ISCI, Volume *15*

Supplemental Information

The Plasticity of Mesenchymal Stem Cells in Regulating Surface HLA-I

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Transparent Methods

Ethics Statement

All experiments were approved by the institutional biosafety committee, institutional animal committee, and institutional review board of Zhejiang University. (No. ZJU2015-004)

Cell culture and Differentiation

The male H1 human ES cell line (ESC) was obtained from WiCell Corporation. Madison, WI, USA (http://www.wicell.org), and was maintained in the undifferentiated state in a feeder-free system. MSCs derived from ESC (hESC-MSCs) were directly differentiated, cultured, and identified as described in our previous study(Wang et al., 2017). In addition, the hESC-MSCs was normally cultured in standard conditions (L-DMEM within 10%FBS). However, for the serum deprivation experiment, the cells were seeded 10000 cells per cm^2 and cultured under standard conditions over night. The following day, cells were rinsed with PBS and further cultured in L-DMEM with 5%FBS or L-DMEM without FBS for 48h, respectively.

Cell stimulation

The hESC-MSCs were stimulated with 100U/mL IFN-γ(PeproTech, Cat.300-02) in MSC culture media (L-DMEM+10%FBS) for 0 days, 1 day, 2 days, and 3 days, and then directly used for the following experiment(Figure S6A). The sample was marked as Day0, Day1, Day2, and Day3. As for the pulsative IFN-γ stimulation, the hESC-MSCs medium was transferred between medium within and without 100U/mL IFN-γ (Figure S6B).

One-way mixed lymphocyte culture

Human PBMCs were prepared from freshly collected, heparinized whole blood samples from donors through Ficoll-Paque PLUS (GE, Cat.17-1440-03). Briefly, whole human blood was diluted 1:2 with PBS and added into the Ficoll solution at 4:3(v/v) ratio. After centrifugation and washing, PBMCs were stained with CFDA SE (Beytime, Cat.C1031) to label the cell proliferation used as responder cells. The hESC-MSCs were then treated with 25μg/mL Mitomycin C used as stimulator cells. 10^4 stimulator cells and 10^5 responder cells were co-cultured into a 96-well round-bottom plate (Corning) for 5 days. PBMCs proliferation was then analyzed with the fluorescence intensity by flow cytometry.

Reconstitution of humanized mice

The B-NDG mice (NOD-scid IL2 receptor gamma null mice) were purchased from Biocytogen

Jiangsu Co., Ltd (Jiangsu, China) at 5 weeks of age. Human $CD34^+$ cells were extracted from the donor blood through Ficoll-Paque PLUS (GE, Cat.17-1440-03) which is supplied by Bone Marrow Transplantation Center, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China. Humanized mice were generated as previously described(Pearson et al., 2008). Briefly, the B-NDG mice were first treated with sublethal irradiation (1.8 Gy), then 5×10^5 human $CD34^+$ cells were intravenously injected into the mice 5 hours later. The $CD34^+$ cells were isolated purified from human PBMCs using a MACS separation system with anti-human $CD34^+$ antibody (Miltenyi Biotec, Cat. 130-046-702). After 6 weeks, the mice were delivered to do the following experiment.

Delayed-type hypersensitivity

Humanized mice were injected subcutaneously with 200 μL 1×10^6 DiI (Beyotime, Cat.C1036) pre-stained hESC-MSCs (Day0, Day1, and Day3) at two sites on the back of the mice (priming). At 13 days after post-implantation, the mice were detected with immunofluorescence through In Vivo Imaging System (PE, IVIS Spectrum). 1×10^6 challenged hESC-MSCs (Day0, Day1, and Day3) in 20μL of PBS were injected into one hind footpad of humanized mice subcutaneously (challenge). Another 20μL of PBS were injected into the other hind footpad of humanized mice subcutaneously as the internal negative control. Evaluation of the responses was performed as previously described(Liu et al., 2012): [footpad swelling (mm)]=[footpad thickness of hESC-MSCs injected footpad (mm)]-[footpad thickness of PBS injected footpad (mm)].

