

Supplemental Materials

Molecular hydrogen triggers TRPC4-TRPC4AP-dependent reversible calcium transients via extracellular influx

Pengxiang Zhao^{1,*}, Han Li^{1,*}, Zisong Cai^{1,*}, Xujuan Zhang¹, Xiaohu Wen¹, Ziyi Liu¹, Shihao Jiang¹, Xue Jiang¹, Jiateng Wang¹, Zheng Dang¹, Mengyu Liu¹, Fei Xie¹, and Xuemei Ma^{1,#}

¹College of Chemistry and Life Science, Beijing University of Technology, Beijing 100124, P. R. China

Correspondence: Xuemei Ma, xmma@bjut.edu.cn

* Pengxiang Zhao, Han Li, and Zisong Cai, contributed equally as first authors

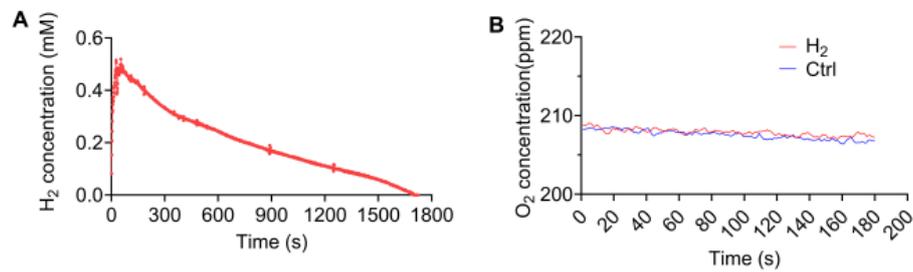


Figure S1. Real-time monitoring of H₂ and O₂ levels in the culture medium after a single administration of H₂.

(A) Real-time monitoring curve of H₂ concentration.

(B) Real-time monitoring curve of O₂ concentration.

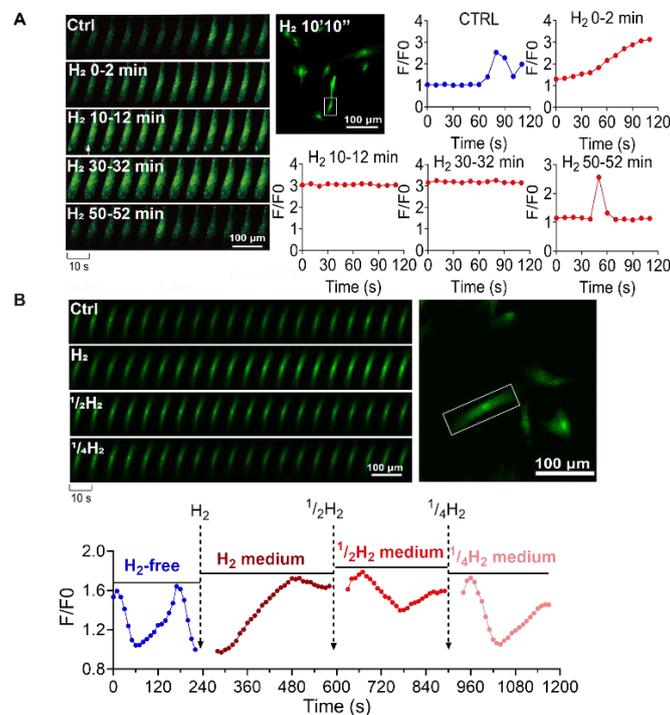


Figure S2. H₂ Induces reversible and concentration-dependent calcium oscillations in MSCs.

- (A) Different time periods of Fluo-4 images and averaged F/F₀ traces in H₂-induced MSCs [Ca²⁺]_i during extended time-lapse imaging.
- (B) Time-lapse Ca²⁺ images and Fluo-4- averaged F/F₀ trace of MSCs in H₂-free, H₂-medium (Saturated H₂ medium), 1/2H₂-medium, and 1/4H₂ medium. Scale bar in A and B= 200 μm.

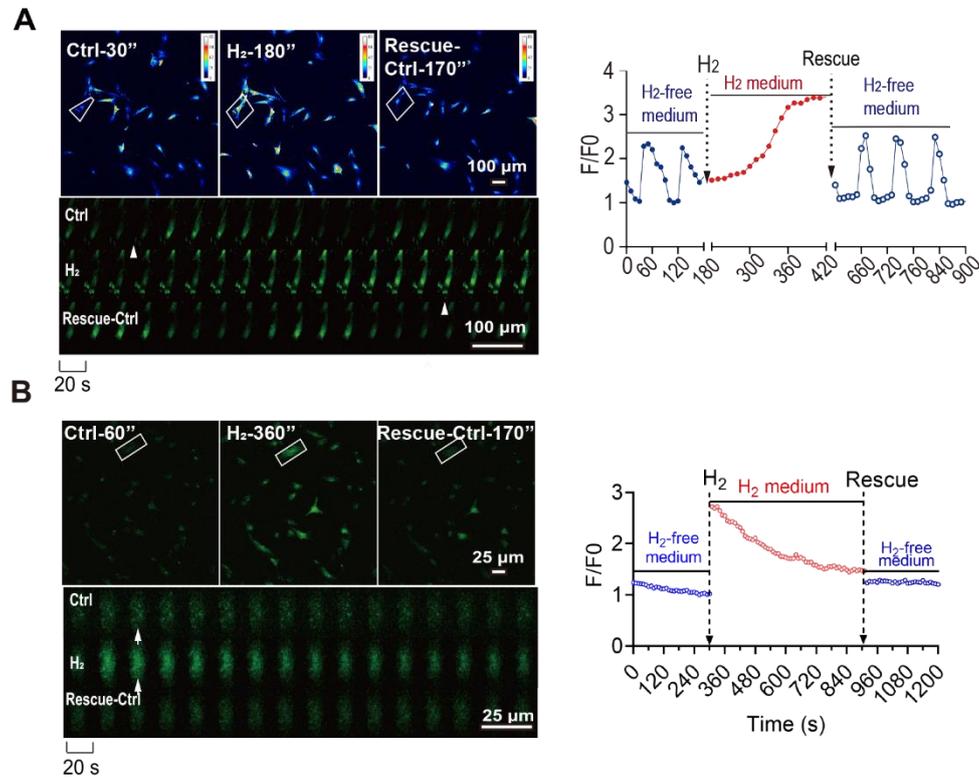


Figure S3. H₂ Induces Intracellular Calcium Elevation in MSCs and HUVECs.

