Supplementary information

Ad5-spike COVID-19 vaccine does not aggravate heart damage after ischemic injury

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Supplementary Material and methods

Supplementary Figures S1 to S10

Material and Methods

Animal ethics statements

All mouse work was done in accordance with the university guidelines (Sun Yat-sen University Animal Care and Use Committee). Adult male C57BL/6 mice were purchased from the Laboratory Animal Center of Sun Yet-Sen University. The C57BL/6 background humanized ACE2 (hACE2) mice (T037659) were purchased from Gempharmatech Co. Ltd (Nanjing, China).

Myocardial ischemia/reperfusion (I/R) model

The myocardial I/R model was induced as previously described [1]. Briefly, the adult male mice aged 10-12 weeks were anesthetized using pentobarbital sodium (50 mg/kg, i.p.) and ventilated using a rodent ventilator. Then we performed a left thoracotomy and ligated the left anterior descending (LAD) coronary artery in the left ventricle (LV) using sterile 7-0 silk sutures with a slipknot. For reperfusion, the ligation was removed at the end of ischemia. For long-term heart function analysis, the hearts were performed ischemia for 1 hour followed by a 4-week reperfusion. The hearts were then harvested for scar area analysis using the Masson's trichrome staining. For short term analysis during acute phage, the time of ischemia was 45 minutes followed by a 24-hour reperfusion. Then the infarct size was analyzed by TTC/Evans blue staining as previously described [1]. Briefly, the LAD was retied for the determination of area at risk with 5% Evans blue (E2129, Sigma) injected to the external iliac vein. Then the hearts were sliced for TTC staining (1% TTC, T8877, Sigma; 37°C, 15 minutes) to visualize the unstained infarcted region. The scar area and infarct area were determined by planimetry with the ImageJ software.

Cardiac function assessment by echocardiography

Mice were anesthetized with inhalation of isoflurane (1 to 1.5%) and examined by transthoracic echocardiography using a Vevo 3100 high-resolution imaging system with 400-MHz (VisualSonics Inc.). The long-axis views in M-model were obtained for calculating the LV ejection fraction and LV fractional shortening.

Construction of Ad5 vectored COVID-19 vaccine

An Ad5 vectored COVID-19 vaccine expressing the spike glycoprotein of SARS-CoV-2 (AdSpike) was prepared as described previously [2]. Briefly, an optimized full length spike gene based on the Wuhan-Hu-1 (GenBank accession number YP_009724390) was fused with a flag epitope sequence, cloned and packaged to Ad5 adenovirus using the Admax system. Then the Ad5 vectored COVID-19 vaccine was purified and prepared as liquid formulation containing 2×10^{10} viral particles per mL before intramuscular injected into the mice.

Construction of recombinant adenoviruses expressing ACE2 and the shRNA for ACE2

Recombinant adenoviruses (Ad) expressing human ACE2 (AdACE2) and short hairpin RNA of ACE2 (AdshACE2) were prepared using a pAdEasy vector system (Qbiogene, USA) and homologous recombined in bacteria BJ5183 as previously described [1]. The recombinant plasmids were then transfected separately into HEK 293 cells for the package of adenovirus. The target sequence of AdshACE2 was: 5-CCGTAACCAGTTGATTGAAGATGTA-3.

Gene delivery *in vivo*

The adenovirus was injected to the hearts as previously described [1]. Briefly, following the thoracotomy on mice anesthetized, the diluted adenoviruses (3×10^{10} pfu/ml, 30μl) in a 30-gauge needle was injected into 3 sites of the left ventricle from the apex to the aortic root.[1,3] The expression analysis of the genes delivered into the heart was performed 3 days later. Then the hearts were subjected to myocardial I/R injury.

Immunoblotting analysis

Myocardium tissues collected from the area at risk of the left ventricle were homogenized with RIPA lysis buffer including 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1% deoxycholate, 10% glycerinum, 150 mM NaCl, 2.5 mM EDTA and 1 mg/ml protease inhibitor cocktail. Then the tissue homogenates were analyzed by standard immunoblotting analysis as described before [1] with specific antibodies against ACE2 (1:1,000, 2280s, Cell Signaling Technology) and GAPDH (1:8,000, 97166, Cell Signaling Technology). After incubated with the secondary antibodies (anti-rabbit, 1:5,000, A0545, Sigma; anti-mouse, 1:5,000, A9309, Sigma),

immunoreaction was visualized with an ECL detection kit (NEL104001EA, PerkinElmer Life), then quantified with the ImageJ software.

Cell culture

H1 human embryonic stem cells (hESCs) (WiCell) were maintained in E8 medium (Stem Cell Technologies) on matrigel-coated dishes (354277, Corning) as described previously [4]. Cells were passaged with 0.5 mM EDTA/PBS EDTA when reached 70%-80% confluency, with the presence of 5 μM Rho-associated protein kinase inhibitor Y27632 (S1049, Selleck) to improve cell viability.

Human umbilical vein endothelial cells (HUVEC, hECs) were purchased from Lonza (CC-2517) and maintained in endothelial medium (CC-3162, Lonza). Human adult cardiac fibroblast cells (hCFs) were purchased from Sciencell (#6330) and maintained in high glucose DMEM (C11995500CP, Gibco) supplemented with 10% fetal bovine serum (FBS).

Differentiation of hESCs into cardiomyocytes

Cardiomyocyte induction of hESCs was performed as described previously [4]. When the hESC confluence reached 70-80% 2-3 days after plating, culture media was replaced to the differentiation basal medium (DMEM/F12 (C11330500BT, Gibco) supplemented with 19.4 μg/ml Insulin (91077C, Sigma), 10.7 μg/ml Transferrin (T0065, Sigma), 71 μg/ml Vitamin C (A8960, Sigma), 14 ng/ml Sodium Selenite (S5261, Sigma), and 1× Chemical Defined Lipid Concentrate (11905031, Gibco)). This day was defined as day 0. From day 0 to day 1, CHIR99021 (6 μM, S1263, Selleck) was added. IWP2 (3 μM, S7085, Selleck) was added from day 2 to day 5. Heparin (3 μg/ml, S1346, Selleck) was added from day 2 to day 7. Insulin (20 μg/ml, 91077C, Sigma) was added to maintain hESC-derived cardiomyocytes (hCMs) from day 7 onward. Beating clusters of hCMs were normally observed at day 7. At day 11 to 13, the hCMs were further metabolically purified by using glucose-free DMEM (11966-025, Gibco) supplemented with 20 mM lactate (L7022, Sigma) as previously described [5].

Differentiation of hESCs into vascular smooth muscle cells

Differentiation of hESCs into vascular smooth muscle cells (hSMCs) was performed as

described previously with minor modification [6]. Briefly, 1 day before differentiation, hESCs were dissociated with 0.5 mM EDTA/PBS (Stem Cell Technologies) and plated onto matrigel-coated dishes at a density of 5×10^4 cells/cm^2 in E8 medium. Upon differentiation, the medium was changed to differentiation basal medium as mentioned in the section of cardiomyocyte differentiation. From day 0 to day 1.5, cells were treated with 20 ng/ml bFGF (AF-100-18B, Peprotech), 10 μM LY294002 and 10 ng/ml BMP4. From day 1.5 to day 5, cells were treated with 20 ng/ml bFGF and 50 ng/ml BMP4 (314-BP/CF, R&D). Medium was renewed at day 3.5. At day 5, cells were dissociated into single cells by Accutase (7920, Stem cells Technology), and then seeded onto gelatin-coated plates at a density of 2×10^4/cm^2. Cells were then treated with 10 ng/ml PDGF-BB (100-14B-10, PeproTech) and 2 ng/ml TGF-β1 (240-B/CF, R&D) for at least another 12 days. Half medium renewal was performed every 2 to 3 days. Confluent cells were passaged onto gelatin-coated plates at a ratio of 1:2. Differentiated hSMCs were maintained in high glucose DMEM supplemented with 10% FBS after 12 days of PDGF-BB and TGF-β1 treatment.

