitative Real-time PCR are as followed (from 5′ to 3′):

ACE2-RT-F: GGTCTTCTGTCACCCGATTT;

ACE2-RT-R: ACCACCCCAACTATCTCTCG;

nCoVN-RT-F: CATTGGCATGGAAGTCACAC;

nCoVN-RT-R: TCTGCGGTAAGGCTTGAGTT;

GAPDH-RT-F: TGGGTGTGAACCATGAGAAG;

GAPDH-RT-R: GTGTCGCTGTTGAAGTCAGA.

The proliferation assay

IPSC and iPSC-nCoVN were seeded in 96-well plates with the same cell number. After 24 hours, CCK-8 reagent was added in the medium to monitor the proliferation rate (Beyotime, C0038). The absorbance at 450 nm was measured at 24 hours, 42 hours, 48 hours, 60 hours and 72 hours. The cell-free medium with CCK-8 reagent were used as blank control sets. The data were analyzed and plotted using GraphPad Prism 6.

Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes and washed three times with 1× PBS. Cells were then permeabilized with PBS containing 0.25% Triton X-100 at room temperature for 10 minutes. After incubating in the blocking buffer (1× PBS with 10% goat serum), cells were stained with different primary antibodies at 4℃ overnight. These primary

antibodies were [target, dilution, species, company, product number]: Troponin T Cardiac Isoform, 1:100, mouse, Thermo Fisher, MA5-12960; alpha-smooth muscle actin, 1:100, mouse, Bioss, bsm-33187M; S100A4, 1:100, rabbit, Bioss, bs-3759R; SSEA4, 1:250, mouse, Invitrogen, 14-8843-80; TRA-1-81, 1:250, mouse, Invitrogen, 14-8883-80. Cells were washed three times with PBS containing 0.1% Triton X-100, then incubated with the Alexa Fluor 488 goat anti-mouse or Alexa Fluor 555 goat anti-rabbit IgG secondary antibodies at room temperature for 1 hour. Nuclei were labeled with DAPI (4′,6-diamidino-2-phenylindole, 1 μg/ml) for 5 min. Images were obtained by using the DMi6000 B inverted microscope (Leica) and analyzed by using ImageJ software.

Statistic

Values were expressed as mean ± SD (standard deviation). Statistical significances were evaluated using one-way ANOVA with Bonferroni correction or Student's T-Test. $P<0.05$ was considered statistically significant.

Figure Legends

Figure 1. *ACE2* was expressed in human stem cells. (A) Expression values of *ACE2* and *GAPDH* derived from the Gene Expression Omnibus database. (B) Images from agarose gel electrophoresis for analyzing the Reverse transcription-PCR products. *ACE2* was expressed in iPSC, iPSC-CM and HCAEC. IPSC, human induced pluripotent stem cell; iPSC-CM, human induced pluripotent stem cell-derived cardiomyocyte; HCAEC, human coronary artery endothelial cell.

Figure 2. nCoVN affected the proliferation and morphology of iPSC. (A) The mRNA expression level of nCoVN was significantly elevated in iPSC-nCoVN for a long-term induction (n=3). **, $p<0.001$. (B) The time course of cellular proliferation from iPSC and iPSC-nCoVN (n=6). #, $p<0.05$; **, $p<0.001$. (C) The morphology of iPSC, iPSC-nCoVN under a 14-day induction, and iPSC-nCoVN under a 28-day induction. The scale bar is 100 μm. (D) Representative immunofluorescent staining images of fibroblast markers alpha-smooth muscle actin (α-SMA, green) and S100A4 (red) in iPSC-nCoVN for a 28-day induction. The cell nuclei were stained by DAPI (blue). IPSC-nCoVN exhibited fibroblast-like morphology. The scale bar represents 50 μm.

Figure 3. IPSC-nCoVN lost the expression of pluripotency markers. Representative immunofluorescent staining images of pluripotency markers (A) SSEA4 (green) and, (B) TRA-1-81 (green) in iPSC and iPSC-nCoVN for a 14-day induction. The cell nuclei were stained by DAPI

(blue). Scale bars represent 50 μm.

Figure 4. IPSC-nCoVN lost the ability for cardiac differentiation. (A) Representative immunofluorescent staining images of cardiomyocyte marker cardiac Troponin T (Red) in iPSC- and iPSC-nCoVN-derived cardiomyocytes. The cell nuclei were stained by DAPI (blue). The scale bar represents 50 μm. (B) The cardiac differentiation efficiency of iPSC and iPSC-nCoVN. Images taken from (A) were analyzed by using ImageJ software. The efficiency was calculated as the portion of cardiac Troponin T positive cells in all the cells. Approximately 6,000 cells were counted in each group. **, $p<0.001$.

Acknowledgement

This work is dedicated to all the medical staff who are still fighting against COVID-19 in China. Your efforts make us safer.

Declaration of conflict of interest: None.