- (A) Pseudocolor (A left, upper) and time series (A left, lower) images and the Fluo-4 averaged F/F₀ trace of the [Ca²⁺]_i changes in H₂-free, H₂-medium, and then back to H₂-free conditions.
- (B) Time-lapse (B) images and the Fluo 4 averaged F/F₀ trace of the [Ca²⁺]_i changes in H₂-free, H₂-medium, and then back to H₂-free conditions in HUVEC. Scale bar in A = 100 μm. Scale bar in B = 25 μm.

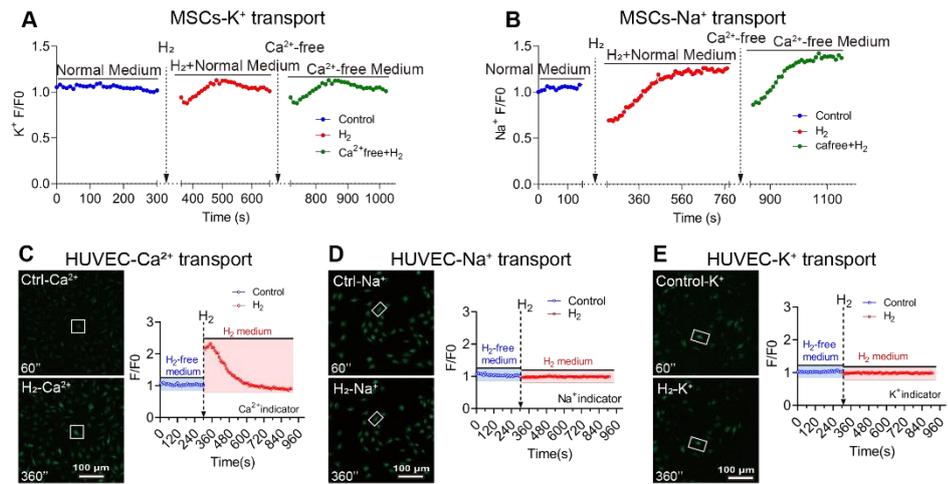


Figure S4. Time-lapse-averaged F/F0 traces showing the influence of H₂ on the influx of different cations in MSCs and HUVECs.
 (A) & (B) H₂-induced K⁺ and Na⁺ transport under normal-medium, H₂-medium and Ca²⁺-free medium conditions in MSCs.
 (C)-(D) & (E) Comparison of the effects of H₂ on Ca²⁺, K⁺, and Na⁺ influx in HUVEC cells. Scale bar in C,D and E= 100 μm;

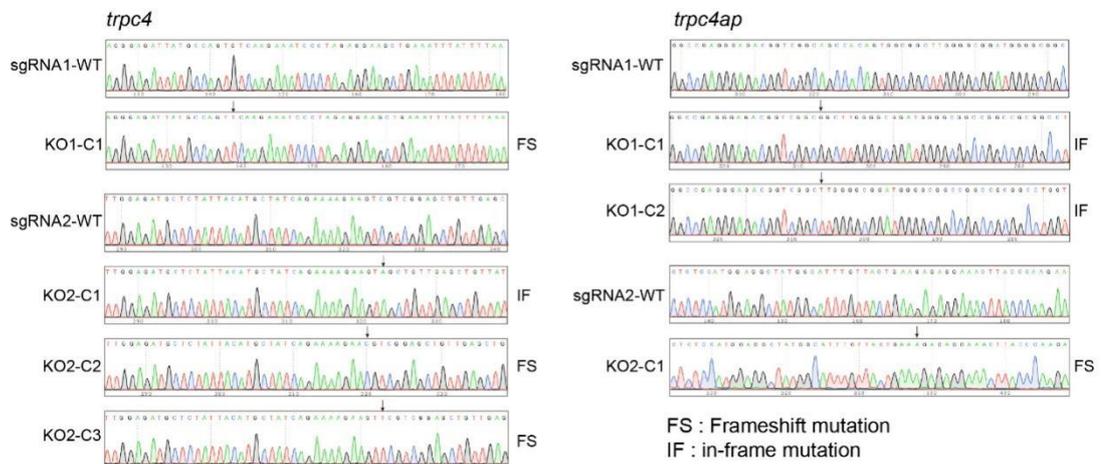


Figure S5. Validation of TRPC4 and TRPC4ap gene knockdown in monoclonal 293T cell lines.

The mutant site is indicated by an arrow. Wild-type (WT) sequences of the corresponding exons were determined by Sanger sequencing and are presented with chromatograms. For each detected sequence variant, the detection frequency and the type of mutation are shown.

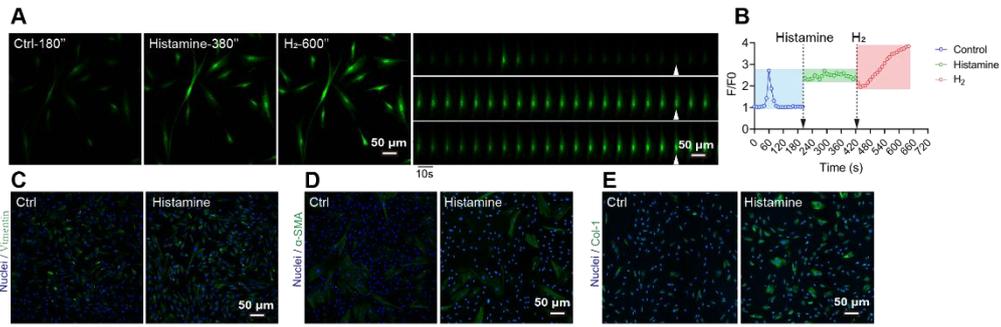


Figure S6. Comparative analysis of the effects of histamine, a Ca^{2+} agonist, and H_2 on $[Ca^{2+}]_i$ in MSCs, and their impact of histamine on the cytoskeleton.