Isolation and culture of primary cardiomyocytes

For isolation of adult rat cardiomyocytes (arCMs), hearts were digested using a langendorff system in perfusion buffer (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose, 10 mM 2,3-butanedione monoxime, 10 mM taurine, at pH 7.5) with 1 mg/ml collagenase II (Worthington, LS004177). The left ventricle became soft and flaccidness after an approximately 20-minute digestion. Cardiomyocytes from the left ventricle were then isolated by gravity for three times and then plated onto laminin-coated culture dishes at a density of 1×10^4 cells/cm^2. Culture of adult cardiomyocytes was performed as previously described [3].

For isolation of neonatal rat cardiomyocytes (nrCMs), ventricles of the hearts were removed from 1-day-old Sprague Dawley rats, and minced, followed by complete digestion with 1 mg/ml collagenase II (LS004177, Worthington) and 0.125% trypsin. nrCMs were separated from fibroblasts by differential plating and then cultured in gelatin-coated tissue culture plates at a density of 2×10^4 cells/cm^2 in medium containing DMEM/F12, 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine.

Cytotoxicity assay

hCMs, hSMCs, hECs and hCFs were dissociated with 0.25% trypsin/EDTA into single cells and then plated onto 96-well plate at a density of 6×10^3 cells/cm^2, 1.5×10^3 cells/cm^2, 3×10^3 cells/cm^2, and 1.5×10^3 cells/cm^2, respectively. Plating of primary nrCMs and arCMs were described above. After 24 hours of culture, cells were infected with the indicated dosage of AdSpike or AdVector adenovirus. 2 hours later, culture medium containing the virus was replaced with fresh culture medium. 48 hours after infection, cells were subjected to hypoxia-reoxygenation treatment. Then the cells viability was determined by a standard calcein-AM/PI/Hoechst cytotoxicity assay and quantified by the Operetta CLS high-content analyses system (PerkinElmer Life).

Hypoxia-reoxygenation modeling for monolayer cultured cells

The hypoxia-reoxygenation modeling was performed in a hypoxia chamber with 1% O_2 and then moved back to normal cell incubator. For hypoxia, cells were cultured in the chamber in serum-free and glucose-free medium for 12 hours (for hCMs, hSMCs, hECs and hCFs) or 6 hours (for nrCMs). For hypoxia of arCMs, cells were cultured in the hypoxia buffer (125 mM NaCl, 8 mM KCl, 1.25 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM HEPES, 5 mM sodium lactate and 1 M $CaCl_2$, pH 6.6) and treated with 1% O_2 for 1 hour.

Spontaneous Ca^{2+} transient measurement

hCMs (at differentiation day 30) and nrCMs were treated with 1 μM Fluo-4 AM (F14201, Thermo Scientific) in Tyrode's solution (140.0 mM NaCl, 5.0 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 1.8 mM $CaCl_2$, 10 mM glucose, pH 7.4 with NaOH) for 10-15 minutes at 37°C. Cells were then washed by Tyrode's solution, and Ca^{2+} imaging was conducted using a Zeiss LSM 710 confocal microscope with a 63× objective and analyzed by the IDL software. All measurements of spontaneous Ca^{2+} transients were obtained at 37°C chamber using the line-scan mode.

Ca^{2+} measurement under electrical field stimulation

Isolated cardiomyocytes were seeded on matrigel-coated glass coverslip and loaded with 1 μM Fura-2 AM (F1221, Invitrogen) in Tyrode's buffer (for hCMs and nrCMs: 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 1.8 mM $CaCl_2$, pH 7.4; for arCMs:129 mM NaCl ,4 mM KCl, 23.8 mM $NaHCO_3$, 0.5 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 11 mM glucose, 1.8 mM $CaCl_2$, pH 7.4) at 37°C for 15 minutes. Cells were then washed with Tyrode's buffer and applied electrical field stimulation at increasing frequencies in a perfusion chamber using the IonOptix System (IonOptix®). Ca^{2+} transients were recorded with 40× objective and all parameters were calculated offline using the IonWizard 6.3.4 software.

Immunostaining

Cells or tissue slices were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.4% (vol/vol) Triton X-100 for 10 minutes, and blocked with 5% bovine serum albumin for 1 hour at room temperature. Then cells or slices were incubated with primary antibody at 4°C overnight followed by the secondary antibodies for 1 hour at room temperature. The following primary antibodies were used: α-actinin (A7811, Sigma), cTnT (MA512960, ThermoFisher), SM22α (ab14106, Abcam), ACE2 (2280s, Abcam), CD31 (ab28346, Abcam), and α-SMA (BM0002, Boster). Images were taken using a Zeiss Cell confocal laser scanning microscope 800 (Zeiss).

Human engineered heart tissue (hEHT) fabrication and adenovirus infection

To generate aligned 3D human cardiac tissue bundle, 14×12 mm^2 polydimethylsiloxane (PDMS, SYLGARD184, Dow Corning) molds with Velcro frame (12 mm long) were used. hEHTs were fabricated and cultured as shown before [7]. Hydrogel solution contains 1×10^6 cells per 120 μl volume. For adenovirus infection, concentrated virus was directly added into the hydrogel solution and the MOI was calculated according to the total cell number.

Hypoxia-reoxygenation for hEHTs

A hypoxic chamber (27310, STEMCELL) was used to simulate anoxic environment on day 8. In the hypoxic environment (2% O_2), hEHTs were cultured in glucose-free DMEM without FBS. After 4 hours, hEHTs were transferred to normoxia environment.

Live and dead staining for hEHTs

Percentage of live and dead cells of hEHTs was determined by calcein-AM/PI Double Stain Kit (40747ES76, Yeasen) together with Hoechst (H21492, Invitrogen). Working solution concentration of calcein-AM, PI and Hoechst is 2 μM, 4.5 μM, and 0.5 μg/ml, respectively. hEHTs were washed with PBS and incubated with the staining working solution for 20 min at 37°C. Confocal microscope (LSM 980, ZEISS) was used for imaging.

hEHT contractility test

hEHTs were paced at 1.5 Hz by the programmable electrical stimulator (YC-2, Chengdu Instrument Factory) in 10V electric field stimulation. Video analysis of the hEHT contraction: videos were recorded with the frame rate of 25 fps, and the plugin MYOCYTER was used to analyze the amplitude, peak time 50%, and contraction frequency of the hEHTs in the video. Assessment of contractile force in stepwise stretching: hEHTs were loaded into the customized mechanical test system with temperature control, electric field stimulator, and force transducer on day 24. The recordings were generated in Tyrode's solution with 1.8 mM Ca^{2+} in 0%-6% stretch with 2% per step. The mechanical stretch data of hEHTs was analyzed by MATLAB.

Statistical analysis

All data are presented as the means ± SEM. Statistical significance was determined using the unpaired, two-tailed Student's t test or Steel-Dwass test (for comparing two groups), and one-way ANOVA (one independent variable) or two-way ANOVA (two independent variables) with Bonferroni post hoc test (for multi-group comparisons). Wilcoxon Rank-Sum test (median, Z-score, p-value) was used for non-normal distributions. A bivariate linear regression analysis was performed to determine significance of linear fits. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Prism software (version 8.0.2).