H&E staining of the footpad

At 24 hours post-challenge, humanized mice footpads of all groups were harvested and fixed in 4% paraformaldehyde. After decalcification in 10% EDTA at 4℃ for 4 weeks, footpads were cross-sectioned at the root of toes before paraffin embedding. Sections of 4μm thickness were made and stained with H&E. Images were captured under the slide scanning machine (Pannoramic MIDI, 3DHISTECH Ltd., Budapest, Hungray).

Transfection of small interfering RNAs

The siRNA sequences used in this study are as follows:siRNA-DNM2 (forward: 5'-GCAACCUGGUGGACUCAUATT-3', reverse: 5'-UAUGAGUCCACCAGGUUGCTT-3'); si-RHOA (forward: 5'-CCAGAAGUCAAGCAUUUCUTT-3', reverse: 5'-

AGAAAUGCUUGACUUCUGGTT-3'); si-Negative Control (forward: 5'-UUCUCCGAACGUGUCACGUTT-3', reverse: 5'-ACGUGACACGUUCGGAGAATT-3'). Double-stranded siRNAs were synthesized by GenePharma. The reaction contained 100nM siRNAs, and transfections were performed using Lipofectamine^{TM}2000 Transfection Reagent (Invitrogen, Cat.11668019) . after 6 hours, the transfected cells were then refreshed with MSC medium.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from hESC-MSCs by lysis in TRIzol (Takara, Shigo, Japan, Cat. #9109). Rever-Tra Ace qPCR RT Master Mix was applied during the reverse transcription process (Toyobo, Osaka, Japan, Cat. #FSQ-201). qRT-PCR was performed utilizing Brilliant SYBR Green QPCR Master Mix (Takara, Cat. # RR420A) with a LightCycler apparatus (480II, Roche, Mannheim, Germany). The amplification efficiencies of primer pairs were validated to enable quantitative comparison of gene expression. All primer sequences (Invitrogen) were designed using Primer 5.0 software (Table S1). Each qRT-PCR was performed three times on at least three different experimental replicates, and results were normalized to those obtained with the endogenous reference gene (GAPDH).

Western blot assay

Cellular protein was extracted with RIPA lysis buffer (Solarbio, Beijing, China, Cat. #R0010), and the total protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA, Cat. #23225). The 20 μg extracted cellular protein was loaded on 10% (w/v) SDS-PAGE-denaturing gels. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and blocked in 5% (w/v) bovine serum albumin (BSA, Sangon Biotech, Shanghai, China, Cat. #9048-4b-8) for 1h at room temperature. The membrane was incubated overnight at 4 °C with mouse anti-HLA-I (1:1000; HLA-ABC, Novus, Cat.NB100-64775), or rabbit anti-GAPDH (1:1000; Beyotime, Cat. #AG019) antibody. After washing in Tris-buffered saline with Tween-20 (TBST), the horseradish peroxidase (HRP) secondary antibodies (HRP-labeled goat anti-mouse IgG (1:1000; Beyotime, Cat. #A0216) and goat anti-rabbit IgG antibody, peroxidase-conjugated (1:1000; EMD Millipore, Cat. #AP132P)) was diluted in 5% (w/v) BSA solution and incubated accordingly with the membrane for 1 h at room temperature (RT). The excessive secondary antibody was washed off by TBST, and a

chemiluminescent signal was generated by the ECL Imaging Kit (Thermo Fisher Scientific, Waltham, MA, USA, Cat. #32209).

Flow cytometry

Cells were harvested by trypsinization (0.05% (w/v); Life Technologies, Cat. #15400-054) for 2 min in a 37℃ incubator, and the cell pellet was re-suspended in PBS to a titer of 10^5/10 μL. The cell suspension was incubated with 10μL anti-HLA-I (1:30; HLA-ABC, Novus, Cat.NB100-64775) on ice for 30min. Cells were washed with PBS completely and incubated with 40μL anti-mouse Alexa Fluor 488(1:250; Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Invitrogen, Cat. A-21202) on ice for 15min. Cells were washed with PBS completely and re-suspended in 1% (w/v) paraformaldehyde. Samples were run on the FC500MPL flow cytometer (Beckman Coulter, Brea, CA, USA) and the data were analyzed by FlowJo vX.0.7 software (FlowJo LLC, Ashland, OR, USA).