(A) & (B) Time-lapse (A) images and the Fluo4 averaged $\Delta F/F_0$ trace (B) of the $[Ca^{2+}]_i$ changes under H_2 -free, Histamine, and then H_2 conditions in MSCs.

(C) - (E) Immunofluorescence results of Vimentin, α -SMA, and Collagen-1 in the Control and Histamine groups of MSCs. Scale bar = 50 μm ;

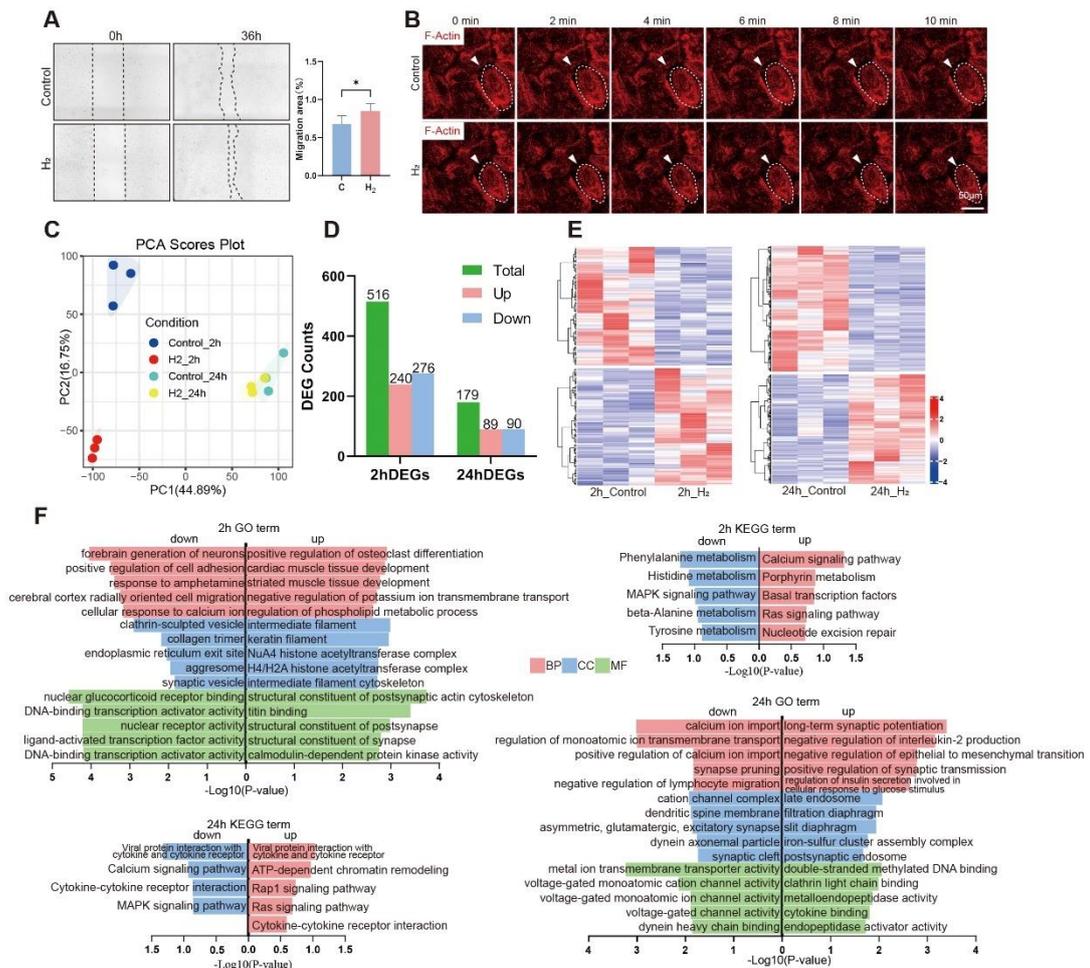


Figure S7. Impact of H_2 on the migratory function of HUVEC cells and its effects on

transcription.

- (A) Effect of H₂ on the motion ability of HUVECs.
- (B) Impact of H₂ on the cytoskeletal structure of HUVECs.
- (C) PCA diagram of sequencing data of each group.
- (D) Differentially expressed genes (DEGs) count results.
- (E) Hierarchical clustering heat map of DEGs in HUVECs following 2 h and 24 h H₂ treatment.
- (F) H₂-2h vs Control-2h and H₂-24h vs Control-24h column charts for GO enrichment analysis and a histogram of KEGG enrichment analysis. For B, using normal culture medium as the control group, changes in the cytoskeleton were captured over a 10-minute period. The medium was then immediately replaced with saturated hydrogen culture medium, to ensure consistent visibility, and changes were recorded for an additional 10 minutes. In the control group, there were no significant changes in the cytoskeleton during the initial 10 minutes. However, after switching to saturated hydrogen culture medium for 10 minutes, intercellular spacing increased, and the cytoskeleton changed, showing a contraction trend. Data A were processed using an unpaired t-test, and were plotted as Mean \pm SEM. *P value < 0.05. Scale bar in B= 50 μ m;