References

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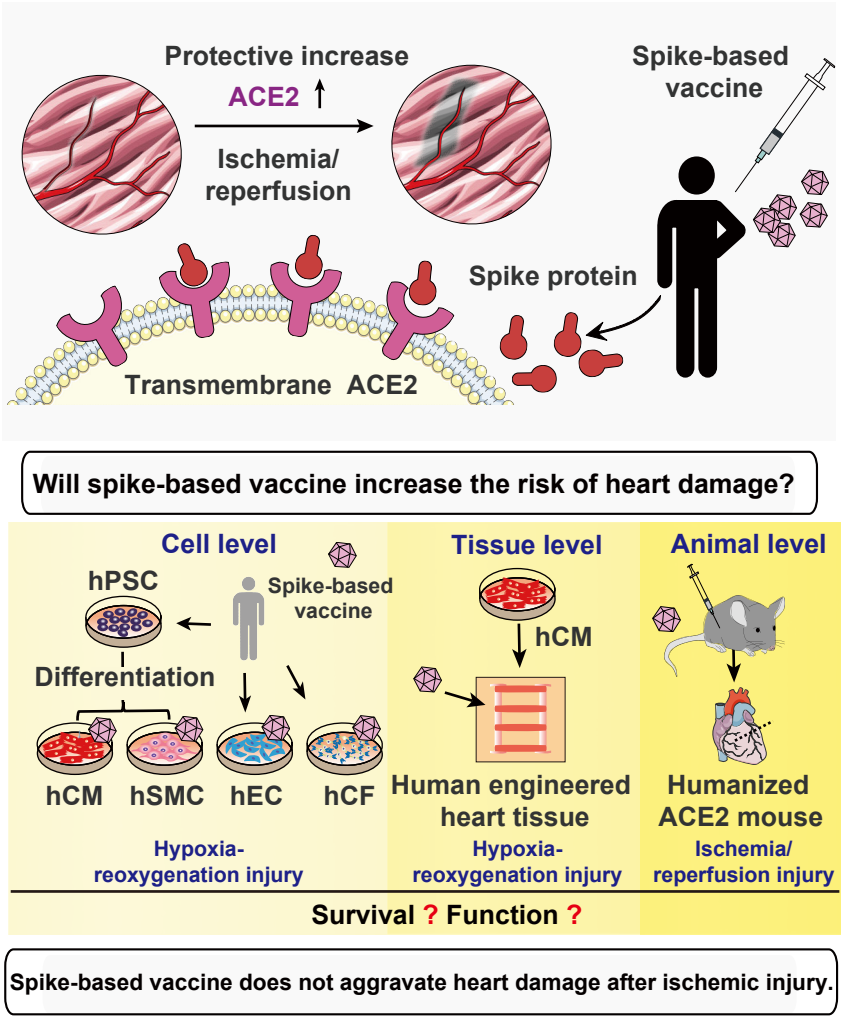
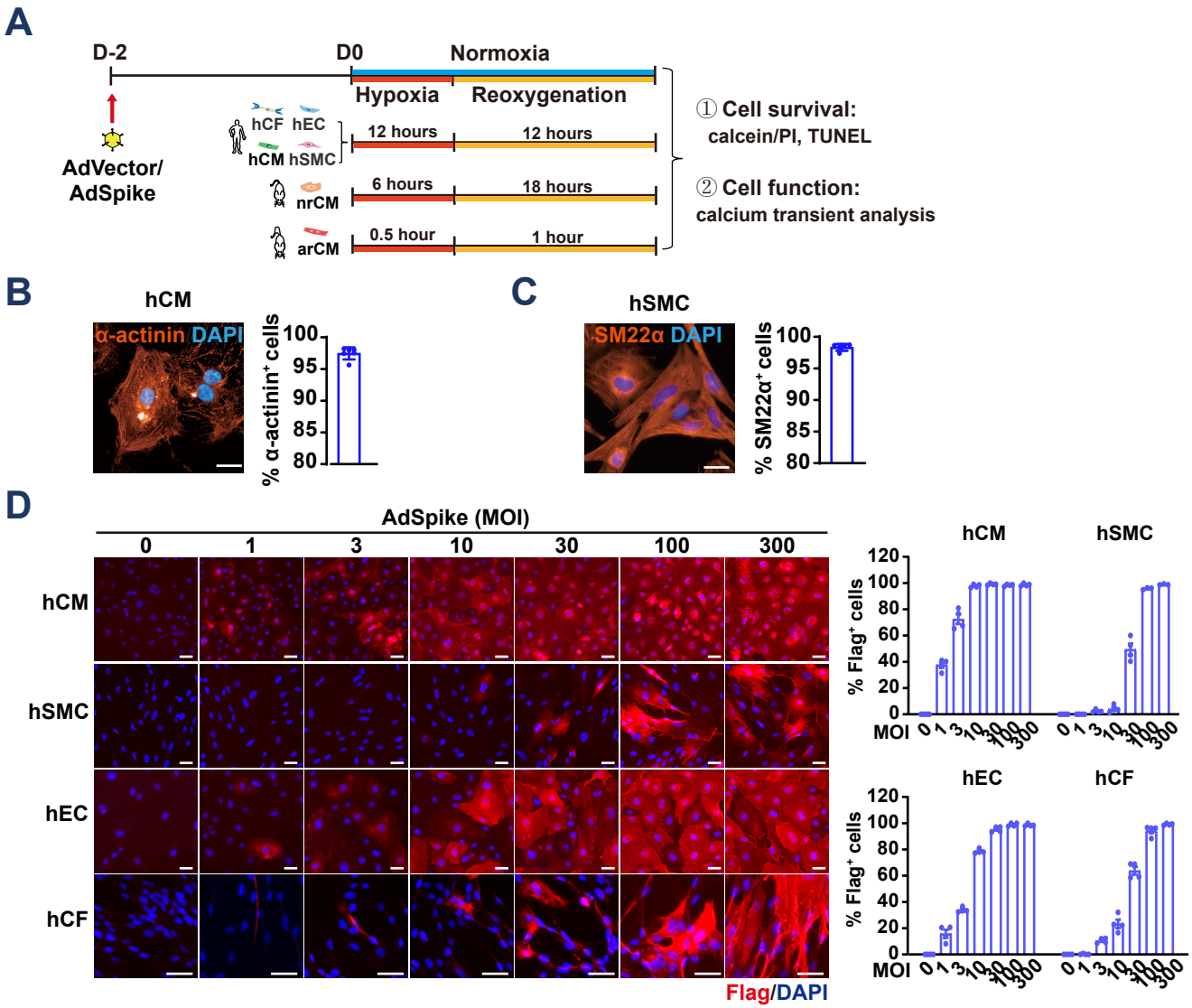
Supplemental Figure 1