RNA extraction and preparation

Total RNA containing small RNA was extracted from IFN-γ-treated hESC-MSC samples using the Trizol reagent (Invitrogen) and purified with mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to manufacturer's protocol. The purity and concentration of RNA were determined from OD260/280 readings using the spectrophotometer (NanoDrop ND-1000). RNA integrity was determined by 1% formaldehyde denaturing gel electrophoresis. Only RNA extracts with RNA integrity number values >6 were used for further study.

Fabrication of DNA microarray

CapitalBio Technology Human LncRNA Array v4 was designed with four identical arrays per slide (4 x 180K format), with each array containing probes interrogating about 41,000 human lncRNAs and about 34,000 human mRNAs. Those lncRNA and mRNA target sequences were merged from multiple databases, 23898 from GENCODE/ENSEMBL, 14353 from Human LincRNA Catalog(Orom et al., 2010), 7760 from RefSeq, 5627 from UCSC, 13701 from NRED (ncRNA Expression Database), 21488 from LNCipedia, 1038 from H-InvDB, 3019 from LncRNAs-a (Enhancer-like), 1053 from Antisense lncRNA pipeline, 407 Hox ncRNAs, 962 UCRs, and 848 from Chen Ruisheng lab (Institute of Biophysics, Chinese Academy of Science). Each RNA was detected by probes twice. The array also contains 4974 Agilent control probes.

Microarray imaging and data analysis

The lncRNA+mRNA array data were analyzed for data summarization, normalization, and quality control using the GeneSpring software V13.0 (Agilent). To select the differentially expressed genes, we used threshold values of ≥2 and ≤−2-fold change and a Benjamini-Hochberg corrected p-value of 0.05. The data was Log2 transformed and median centered by genes using the Adjust Data function of CLUSTER 3.0 software, then further analyzed with hierarchical clustering with average linkage. Finally, we selected mRNAs with differentiated expression to perform short time series expression miner (STEM) analysis(Ernst and Bar-Joseph, 2006). Genes classified in eight model profiles were further analyzed with DAVID.

Flow cytometry analysis to assay endocytosis rate

To detect the HLA-I endocytosis rate of hESC-MSCs, cells were harvested by trypsinization (0.05% (w/v); Life Technologies, Cat. #15400-054) for 2 min, and the cell pellet was re-suspended in PBS to a titer of 10^6/100μL. The cell suspension was incubated with 100μL of HLA-ABC (1:25, Novus, Cat.NB100-64775) for 30min on ice. Cells were washed with PBS completely and resuspended in 50μL. The sample was divided into 5 parts: 0h, 1h, 2h, 5h, 7h. Cells were added in 1000μL cell medium and put back into the incubator to initiate endocytosis (37℃,5% CO2). At a certain time point, cells were put on the ice to stop the endocytosis process. Cell samples were harvested and centrifuged at 200× g, 5min, before resuspension in 40μL anti-mouse Alexa Fluor 488(1:250; Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Invitrogen, Cat. A-21202) on ice for 15min. Cells were then washed with PBS and re-suspended in 1% (w/v) paraformaldehyde. Samples were run on an FC500MPL flow cytometer (Beckman Coulter, Brea, CA, USA) and the data were analyzed by FlowJo vX.0.7 software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis

All data were expressed as mean ± SEM unless otherwise stated. All experiments in vitro were repeated independently at least twice in addition to the triplicates applied in each experiment. Statistical results were analyzed and bar charts were constructed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Statistical results were considered significant when the p-value was less than 0.05. Two-tailed Student's t-test was used to compare two groups at the same time point. One-way ANOVA including the Tukey-Kramer post hoc test was

used to compare multiple time point groups.

Data and software available

The accession number for microarray data reported in this paper is GEO Database: GSE122091.

Reference

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Supplemental Figure Titles and Legends

Figure S1. The HLA-I surface expression on hBMSCs under IFN-γ treatment, related to Figure 1.

(A) HLA-I surface expression on hBMSCs (red line) within IFN-γ treatment for 0 day, 1 day, 2 days and 3 days were compared by flow cytometry analysis. Blue line demarcates isotype control.

(B) The flow cytometry results quantified analysis of HLA-I surface expression level on hBMSCs with IFN-γ treatment for 0 day, 1 day, 2 days and 3 days by mean fluorescence index. Data are shown as means±SEM.