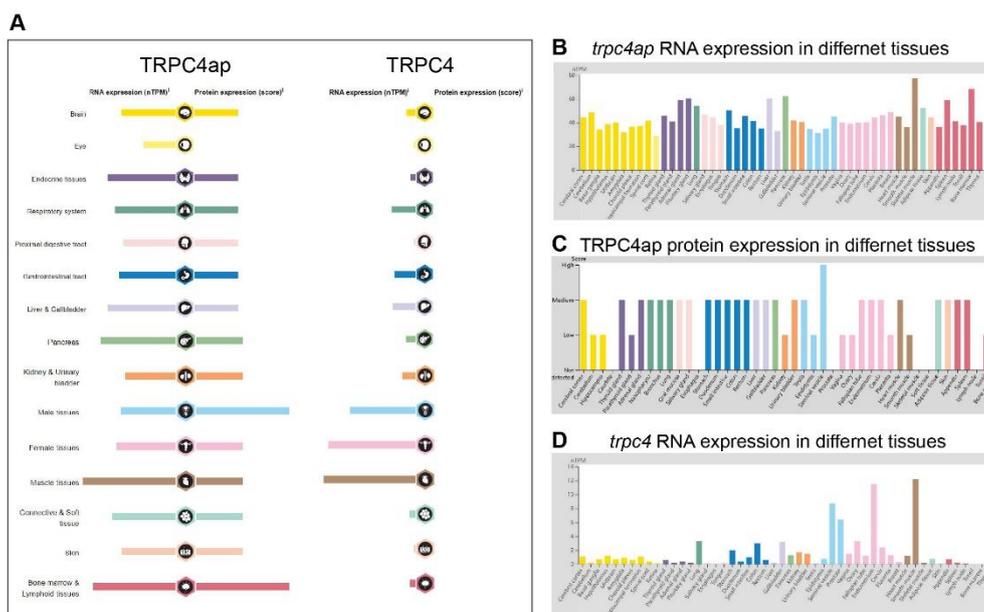


Figure S8. Overview of TRPC4 and TRPC4ap expression based on data from the Human Protein Atlas.

- (A) Expression levels of TRPC4ap and TRPC4 across various human organs, with RNA levels shown on the left and protein levels on the right.
- (B) and (D) RNA expression patterns of TRPC4ap and TRPC4 in distinct tissue types. (C) Distribution of TRPC4ap protein expression across multiple tissues. The images have been sourced, downloaded, and reorganized from the following URLs:

<https://v19.proteinatlas.org/ENSG00000100991-TRPC4AP/tissue>

<https://v19.proteinatlas.org/ENSG00000133107-TRPC4/tissue>

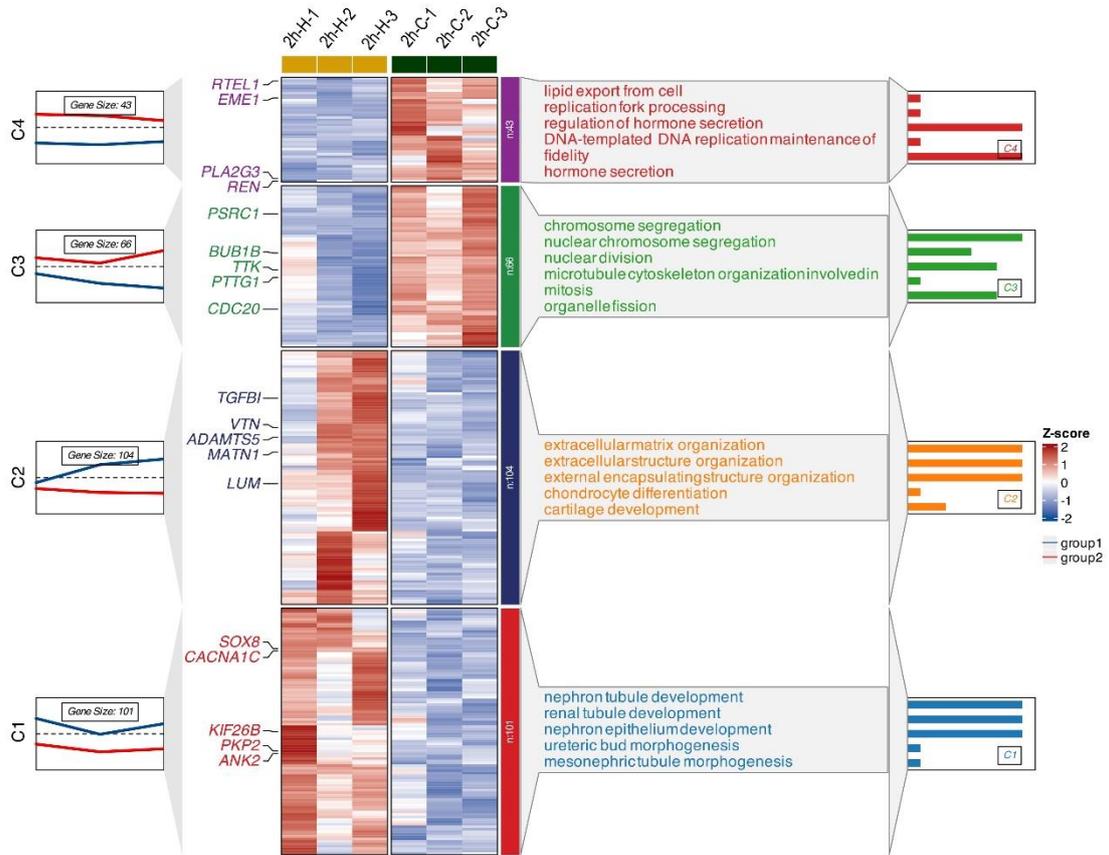


Figure S9. Expression trends, heat maps, and significantly enriched Biological Process terms for each cluster of DEGs in MSCs after H₂ treatment for 2 h.