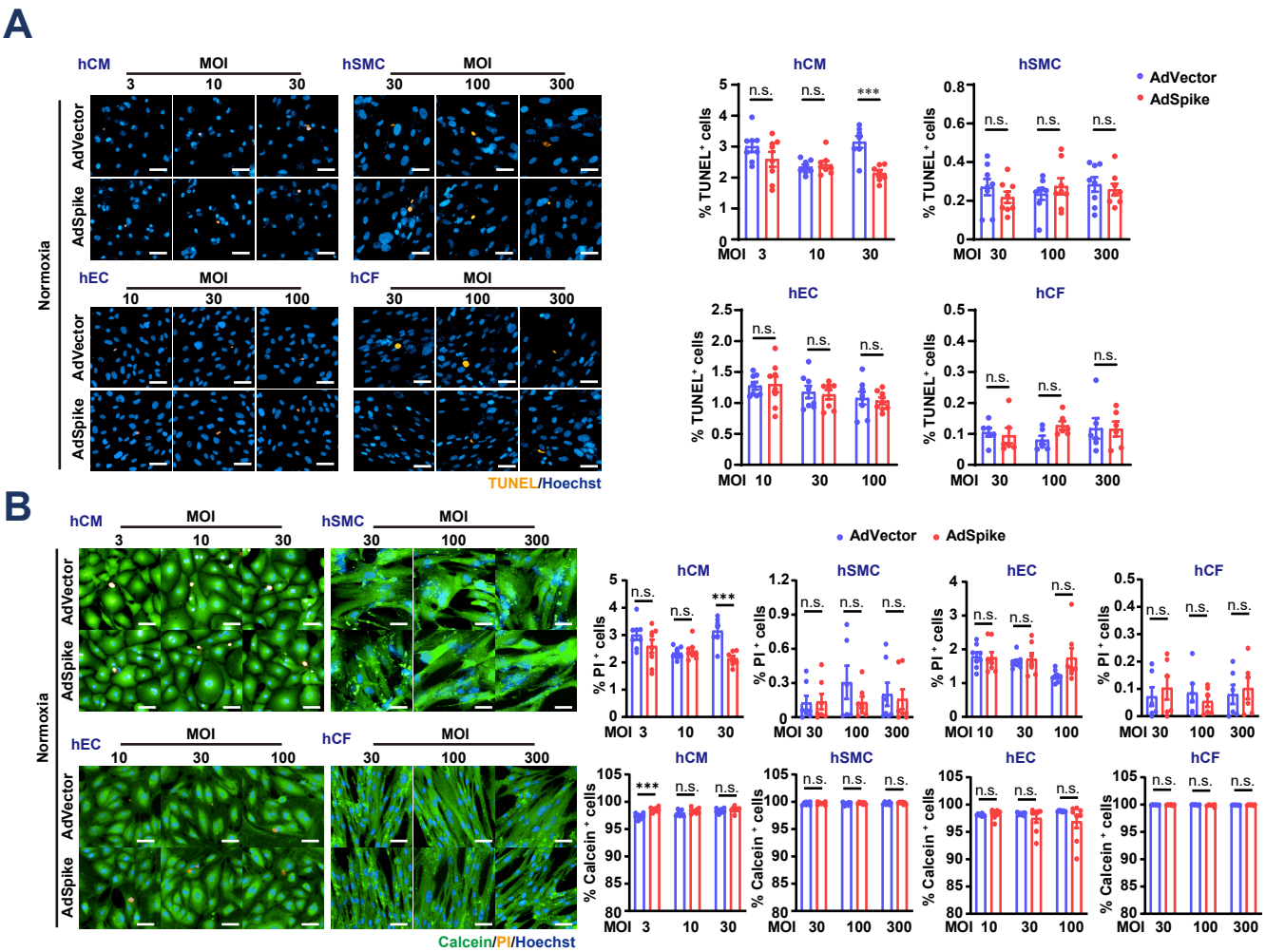
Supplemental Figure 1. Rationale and strategy of this study for evaluating the effects of spike-based vaccine on the ischemic heart.

Supplemental Figure 2



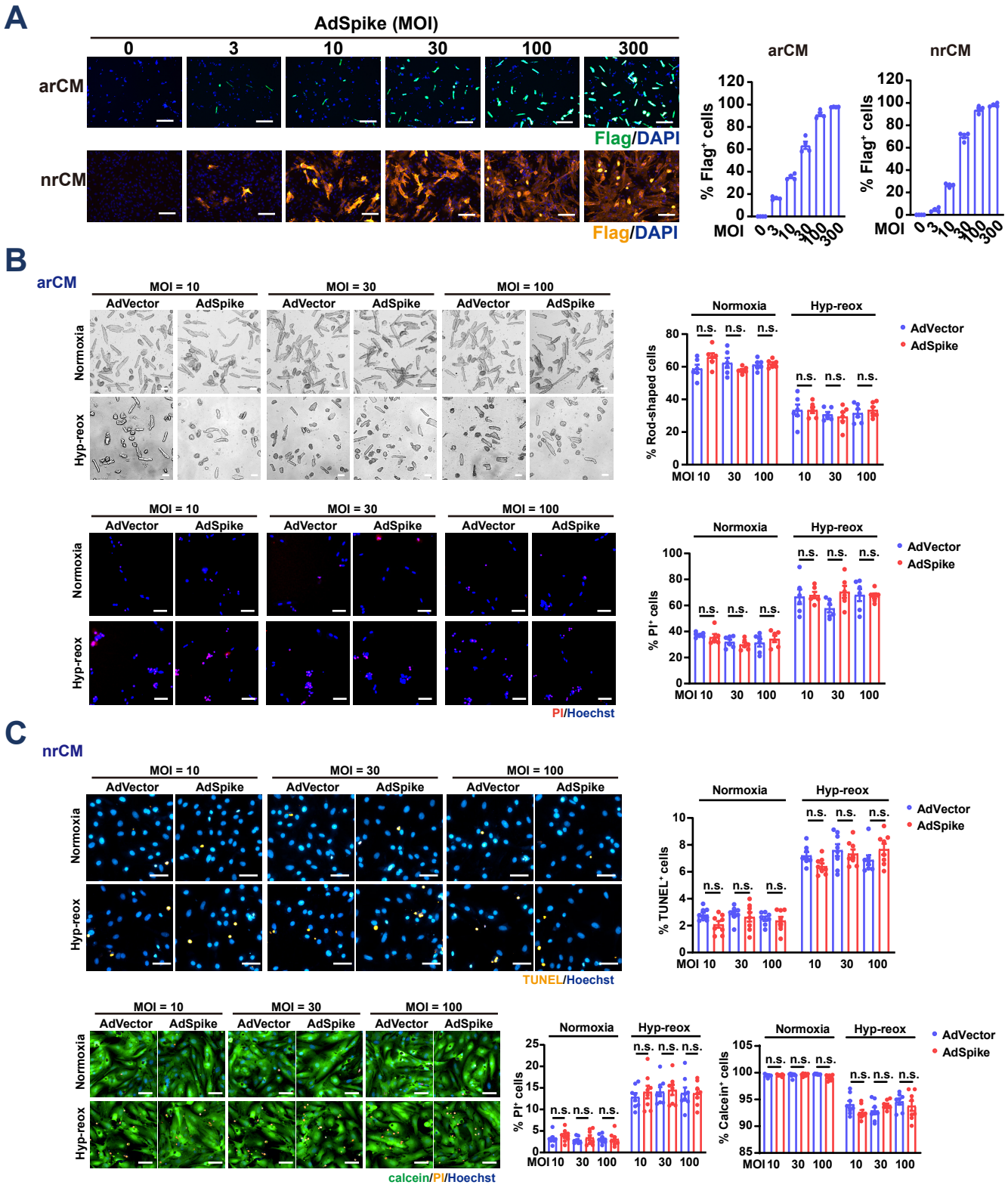
Supplemental Figure 2. The infection of AdSpike in human cardiac cells.
(*A*) Schematic for ex vivo assessment of the effect of AdSpike on cardiac cells with or without hypoxia-reoxygenation (hyp-reox) injury. hCF, human cardiac fibroblast; hCM, human cardiomyocyte; hEC, human endothelial cell; hSMC, human smooth muscle cell; nrCM, neonatal rat cardiomyocyte; arCM, adult rat cardiomyocyte. (*B-C*) Representative and quantitative immunostaining analysis of α-actinin⁺ hCMs (*B*) and SM22α⁺ hSMCs (*C*). n=5-6 for each group. Scale bar, 20 μm. (*D*) Representative immunostaining analysis of spike expression with quantification of the spike-flag⁺ cells in hCMs, hSMCs, hECs, hCFs infected with AdSpike at different MOI (multiplicity of infection). n=4 for each group. Scale bar, 50 μm. Scale bar, 200 μm.