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Figure 1

A

Dataset	Sample	Cell Line	ACE2 Ave Value	GAPDH Ave Value
GDS5408	GSM1309417	human embryonic stem cell H1 1	25.61	24162.72
	GSM1309418	human embryonic stem cell H1 2	30.16	26123.47
	GSM1309421	human embryonic stem cell H9 1	39.93	24325.00
	GSM1309422	human embryonic stem cell H9 2	27.90	23177.18
GDS5638	GSM1235179	human induced pluripotent stem cell 1	62.92	29904.83
	GSM1235180	human induced pluripotent stem cell 2	76.38	30926.80
	GSM1235184	human epithelial stem cell 1	67.86	15493.84
	GSM1235185	human epithelial stem cell 2	70.71	23540.97
GDS5056	GSM1187676	human adipose stem cell 1	5.77	14.29
	GSM1187677	human adipose stem cell 2	5.72	14.31
	GSM1187678	human adipose stem cell 3	5.87	14.34
GDS3942	GSM812988	human hematopoietic stem cell 1	3.77	12.47
	GSM812989	human hematopoietic stem cell 2	4.71	12.54
	GSM812990	human hematopoietic stem cell 3	4.20	12.61
GDS3785	GSM490983	human mesenchymal stem cell	5.47	12.29

B

Figure 2

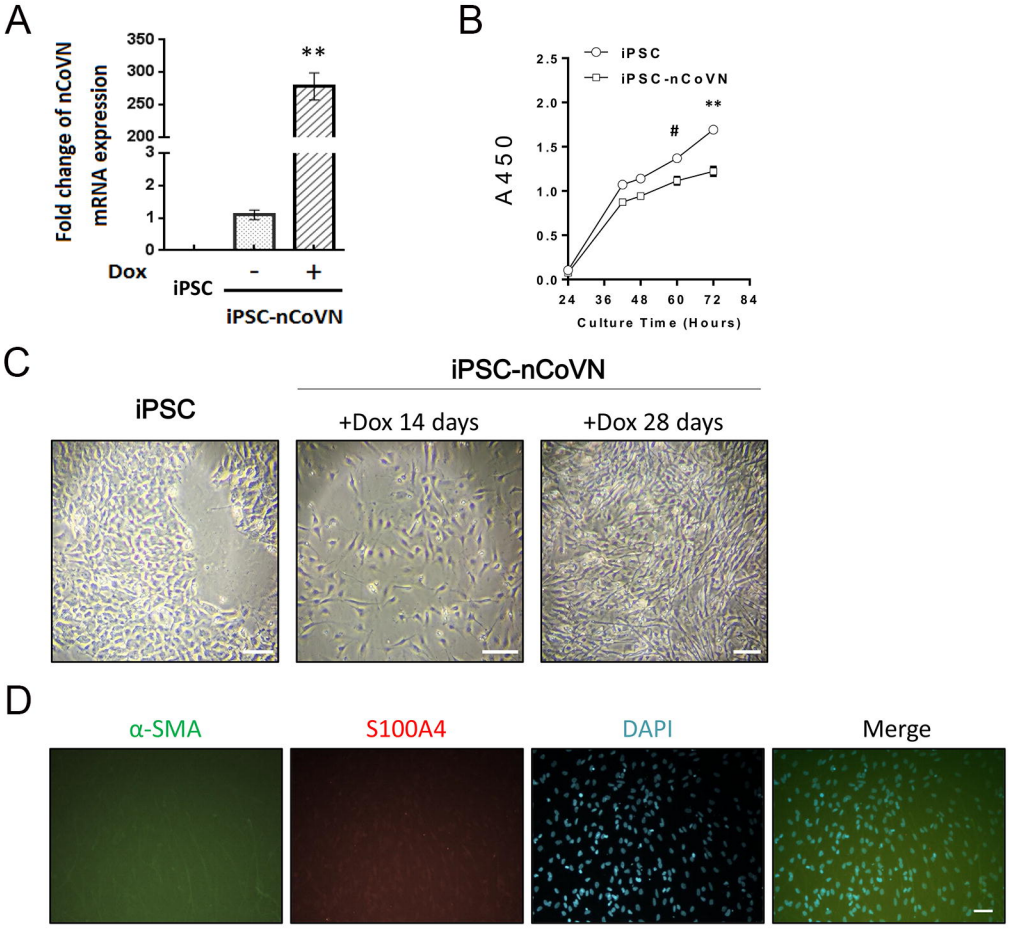

A
Fold change of nCoVN mRNA expression
350
300
250
200
3
2
1
0
**
Dox
iPSC
-
+
iPSC-nCoVN
B
iPSC
iPSC-nCoVN
A450
2.5
2.0
1.5
1.0
0.5
0.0
#
**
24
36
48
60
72
84
Culture Time (Hours)
C
iPSC
iPSC-nCoVN
+Dox 14 days
+Dox 28 days
D
α-SMA
S100A4
DAPI
Merge