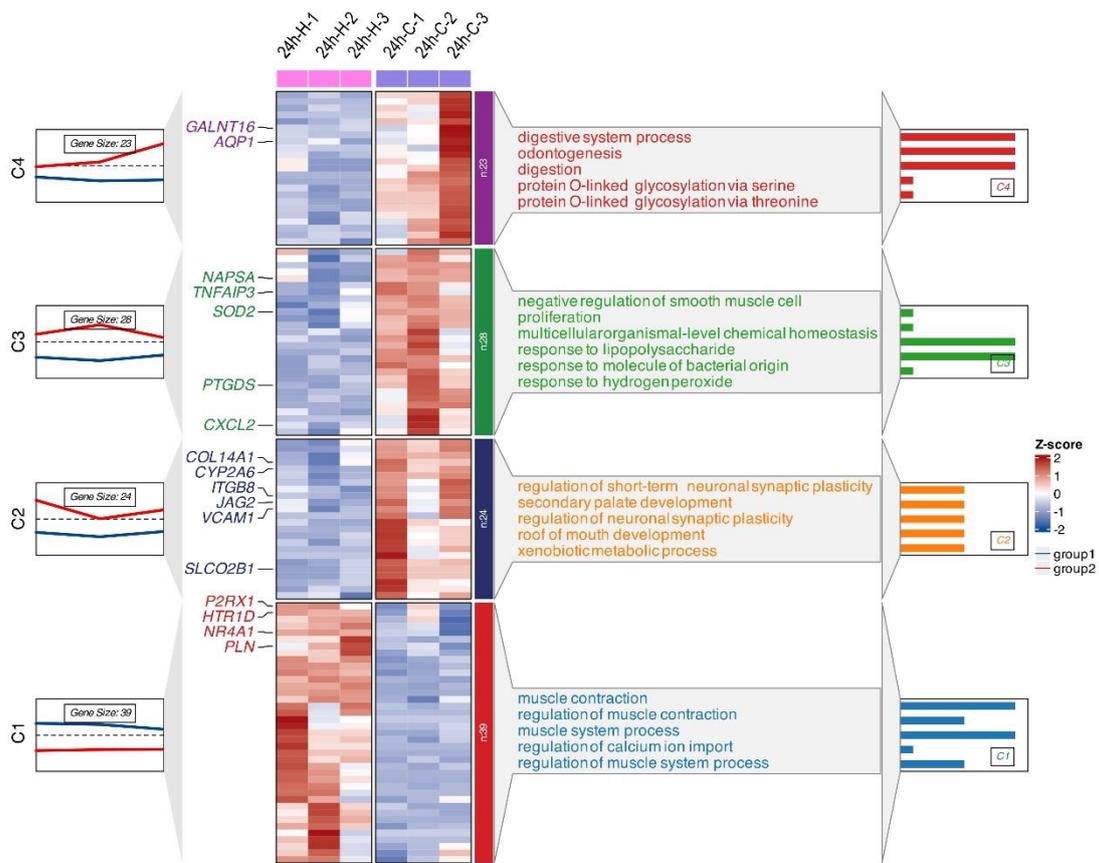


Figure S10. Expression trends, heat maps, and significantly enriched Biological Process terms for each cluster of DEGs in MSCs after H₂ treatment for 24 h.

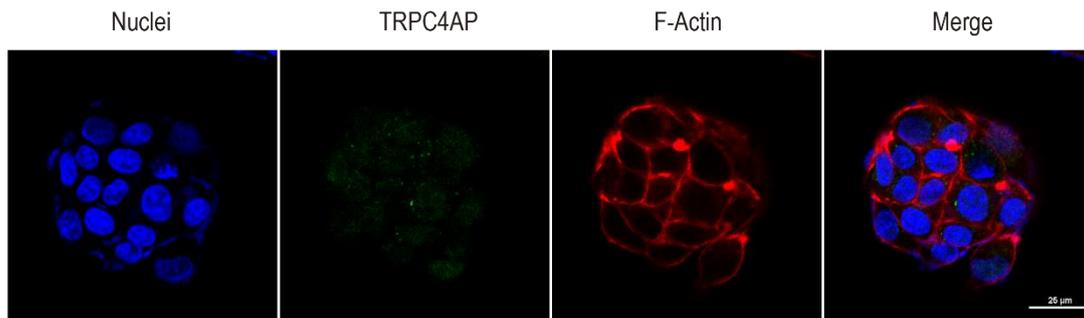


Figure S11. Fluorescence co-localization results of F-Actin (red) and the TRPC4AP (green) of HepG2. Scale bar = 25 μm;

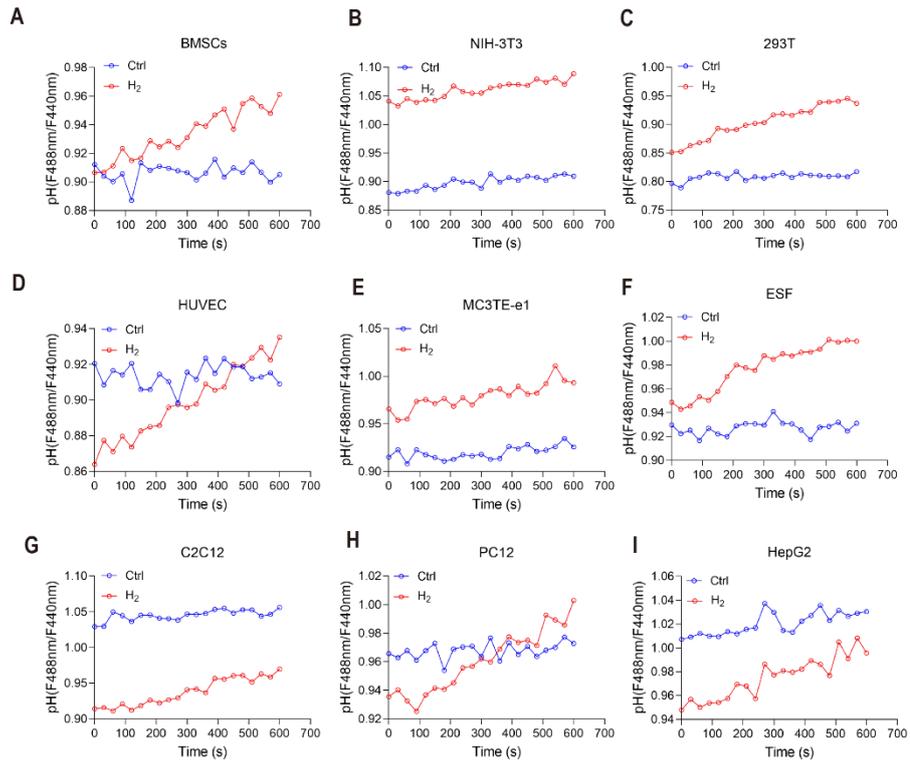


Figure S12 . Intracellular pH variation under the influence of H₂ in different cells.