Supplemental Figure 3



Supplemental Figure 3. AdSpike has little effects on cell survival of cardiac cells in nomoxia condition. (*A*) Representative and quantitative immunostaining analysis of TUNEL⁺ cells in hCMs, hSMCs, hECs, hCFs infected with AdVector or AdSpike in normoxia condition. n=6-8 for each group. (*B*) Representative and quantitative calcein-AM/propidium iodide (PI) double staining in hCMs, hSMCs, hECs, hCFs infected by AdVector or AdSpike in normoxia condition. n=6-8 for each group. Scale bar, 50 μm. Data are presented as mean ± SEM; ***$p < 0.001$; n.s., not significant.

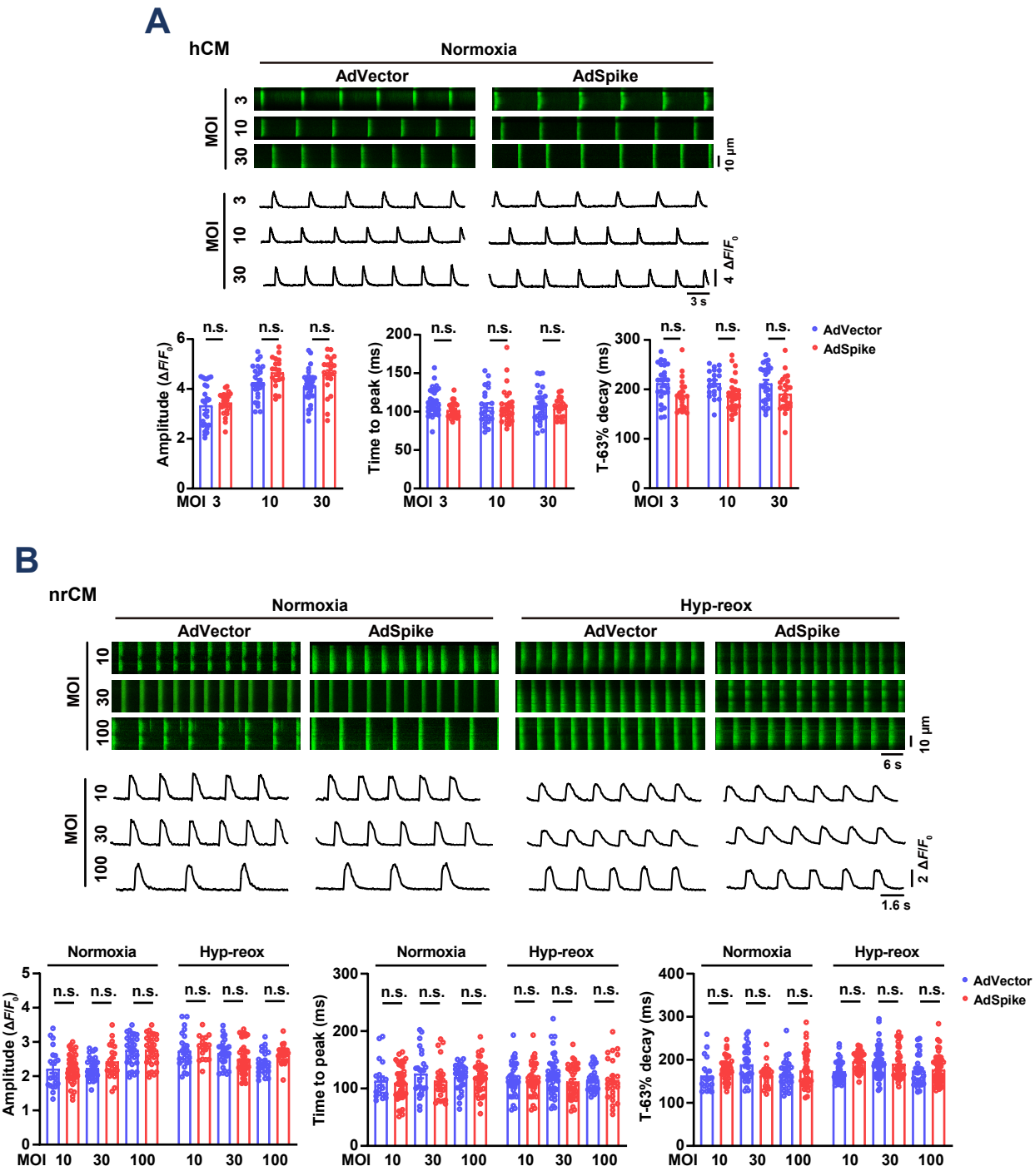
Supplemental Figure 4



Supplemental Figure 4. AdSpike has little effects on cell survival of primary rat cardiomyocytes with or without hyp-reox injury.

(*A*) Representative immunostaining analysis of spike expression with quantification of the spike-flag⁺ cells in arCMs and nrCMs infected with AdSpike at various MOI. n=4 for each group. Scale bar, 200 μm. (*B*) Representative morphology and PI staining analysis of arCMs infected by AdVector or AdSpike with or without hyp-reox injury, with quantification of the rod-shaped healthy cells and PI⁺ apoptotic cells. n=6 for each group. Scale bar, 50 μm. (*C*) Representative staining images with quantification of TUNEL⁺, PI⁺, or calcein⁺ nrCMs infected by AdVector or AdSpike with or without hyp-reox injury. Scale bar, 50 μm. n=8 for each group. Data are presented as mean ± SEM; n.s., not significant.