Figure S2. HLA-I levels are lower in hESC-MSCs than hFF, related to Figure 1.

(A) qRT-PCR expression analysis of HLA-I related genes (include HLA-A, HLA-B, HLA-C, and B2M) on hESC-MSCs and hFF. RNA expression levels were normalized to the level of GAPDH expression. Data are shown as means±SEM.

(B) HLA-I expression in hESC-MSCs and hFF were compared by western blotting.

(C) HLA-I surface expression level on hESC-MSCs (red line) and hFF (blue line) were compared by flow cytometry analysis. Dashed lines demarcate isotype control.

(D) The flow cytometry results quantified analysis of HLA-I surface expression level on hESC-MSCs (red bar) and hFF (blue bar) with mean fluorescence index. Data are shown as means±SEM.

Figure S3. HLA-I levels are lower in hESC-MSCs at early passage than hESC-MSCs at late passage, related to Figure 1.

(A) hESC-MSCs growth curve at different passage(from passage0 to passage9).

(B) The flow cytometry results quantified analysis of HLA-I surface expression level on passage3 hESC-MSCs (red bar) and passage8 hESC-MSCs(blue bar) with mean fluorescence index. Data are shown as means±SEM.

Figure S4. HLA-I levels are higher in hESC-MSCs after the serum deprivation, related to Figure 1.

(A) HLA-I surface expression level on hESC-MSCs under different concentration of FBS treatment for 48h was compared by flow cytometry analysis. The blue lines demarcate isotype control.

(B) The flow cytometry results quantified analysis of HLA-I surface expression level on 10%FBS treated hESC-MSCs (red bar), 5%FBS treated hESC-MSCs(blue bar), and 0%FBS treated hESC-MSCs(green bar) with mean fluorescence index. Data are shown as means±SEM.

Figure S5. HLA-I levels in hESC-MSCs within pulsed IFN-γ treatment, related to Figure 1.

(A) HLA-I surface expression level on hESC-MSCs under pulsed IFN-γ treatment was compared by flow cytometry analysis. The blue lines demarcate isotype control.

(B) The flow cytometry results quantified analysis of HLA-I surface expression level on hESC-MSCs under pulsed IFN-γ treatment with mean fluorescence index. Data are shown as means±SEM.

Figure S6. Schematic diagram of the collection about hESC-MSCs under IFN-γ treatment, related to Figure 1 and Figure S5.

(A) The Schematic diagram of the collection about hESC-MSCs under continuous constant IFN-γ treatment.

(B) The Schematic diagram of h the collection about hESC-MSCs under pulsed IFN-γ treatment.

Table S1. The primers designed for qRT-PCR, related to Figure 1 and Figure S2.

Gene	Sequence(5'-3')
HLA-A-F	TCCTTGGAGCTGTGATCACT
HLA-A-R	AAGGGCAGGAACAACTCTTG
HLA-B-F	ATTACATCGCCCTGAACGAG
HLA-B-R	ATCTCCGCAGGGTAGAAACC
HLA-C-F	TCCTGGTTGTCCTAGCTGTC
HLA-C-R	CAGGCTTTACAAGTGATGAG
B2M-F	GATGAGTATGCCTGCCGTGTG
B2M-R	CAATCCAAATGCGGCATCT
GAPDH-F	TGACGCTGGGGCTGGCATTG
GAPDH-R	GGCTGGTGGTCCAGGGGTCT

Figure S1

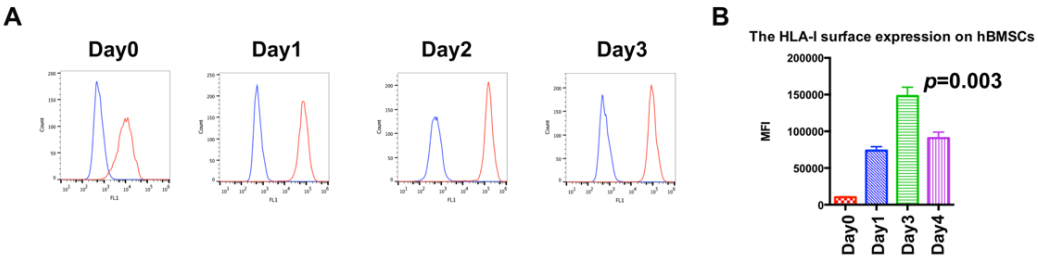

A
Day0
Day1
Day2
Day3
B
The HLA-I surface expression on hBMSCs
MFI
200000
150000
100000
50000
0
p=0.003
Day0
Day1
Day3
Day4