ARG-730 under pH 7.4 ARG-730 under pH 8.0 ARG-731 under pH 7.4 ARG-731 under pH 8.0

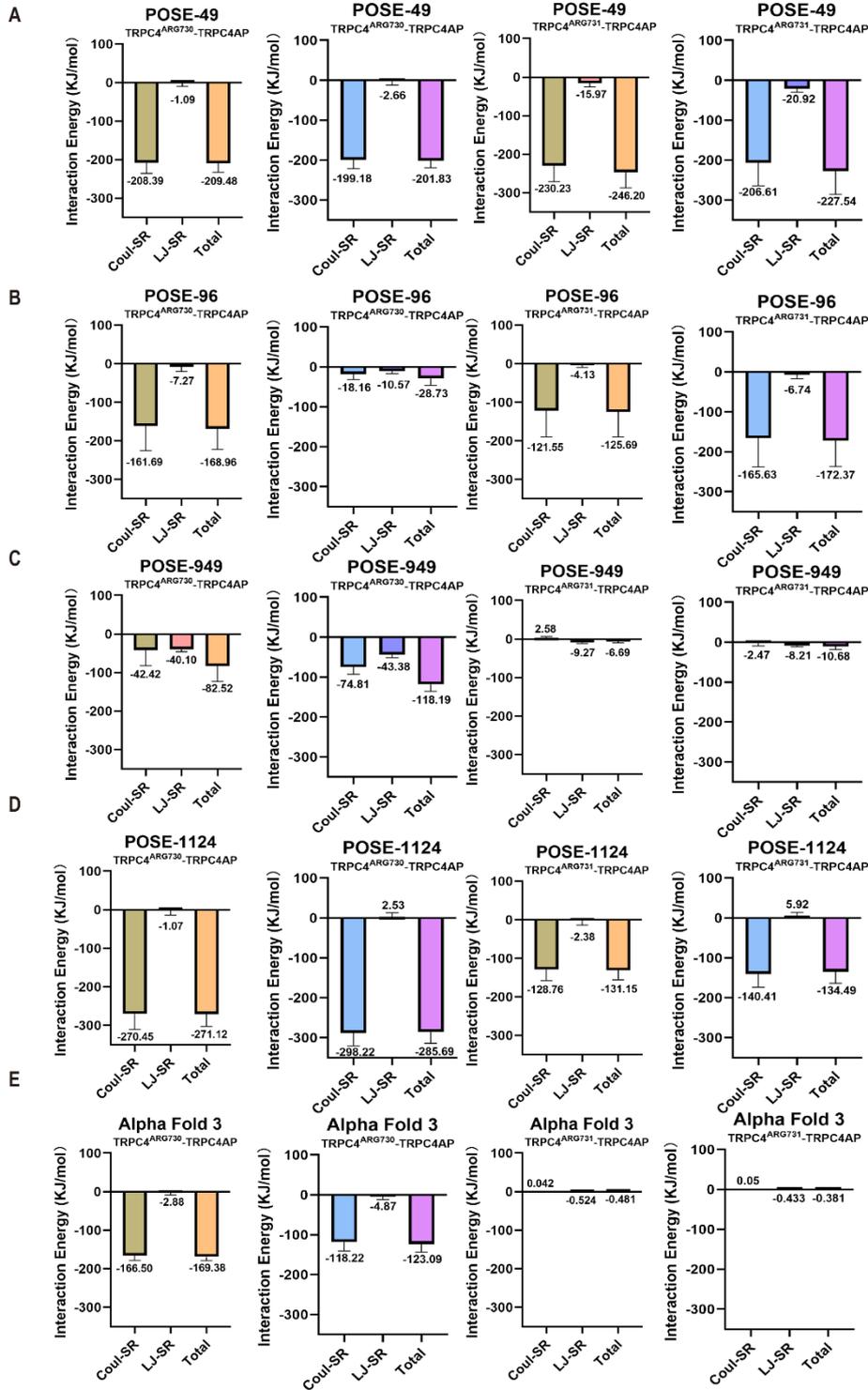


Figure S13. Interaction forces between arginine residues at positions 730 and 731 of the TRPC4 protein and the TRPC4AP protein in protonated states under pH 7.4 and pH 8.0 in molecular dynamics simulations.

(A).pose-49;(B).pose-96; (C).pose949; (D).pose1124.(E) .Alpha Fold 3. From left to right: Interaction forces between TRPC4, specifically the arginine at position 730, and the TRPC4AP protein at pH 7.4; Interaction between TRPC4, specifically the arginine at

position 730, and the TRPC4AP protein at pH 8.0; Interaction between TRPC4, specifically the arginine at position 731, and the TRPC4AP protein at pH 7.4; Interaction between TRPC4, specifically the arginine at position 731, and the TRPC4AP protein at pH 8.0. Coul-SR (short-range Coulomb force, representing electrostatic interactions), LJ-SR (short-range Lennard-Jones potential, representing van der Waals forces), and Total (total interaction force).