Figure 3

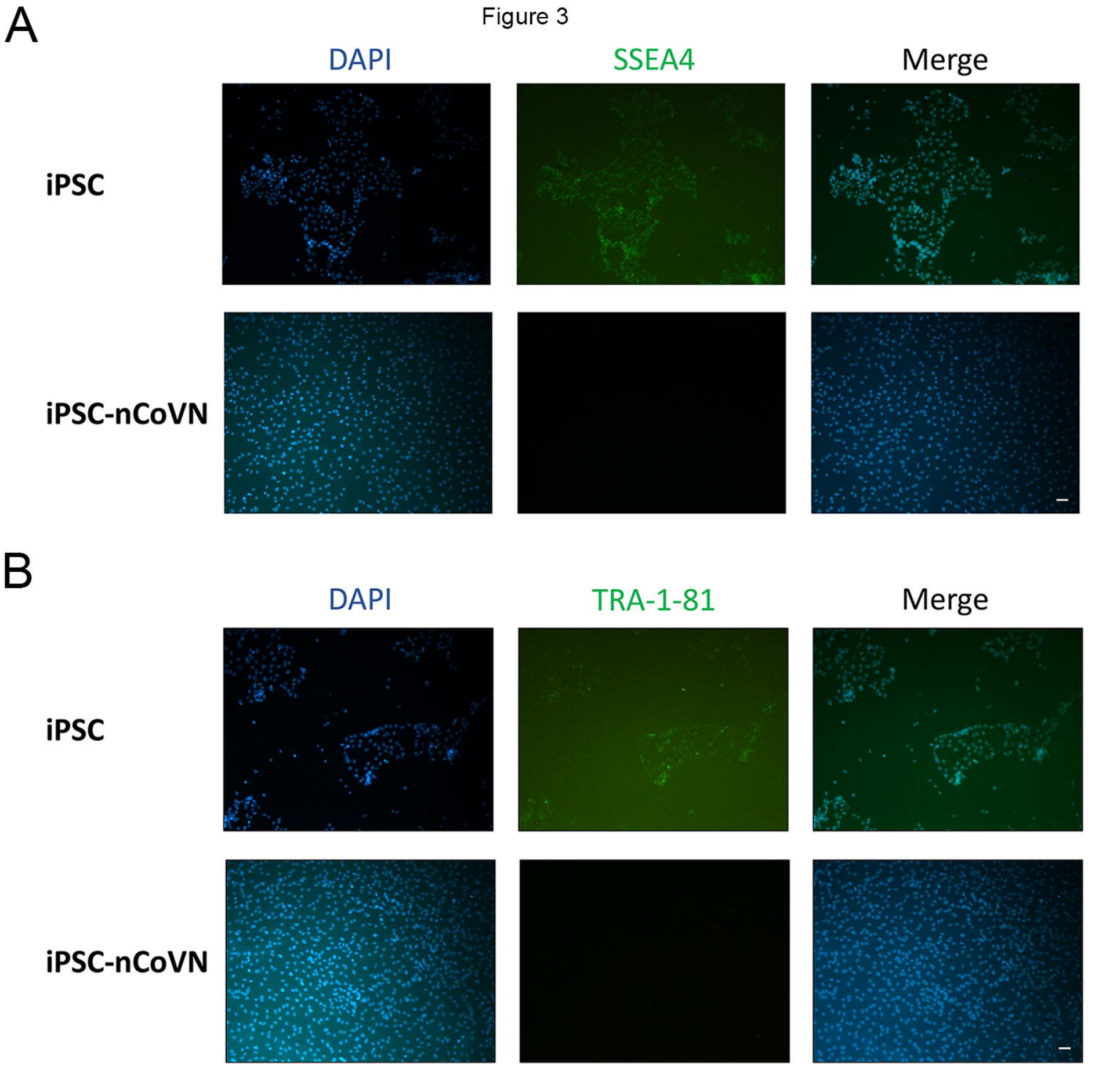
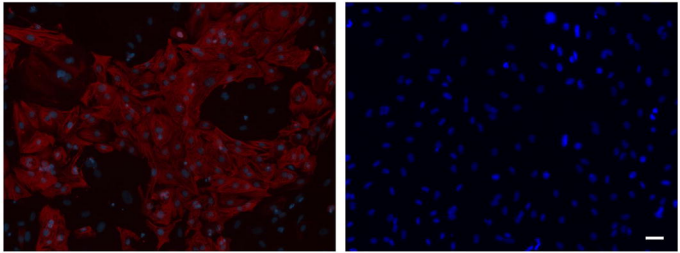

A
DAPI
SSEA4
Merge
iPSC
iPSC-nCoVN
B
DAPI
TRA-1-81
Merge
iPSC
iPSC-nCoVN

Figure 4

A

cardiac Troponin T/DAPI



iPSC-derived cardiomyocytes

iPSC-nCoVN-derived cardiomyocytes

B