**Virologica Sinica**

**Supplementary Data**

**Direct Infection of SARS-CoV-2 in Human iPSC-Derived 3D Cardiac Organoids Recapitulates COVID-19 Myocarditis**

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**Supplementary Information**

**Materials and Methods**

***Generation of Cardiac organoids***

The hiPSC were harvested at approximately 70% confluency. Then hiPSC were seeded at 5,000 cells/ well in a volume of 200 μL into U bottom ultra-lowattachment 96-well plates containing hiPSC medium + 10 μmol/L Y27632 for 24 h. Then cells were induced for 2 days with the Stage 1 medium. After 2 days, cells were induced with Stage 2 medium for 4 days with medium change every 2 days. Subsequently, the medium was changed to Stage 3 medium with medium change every 2 days (Hofbauer et al., 2021). Until day 20, matured cardiac organoids were subjected to the corresponding experiments. The medium compositions were shown in Supplementary Table S1.

***Cryosectioning of cardiac organoids***

Cardiac organoids (COs) were fixed with 4% PFA (Servicebio, G1101) for overnight at 4℃ and washed three times in 1×PBS (Gibco, #14190094). Fixed COs were then soaked with 30% sucrose for overnight at 4 °C and embedded in O.C.T. cryoembedding medium (SAKURA, #4583) using a plastic mold (Bagley et al., 2017). After complete freezing under –80 °C, cardiac organoids were sectioned on CryoStar NX50 and the cryosections were collected on Ultra Plus slides and kept at –20 °C or –80 °C until immunostaining.

***Immunofluorescence staining***

For whole mount staining, 4% PFA-fixed COs was washed twice in 1x PBS and once in PBST for 15 min each. For crysectioned slides staining, O.C.T. was removed by washing with PBS before the immunostaining steps (Bagley et al., 2017). Then samples were incubated in blocking solution consisting of PBS with 4% goat serum (Beyotime, C0265) and 0.2% Triton X-100 (Sigma-Aldrich, #T8787) for 1 h (Hofbauer et al., 2021). The primary antibody was subsequently applied in above blocking buffer for overnight at 4℃ in the case of the 2D samples and 2 days at 4 °C on a shaker for 3D samples (Drakhlis et al., 2021; Lewis-Israeli et al., 2022). Following washing twice with PBS/Tween20, 3D tissues were incubated with the secondary antibody solution at 4 °C on a shaker for 2 h while 2D samples were incubated up to 1 hour at room temperature. Following these washing steps and an additional PBS wash, tissues were ready for analysis, while slides were mounted using fluorescence mounting medium (Beyotime, P0128S)

***Virus infection and detection***

COs were used to study replication dynamic of SARS-CoV-2, including wild-type, Omicron BA.5. COs were infected with SARS-CoV-2 at an MOI of 5. After incubation 1–2 h, cardiac organoids were washed and fresh medium was supplemented to COs (Zhao et al., 2021; Khan et al., 2022; Monteil et al., 2022). At different time points after infection, the cell supernatants were collected to extract viral RNA using viral DNA/RNA extraction kit (Tianlong, T050). COs were harvested to extract total RNA using RNAiso plus (Takara, 9109). Subsequently, the RNA was subjected to one-step qPCR (Vazyme, P611-01). Primers used were listed in Supplementary Table S2.

***Real-time qPCR***

Total RNA was isolated from organoids by RNAiso Plus (Takara, 9109) and reverse-transcribed into cDNA with M-MLV Reverse Transcriptase (Invitrogen, 28025013) in biosafety level 2 facility strictly following the regulations. Quantitative real-time PCR was performed on CFX384 Touch System (Bio Rad). Primers used were listed in Supplementary Table S2.

***RNA-Seq and transcriptome bioinformatic analysis***

Total RNA was extracted from COs by RNAiso Plus (Takara, 9109) in Biosafety level 3 facility according to strict regulations and then sent to the company (BIOLINKER) for quality control and sequencing. Libraries were sequenced on an Illumina NovaSeq 6000 platform. After quality control, clean reads were aligned to human reference genome (GRCh38) using HISAT2 (version 2.2.1). The alignments were then passed to StringTie (version 2.2.1) to assemble and quantify the transcripts in each sample. Differentially expressed genes (DEGs) were identified by the R package edgeR (version 1.0.12). Genes were defined as DEGs if it possesses the following characteristics: 1) gene expression (FPKM) > 1 in any sample, 2) absolute log2 (fold change) > 1.5) *P*-value < 0.05. Visualization and hierarchical clustering of log2-transformed FPKM was generated by pheatmap (version 1.0.12). GO/KEGG analysis were conducted using enrichGO/enrichKEGG function from ClusterProfiler package (version 4.7.1.00), GSEA analysis was conducted using the ClusterProfiler, with the hallmark gene sets acquired from GSEA database (http://www.gsea-msigdb.org/gsea/index.jsp).