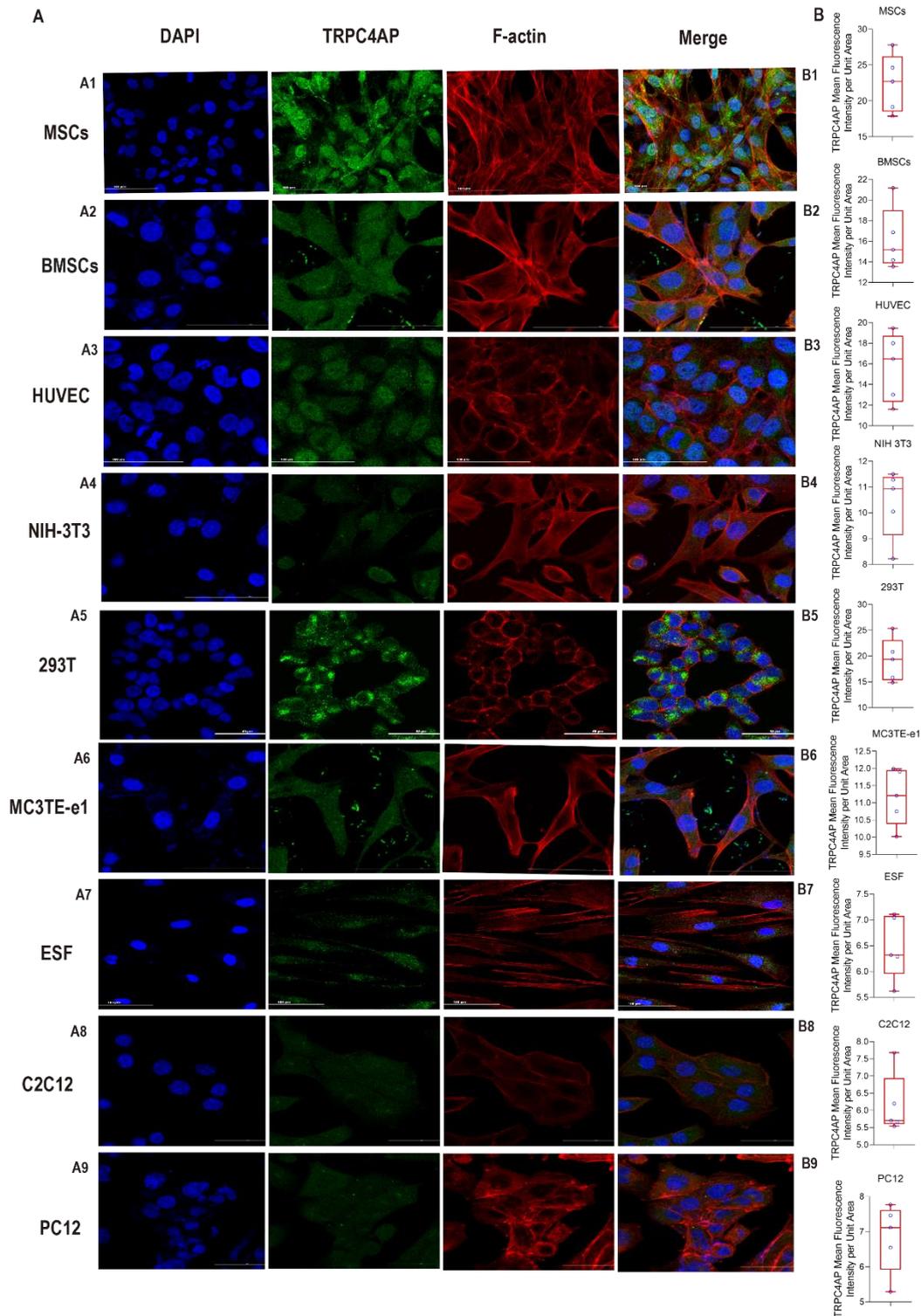


Figure. S14. Fluorescence co-localization results of F-Actin (red) and TRPC4AP (green) in cells. Scale bar = 50 μm ;

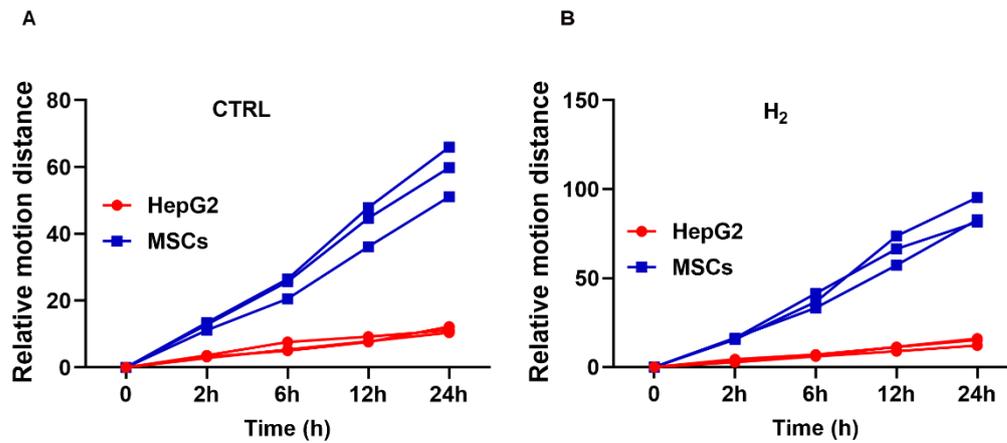


Figure S15. Corresponding migration distance of tumor cells HepG2 and MSCs under (A).H₂-free and (B).H₂-medium using a live-cell imaging system .

TABLE**Table S1. Comparative analysis of Ca²⁺ response to H₂ stimulation across different cell types.**

| Cell Types | F ₀ | Peak F/F ₀ | F(average) under H ₂ | Flow velocity amplitude | Flow velocity amplitude under H ₂ |
|------------|----------------|-----------------------|---------------------------------|-------------------------|--|
| MSCs | 296.07 | 2.819 | 873.59 | 0.0107 | 0.0216 |
| BMSc | 167.80 | 3.908 | 662.63 | 0.0373 | 0.0870 |
| HUVEC | 202.59 | 1.700 | 399.51 | 0.0135 | 0.0330 |
| 293T | 478.88 | 2.035 | 1061.49 | 0.0112 | 0.015 |
| NIH3T3 | 286.72 | 1.67 | 552.34 | 0.0075 | 0.0209 |
| MC3TE-e1 | 108.04 | 3.675 | 384.39 | 0.0296 | 0.0631 |
| ESF | 240.63 | 1.908 | 492.54 | 0.0045 | 0.0245 |
| C2C12 | 242.92 | 1.725 | 418.82 | 0.0141 | 0.0162 |
| PC12 | 90.04 | 2.512 | 222.46 | 0.0460 | 0.0587 |