Figure S2

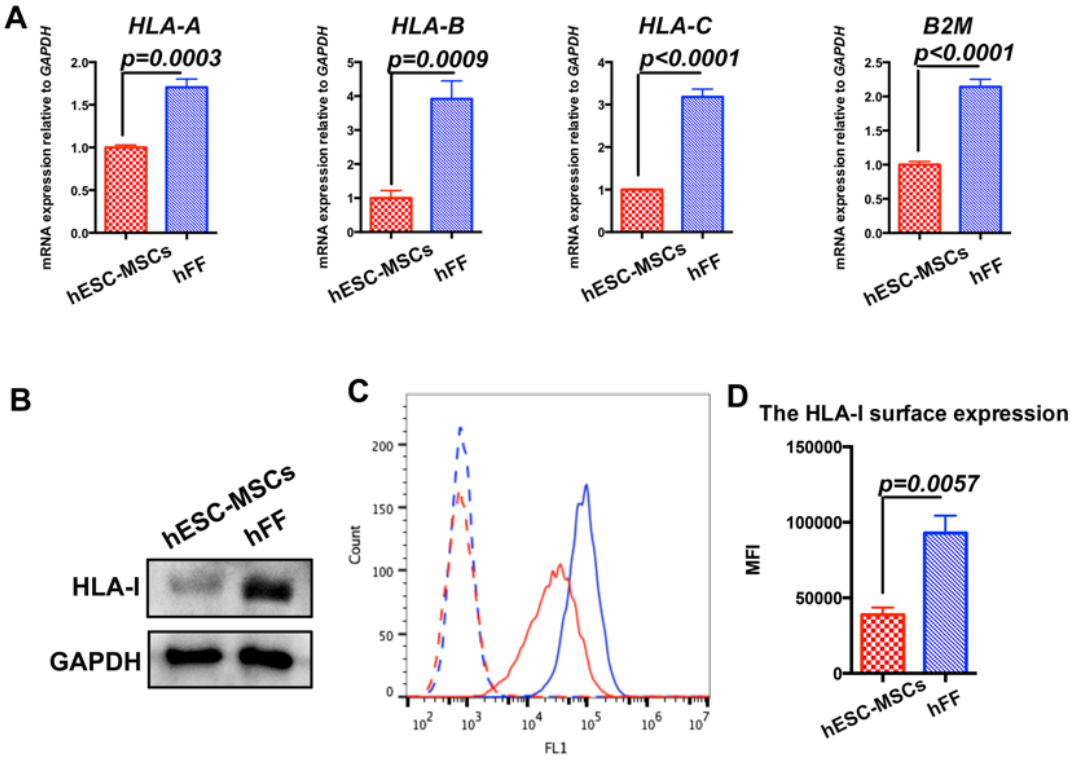

A
HLA-A
p=0.0003
HLA-B
p=0.0009
HLA-C
p<0.0001
B2M
p<0.0001
mRNA expression relative to GAPDH
hESC-MSCs
hFF
B
hESC-MSCs
hFF
HLA-I
GAPDH
C
Count
FL1
D
The HLA-I surface expression
MFI
p=0.0057