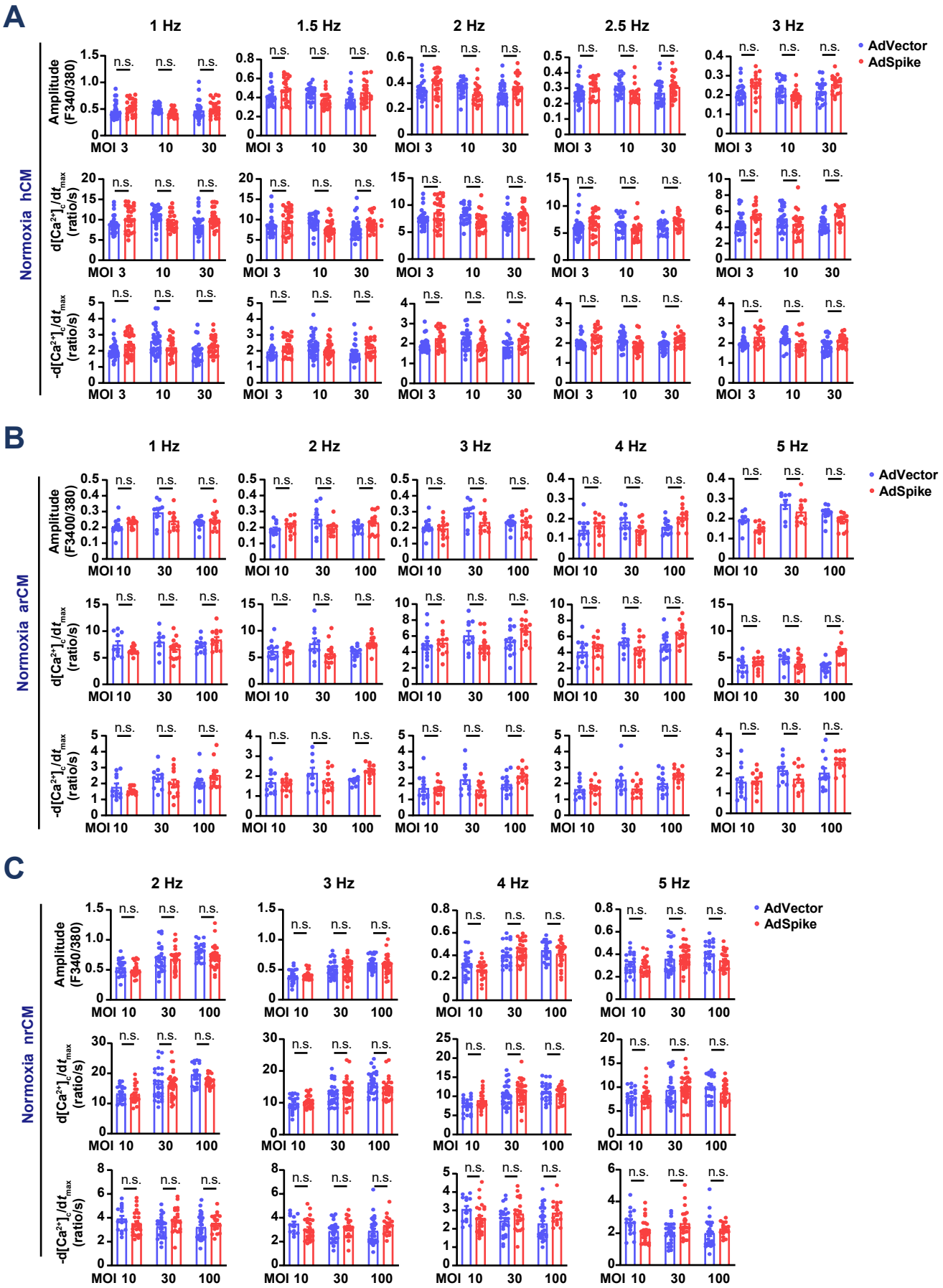
Supplemental Figure 5



Supplemental Figure 5. AdSpike has little effects on spontaneous calcium handling properties of cultured cardiomyocyte.

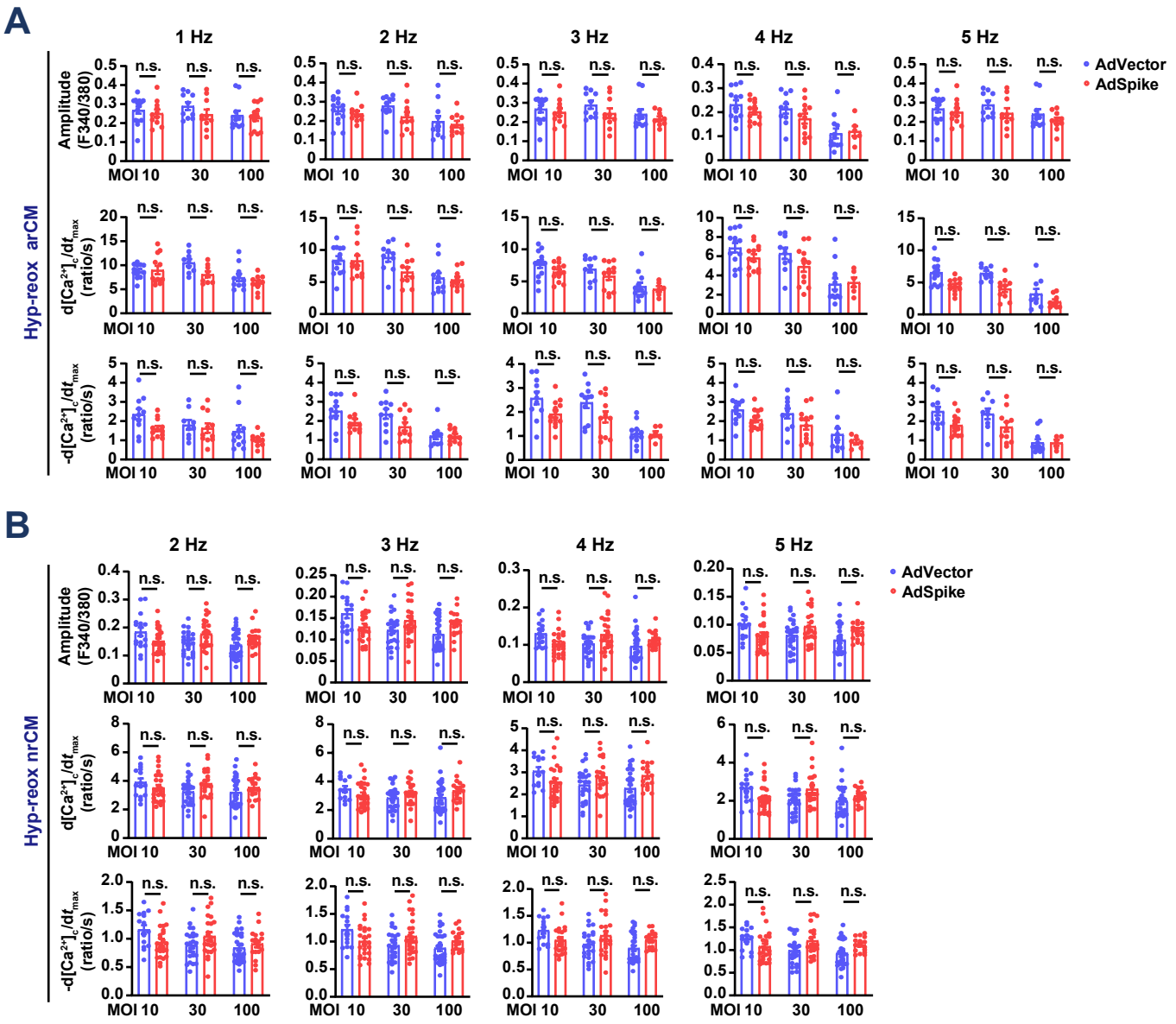
(*A*) Representative traces and averaged parameters of spontaneous intracellular calcium transient of hCM infected with AdVector or AdSpike in normoxia condition. n=15-30 for each group. (*B*) Representative traces and averaged parameters of spontaneous intracellular calcium transient of nrCMs infected with AdVector or AdSpike with or without hyp-reox injury. n=18-40 for each group. Data are presented as mean ± SEM; n.s., not significant.