Table S2 Materials and Reagents

| RAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|--------------------------|---------------------|
| Antibodies | | |
| Rabbit anti-TRPC4AP | Proteintech | 29310-1-AP |
| Mouse anti- α SMA | Cell SignalingTechnology | 19245 |
| Rabbit anti-Vimentin | Cell SignalingTechnology | 5741 |
| Mouse anti-Beta actin | Proteintech | 66009-1-1g |
| Rabbit anti- Collagen I | Abcam | AB254113 |
| secondary antibodies (Alexa Fluor 488) | Cell SignalingTechnology | 4412 |
| Chemicals | | |
| Ca ²⁺ -GPCR,Fluo-4,AM (5 μ M,for Live-cell) | KeyGEN | KGAF024 |
| Na ⁺ -ENG-2,AM, (5 μ M, for Live-cell) | Maokang | MX4514 |
| K ⁺ -EPG-4,AM, (5 μ M , for Live-cell) | Maokang | MX4521 |
| DAPI | ThermoFisher | R37606 |
| ER-tracker (1 μ M) | YEASEN | 40764ES20 |
| Mito-tracte r(100 nM) | YEASEN | 40741ES50 |
| Stem cell culture-medium | iCell | PriMed-icell-012-sf |
| JC-1 (2 μ M) | ThermoFisher Scientific | T3168 |
| CCK-8 | Beyotime | C0038 |
| Trypsin EDTA(0.25%) | ThermoFisher Scientific | 25200056 |
| DMEM high | Gibco | 11865092 |
| 1640 culture-medium | Gibco | 11875119 |
| Ca ²⁺ -free medium | iCell | PriMed-icell-012-sf |

| | | |
|---|--------------------------|-------------|
| Phosphate buffered saline,PH=7.4 | Solarbio | P1003-2L |
| Lipofectamine™ 2000 Transfection Reagent | ThermoFisher Scientific | 11668019 |
| Triton TM X-100 | Solarbio | T8200 |
| CoraLite®594 F-Actin | Proteintech | PF00003 |
| 4% paraformaldehyde (PFA) | ThermoFisher | J61899.AP |
| Bovine Serum Albumin | Gibco | A5256701 |
| BCA protein concentration determination kit | Beyotime | P0010 |
| TRIzol™ Reagent | ThermoFisher Scientific | 15596018CN |
| OPTI-MEM | Gibco | 2898884 |
| SKF-96365 hydrochloride(10μM) | MedChemExpress | HY-100001 |
| ML204(0.5μM) | MedChemExpress | HY-12949 |
| Mibefradil dihydrochloride T(2.7μM) | MedChemExpress | HY-15553A |
| Amlodipine L(30μM) | MedChemExpress | HY-B0317 |
| PD173212 N (10μM) | MedChemExpress | HY-103318 |
| Histamine (10μM) | MedChemExpress | HY-B1204 |
| 2-APB(100μM) | MedChemExpress | HY-W009724 |
| Penicillin-Streptomycin | Gibco | 15070063 |
| Total Protein Extraction (TPE(TM)) | Sangon | C006225 |
| Ca ²⁺ -Rhod-2, AM, (5μM,for Live-cell) | YEASEN | 40776ES72 |
| Ionomycin calcium (2μM) | MedChemExpress | HY-13434A |
| Ca ⁺⁺ Mg ⁺⁺ -ATPase Assay Kit | BIOSS | AK266 |
| Hank's Balanced Salt Solution | ThermoFisher Scientific | 14175095 |
| Total Protein Extraction | Sangon | C006225 |
| NucGreen™ Dead 488 ReadyProbes™ | ThermoFisher Scientific | S7020 |
| PCR | Novoprotein | E035 |
| Experimental models: Cell lines | | |
| C57 | Vitalriver | N/A |
| MSCs | BeijingMaternityHospital | N/A |
| MC3TE-e1 | Peking Union Cell Bank | N/A |
| C2C12 | Peking Union Cell Bank | N/A |
| iBMSCs | icell | N/A |
| NIH-3T3 | Peking Union Cell Bank | N/A |
| ESF | Peking Union Cell Bank | N/A |
| PC12 | Peking Union Cell Bank | N/A |
| HUVEC | Peking Union Cell Bank | N/A |
| 293T | Peking Union Cell Bank | N/A |
| PX459 | Peking Union Cell Bank | N/A |
| SiRNASequence (5'-3') | | |
| si-TRPC4AP-homo GAGGAAACUUACCCAAGAATT | This Study | Gene Pharma |
| si-TRPM7-homo AACAUCAAGACGAACAGAAUUAGUUG | This Study | Gene Pharma |

| | | |
|--|-------------------------|-------------|
| si-TRPM4-homo AUGUGU AACUGAACAUGGCTT | This Study | Gene Pharma |
| si-TRPV2-homo GCUUCCUUCUGAUCUACUUTT-3 | This Study | Gene Pharma |
| si-ORAI1-homo CGUGCACAAUCUCAACUCGTT | This Study | Gene Pharma |
| si-ORAI2-homo AACCGUUUGGUUCA AUGAGG | This Study | Gene Pharma |
| si-STIM1-homo GGCUCUGGAUACAGUCUCTT | This Study | Gene Pharma |
| si-TRPC1-homo GCGACAAGGGUGACUAUUATT | This Study | Gene Pharma |
| si-TRPC4-homo GGUCAGACUUGAACAGGCATT | This Study | Gene Pharma |
| si-TRPC5-homo UACCAAGGUUAUGAAAUGACUUGGG | This Study | Gene Pharma |
| Others | | |
| 96-well cell culture plate | Corning | 3548 |
| 6-well cell culture plate | Corning | 3335 |
| Nunc™ EasYFlask™ Cell Culture Flasks-T25 | ThermoFisher Scientific | 156367 |
| Nunc™ EasYFlask™ Cell Culture Flasks-T75 | ThermoFisher Scientific | 156499 |
| Glass bottom cell culture dish (20mm) | NEST | 801001 |
| Centrifuge tube-15ml | NEST | 601001 |
| Centrifuge tube-50ml- | NEST | 602002 |