Figure S3

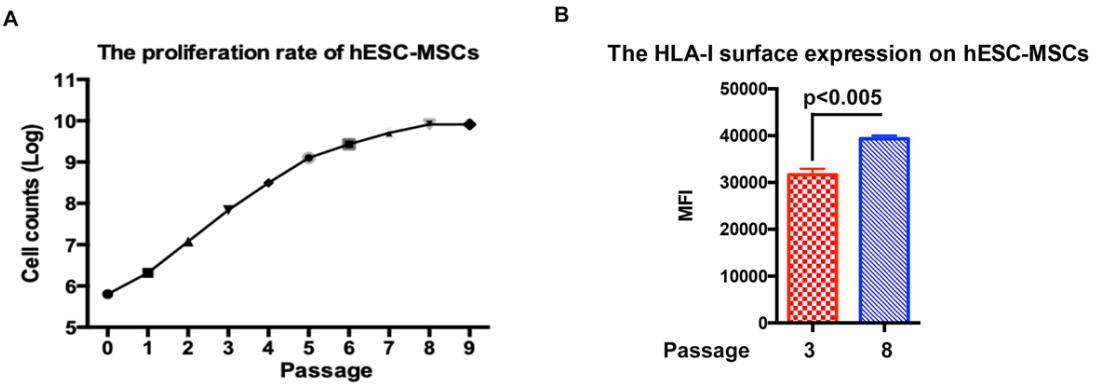

A
The proliferation rate of hESC-MSCs
Cell counts (Log)
11
10
9
8
7
6
5
0 1 2 3 4 5 6 7 8 9
Passage
B
The HLA-I surface expression on hESC-MSCs
p<0.005
MFI
50000
40000
30000
20000
10000
0
Passage
3
8

Figure S4

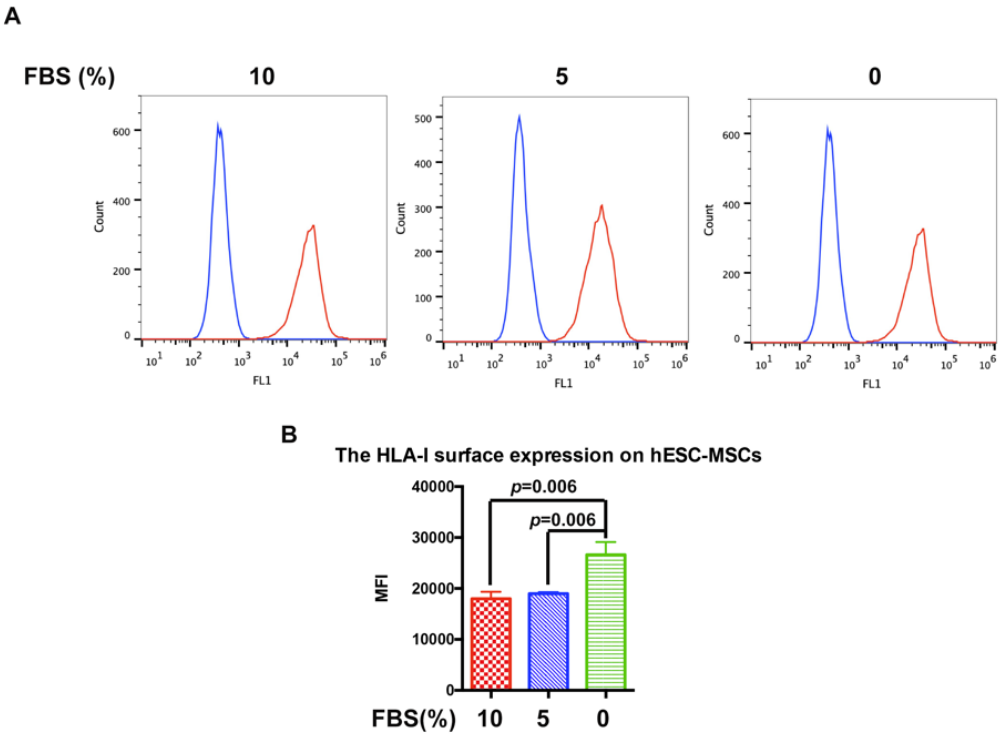

A
FBS (%)
10
5
0
Count
FL1
B
The HLA-I surface expression on hESC-MSCs
p=0.006
p=0.006
MFI
FBS(%) 10 5 0