Supplemental Figure 6



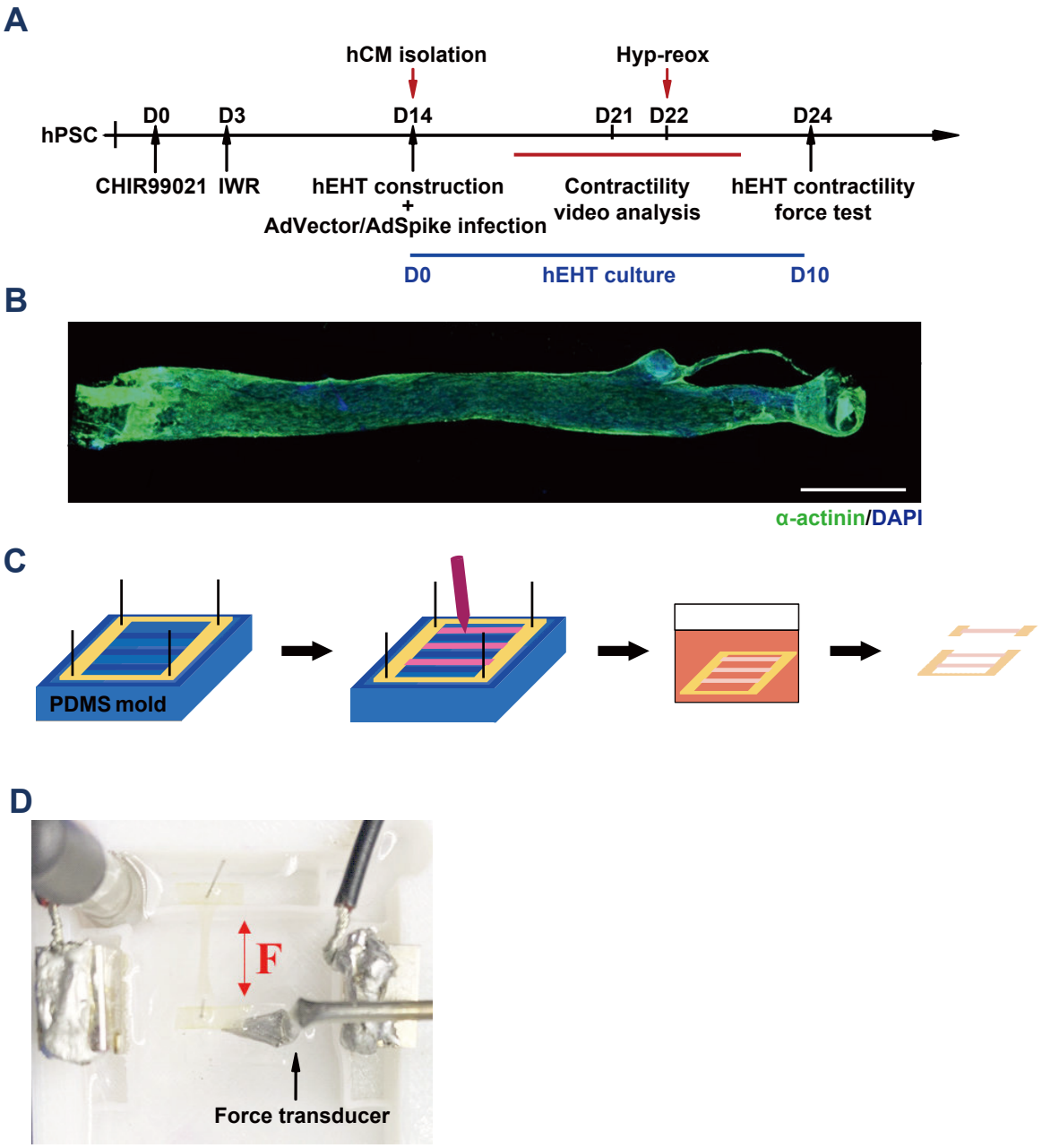
Supplemental Figure 6. AdSpike has little effects on calcium handling properties of cultured cardiomyocytes subjecting to electrical field stimulation in normoxia condition.
(*A-C*) Averaged parameters of calcium transient of the cardiomyocytes infected by AdVector or AdSpike with different frequency of electrical field stimulation. n=15-30 for each group in (*A*) and (*B*); n=10-14 for each group in (*C*). Data are presented as mean ± SEM; n.s., not significant.

Supplemental Figure 7



Supplemental Figure 7. AdSpike has little effects on calcium handling properties of cultured cardiomyocytes subjecting to electrical field stimulation after hyp-reox injury.
Averaged parameters of calcium transient of the cardiomyocytes infected with AdVector or AdSpike with different frequency of electrical field stimulation. n=15-30 for each group in (*A*); n=10-14 for each group in (*B*); Data are presented as mean ± SEM; n.s., not significant.

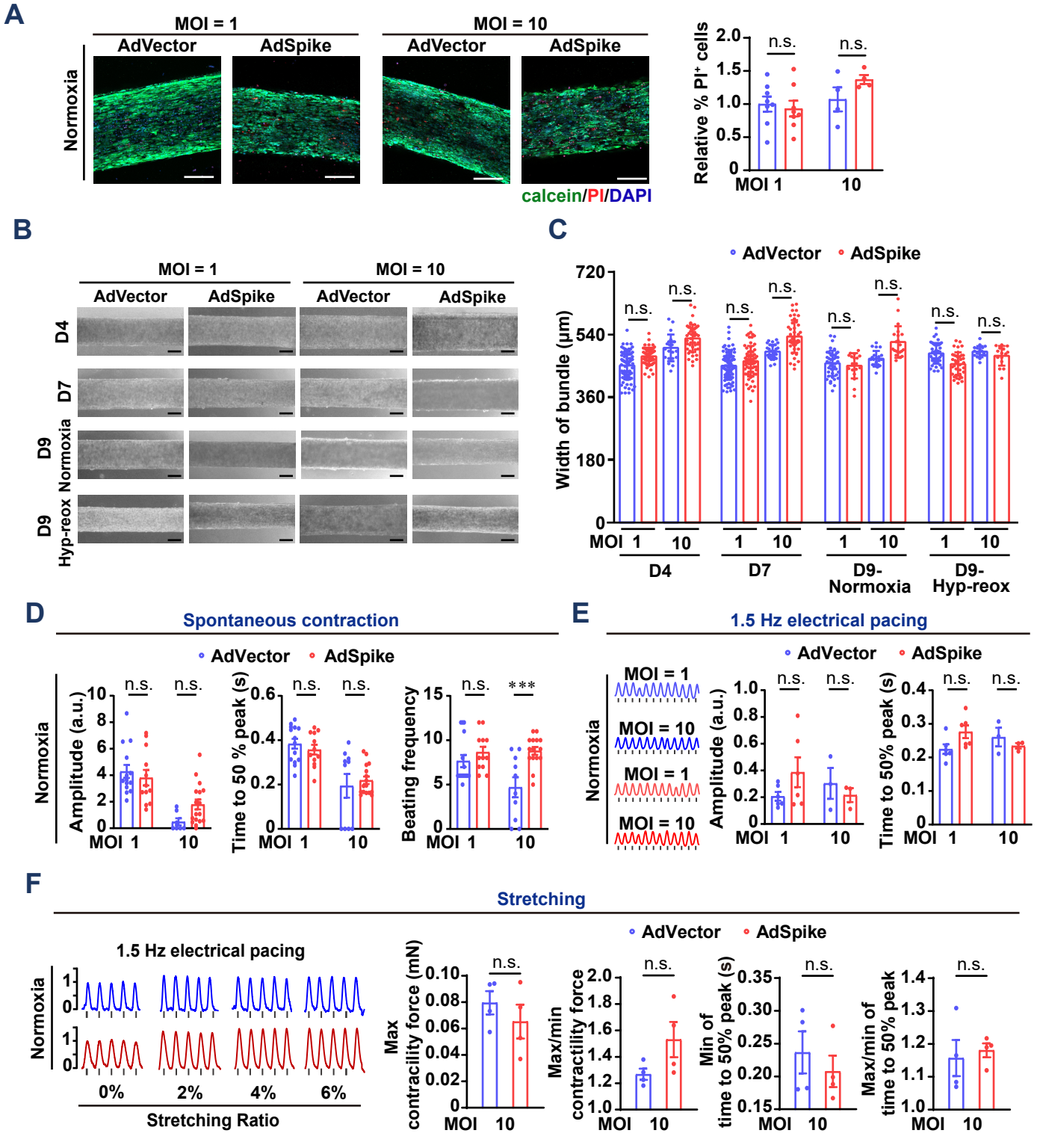
Supplemental Figure 8



Supplemental Figure 8. The principle of the human engineered heart tissue (hEHT) production and the steps of functional test.