***Statistical analysis***

We employed Student’s *t*-test or ANOVA test to analyze the parametric experimental results. Significant differences were noted with asterisks.

**Table S1** Composition of cardiac organoids differentiation medium

|  |  |  |  |
| --- | --- | --- | --- |
| **Composition** | **Stage 1** | **Stage 2** | **Stage 3** |
| Advanced DMEM F12 | + | + | + |
| BSA (5 mg/mL) | + | + | + |
| Monothioglycerol (MTG) (450 μmol/L) | + | + | + |
| CD lipid concentrate (100× ) | + | + | + |
| Insulin-transferrin-Selenium (100× ) | + | + | + |
| L-Ascorbic Acid 2-phosphate (50 μg/mL) | + | + | + |
| FGF2 | 30 ng/mL | 8 ng/mL | 8 ng/mL |
| LY294002 | 5 μmol/L | - | - |
| Activin A | 50 ng/mL | - | - |
| BMP4 | 10 ng/mL | 10 ng/mL | 10 ng/mL |
| CHIR99021 | 4 μmol/L | - | - |
| insulin | 1 μg/mL | 10 μg/mL | 10 μg/mL |
| Y27632 | 5 μmol/L | - | - |
| IWP2 | - | 5 μmol/L | - |
| Retinoic acid | - | 0.5 μmol/L | - |
| VEGF165 | - | - | 100 ng/mL |

**Table S2** Primers for qPCR

|  |  |  |
| --- | --- | --- |
| qPCR primers | Forward (5'–3') | Reverse (5'–3') |
| RBD  | CAATGGTTTAACAGGCACAGG | CTCAAGTGTCTGTGGATCACG |
| GAPDH  | GAAGGTGAAGGTCGGAGTC | GAAGATGGTGATGGGATTT |
| MYH6  | CGGTGCTTTTCAACCTCAAGG | GGACTGGTTCTCCCGATCTGT |
| MYL7  | GCCCAACGTGGTTCTTCCAA | CTCCTCCTCTGGGACACTC |
| TNNT2  | TTCACCAAAGATCTGCTCCTCGCT | TTATTACTGGTGTGGAGTGGGTGTGG |
| ACTC1  | GCTGTCTTCCCGTCCATC | CATGCTCGATGGGATACTTCAG |
| PLN  | ACCTCACTCGCTCAGCTATAA | CATCACGATGATACAGATCAGCA |
| TRDN  | TCACAGAAGACATAGTGACGACG | TGGCAATAGAGCTTGCTGAAA |
| ICAM1  | ATGCCCAGACATCTGTGTCC | GGGGTCTCTATGCCCAACAA |
| ICAM4  | ATGCCCAGACATCTGTGTCC | GGGGTCTCTATGCCCAACAA |
| GDF2  | AGAACGTGAAGGTGGATTTCC | CGCACAATGTTGGACGCTG |
| A2M  | CGGAGAATGACGTACTCCACT | TGGGTTGGTCCTTTCACTTGG |
| VEGFA  | AGGGCAGAATCATCACGAAGT | AGGGTCTCGATTGGATGGCA |
| THBD  | ACCTTCCTCAATGCCAGTCAG | CGTCGCCGTTCAGTAGCAA |
| IL6  | ACTCACCTCTTCAGAACGAATTG | CCATCTTTGGAAGGTTCAGGTTG |
| IL33  | GTGACGGTGTTGATGGTAAGAT | AGCTCCACAGAGTGTTCCTTG |
| IL2RB  | CAGCGGTGAATGGCACTTC | GGCATGGACTTGGCAGGAA |
| CCL2  | CAGCCAGATGCAATCAATGCC | TGGAATCCTGAACCCACTTCT |
| TLR6  | AGACCTACCGCTGAAAACCAA | ACTCACAATAGGATGGCAGGA |
| TGFβ1  | GGCCAGATCCTGTCCAAGC | GTGGGTTTCCACCATTAGCAC |
| IFNβ1 | CTTGGATTCCTACAAAGAAGCAGC | TCCTCCTTCTGGAACTGCTGCA |
| IFNL1 | GTTCAAATCTCTGTCACCAC | TTCAGCTTGAGTGACTCTTC |
| ISG15 | GAGGCAGCGAACTCATCTTT | AGCATCTTCACCGTCAGGTC |
| BST2 | CACACTGTGATGGCCCTAATG | GTCCGCGATTCTCACGCTT |
| ACE2 | CATTGGAGCAAGTGTTGGATCTT | GAGCTAATGCATGCCATTCTCA |
| TMPRSS2 | CTTTGAACTCAGGGTCACCA | TAGTACTGAGCCGGATGCAC |