Figure S5

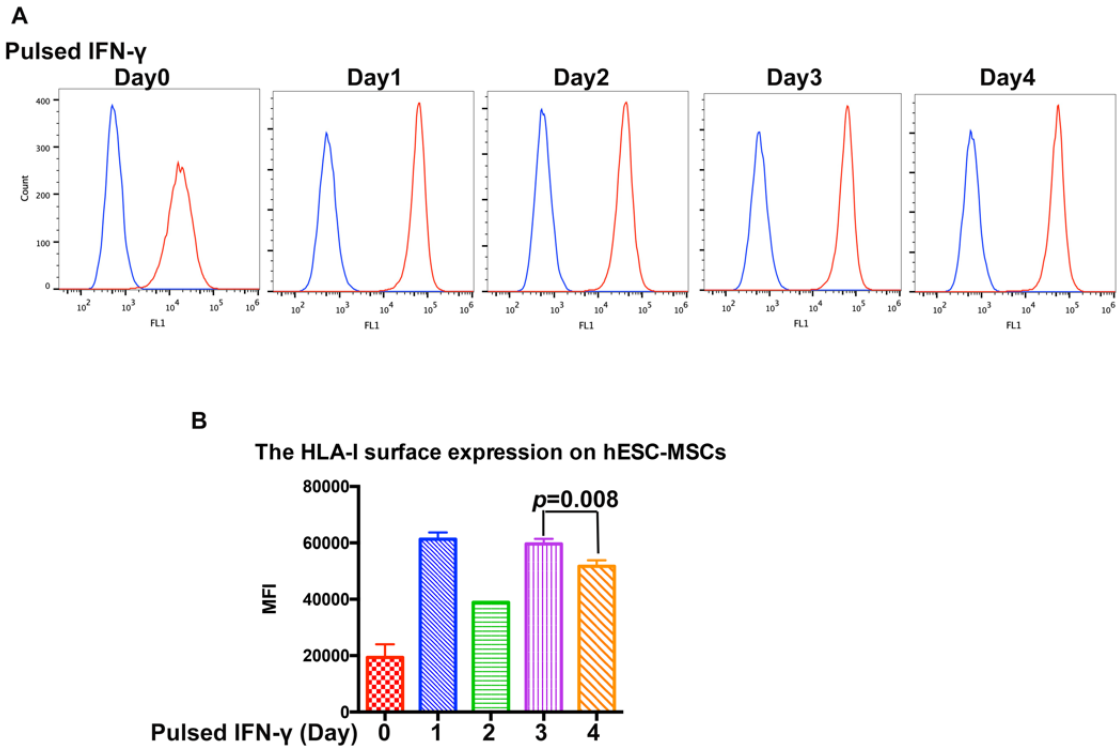
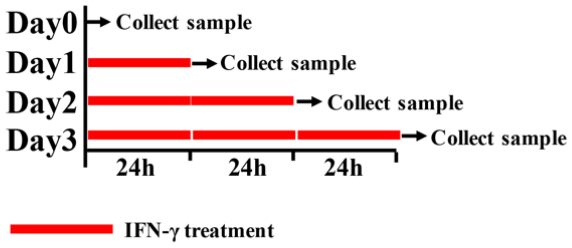
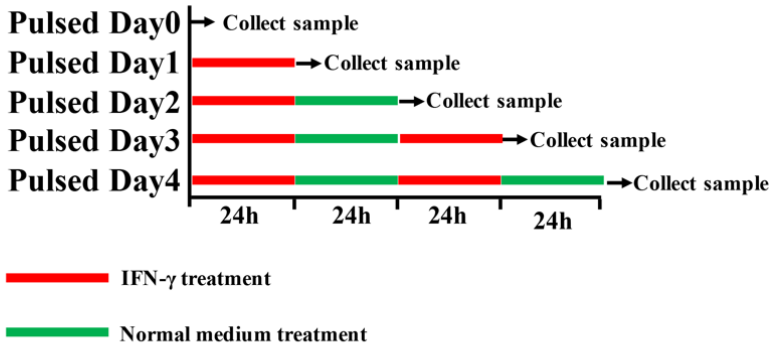

A
Pulsed IFN-γ
Day0
Day1
Day2
Day3
Day4
Count
FL1
B
The HLA-I surface expression on hESC-MSCs
MFI
p=0.008
80000
60000
40000
20000
0
Pulsed IFN-γ (Day) 0 1 2 3 4

Figure S6

A


Day0
Collect sample
Day1
Collect sample
Day2
Collect sample
Day3
Collect sample
24h
24h
24h
IFN-γ treatment

B


Pulsed Day0
Collect sample
Pulsed Day1
Collect sample
Pulsed Day2
Collect sample
Pulsed Day3
Collect sample
Pulsed Day4
Collect sample
24h
24h
24h
24h
IFN-γ treatment
Normal medium treatment