(*A*) The timeline of the assay based on the hEHT model, including the hPSC (human pluripotent stem cell) differentiation into cardiomyocytes (CM), hEHT construction, AdVector/AdSpike infection, contractility video analysis, and contractility force test. (*B*) Multi stitching immunofluorescence image of the whole hEHT bundle. Cardiomyocytes are labeled with anti-α-actinin (green) and nucleus with DAPI (blue). Scale bar, 1 mm. (*C*) Schematic diagram of hEHT construction. (*D*) The equipment image of mechanical contractility force test for hEHTs.

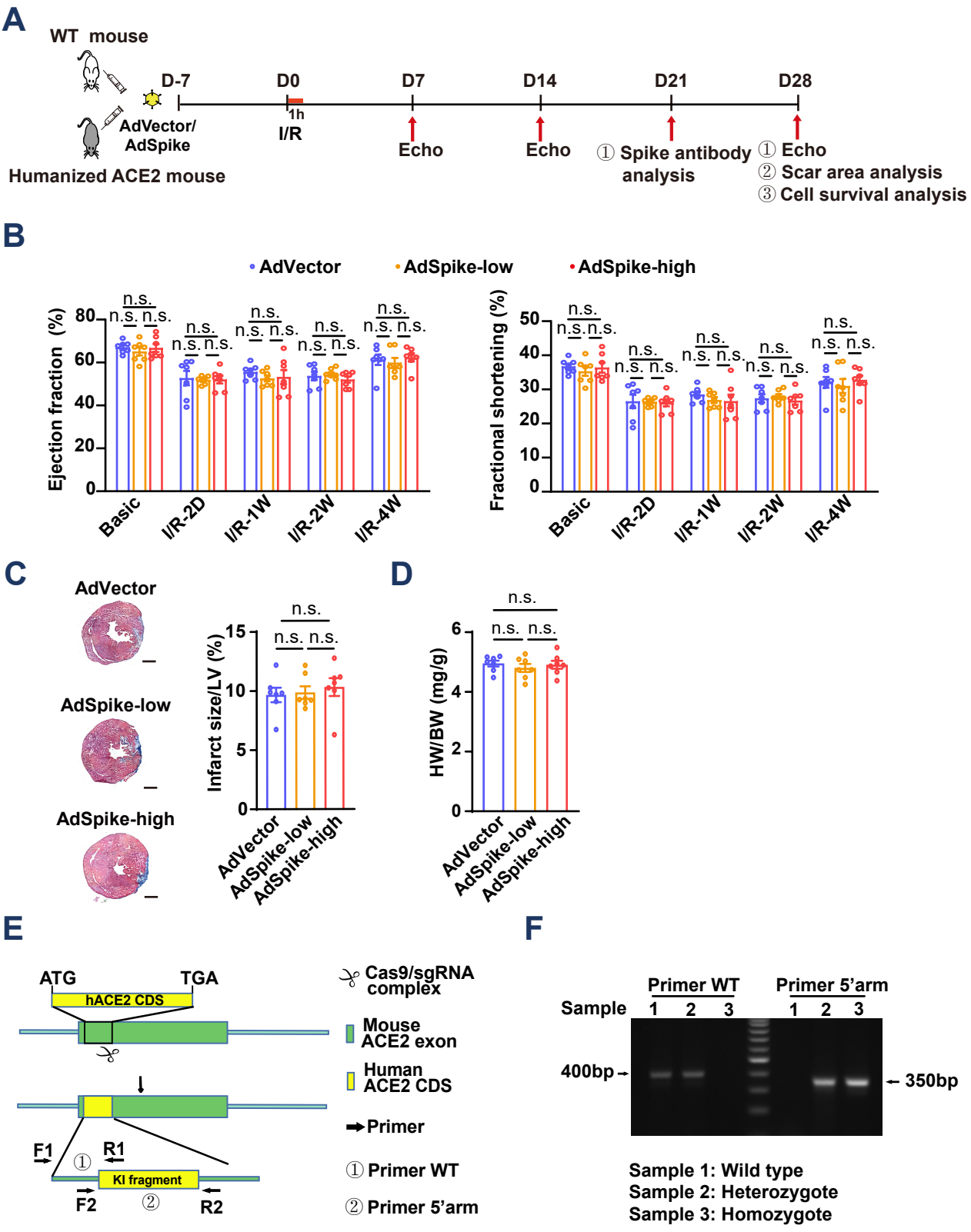
Supplemental Figure 9



Supplemental Figure 9. AdSpike has little effects on hEHTs that underwent spontaneous contraction, electrical field stimulation, and mechanical tensile test in normoxia condition.

(*A*) The morphology of hEHTs at various days after viral infection. Scale bar, 200 μm. (*B*) Quantification of the width of hEHTs at various days after viral infection. n=15-78 for each group. (*C*) Representative calcein-AM/PI double staining in hEHT (left) with quantification (right). n=4-8 for each group. Scale bar, 200 μm. (*D*) Video analysis of the hEHTs for spontaneous contraction amplitude, time to 50% peak, and beating frequency per 10 seconds. a.u., absolute units. n=7-17 for each group. (*E*) Representative contractile traces and contraction parameters' video analysis in hEHTs under 1.5 Hz electrical pacing. n=3-6 for each group. (*F*) Representative contractile force traces and contractile force parameters during progressive stretch (0%, 2%, 4%, 6% tissue length) of hEHTs cultured in normoxia condition on day 24. n=4 for each group. All the hEHTs were infected with AdVector (MOI=1 or 10) or AdSpike (MOI=1 or 10) on day 14. Data are presented as mean ± SEM; ***$p < 0.001$; n.s., not significant.

Supplemental Figure 10



Supplemental Figure 10. AdSpike has little effects on heart damage repair after myocardial I/R on wild-type (WT) mice *in vivo*.

(*A*) Schematic for evaluating the effect of AdSpike in ischemic heart in vivo. I/R, ischemia/reperfusion; Echo, echocardiography. (*B*) Ejection fraction and fractional shortening of WT mouse hearts at various time points post-I/R measured by echocardiography. D, day; W, week. n=7 for each group. (*C*) Masson-Trichrome staining of heart cross sections of WT mice 4 weeks post-I/R (left) with quantification of scar size (right). n=7 for each group. Scale bar, 1 mm. (*D*) Quantification of heart weight (HW) relative to body weight (BW) of WT mice at 4 weeks post-I/R. n=7 for each group. (*E*) Strategy for generating humanized ACE2 (hACE2) mouse model. **F**. Genotyping of hACE2 mouse. Data are presented as mean ± SEM; ***$p < 0.001$; n.s., not significant.