**Table S3** Reagent information

|  |
| --- |
| **Antibodies** |
| TNNT2 | Thermo, MA5-12960 |
| CD31 | Abcam, ab28364 |
| CD31 | Abcam, ab9498 |
| ACE2 | CST, 4355S |
| ACE2 | Proteintech, 21115-1-Ap |
| SARS-COV-2 N protein | SinoBiological, 40143-R019 |
| Goat anti-mouse IgG, Dylight 488 | A23210 |
| Goat anti-rabbit IgG, Dylight 488 | A23220 |
| Goat anti-mouse IgG, Dylight 594 | A23410 |
| Goat anti-rabbit IgG, Dylight 594 | A23420 |
| **Chemicals, peptides, and recombinant proteins** |
| FGF2 | MCE, HY-P7331 |
| FGF2 | SinoBiological, 10014-HNAE |
| LY294002 | MCE, HY-10108  |
| Activin A | MCE, HY-P70311 |
| BMP4 | MCE, HY-P7007 |
| BMP4 | R&D Systems, 314-BP-050 |
| CHIR99021 | MCE, HY-10182  |
| CHIR99021 | R&D Systems, RD-4423/50 |
| insulin | MCE, HY-P0035 |
| Y27632 | MCE, HY-10583 |
| IWP2 | MCE, HY-13912  |
| Retinoic acid | MCE, HY-12238 |
| VEGF165 | MCE, HY-P70458 |
| VEGF165 | Peprotech, AF-100-20 |
| **Other** |
| Advanced DMEM F12 | GIBCO, 12634028 |
| Bovine Serum Albumin (BSA)  | Sigma, A1933  |
| Monothioglycerol (MTG)  | Sigma-Aldrich, M6145 |
| Concentrated Lipids  | GIBCO, #11905031 |
| L-Ascorbic Acid 2-phosphate  | Sigma-Aldrich, A8960 |
| Insulin-Transferrin-Selenium  | GIBCO, #41400045 |
| mTeSR TM 1 | Stemcell, 85850 |
| ReLeSR | Stemcell, 05872 |
| **Experimental models: Cell line** |
| iPSC | DYR0100, ATCC |

Supplementary Video S1 Representative beating of cardiac organoids (see separate video file).

**Supplementary Figures**

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Fig. S1The cardiac organoids and hiPSCs were tested by RT–qPCR to detect the transcriptional level of ACE2, TMPRSS2. GAPDH was used as an internal control. Data were presented as mean ± S.D. \* indicates *P* < 0.05; \*\* indicates *P* < 0.01; \*\*\* indicates *P* < 0.001.



Fig. S2 **A** Immunofluorescence staining for SARS-CoV-2 N protein and TNNT2 in the cardiomyocytes、SARS-CoV-2 N protein and CD31 in the endothelial cells. Scale bar: 10 um. **B** Viral RNA copies were quantified in viral infected cardiac organoids in supernatant and cells.

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Fig. S3 The cardiac organoids after SARS-CoV-2 infection were harvested to examine the expression of indicated genes using qRT-PCR. GAPDH was used as an internal control. Data were presented as mean ± S.D. \* indicates *P* < 0.05; \*\* indicates *P* < 0.01; \*\*\* indicates *P* < 0.001.



Fig. S4 GSEA enrichment analysis of SARS-CoV-2-infected organoids (Inf) (24 h) versus the mock (Mo).

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