Supplementary Table 1. Antibodies used in FACS

Primary antibodies	Host	Cat. No.	Supplier
CD24-PE	Mouse	555428	BD Biosciences
CD29-APC	Mouse	559883	BD Biosciences
CD34-FITC	Mouse	555821	BD Biosciences
CD44-APC	Mouse	559942	BD Biosciences
CD45-FITC	Mouse	555482	BD Biosciences
CD73-PE	mouse	550257	BD Biosciences
CD90-APC	Mouse	559869	BD Biosciences
CD166-PE	Mouse	559263	BD Biosciences

Supplementary Table 2. Primary antibodies used in immunostaining

Primary antibodies	Host	Dilution	Cat. No.	Supplier
T	goat	1:200	AF2085	R&D Systems, Minneapolis, MN, USA
SOX2	Rabbit	1:500	ab97959	Abcam, Cambridge, UK
PAX6	Rabbit	1:300	ab195045	Abcam, Cambridge, UK
NESTIN	mouse	1:200	MAB5326	Millipore，Merck KGaA, Darmstadt, Germany
TBX6	goat	1:200	AF4744	R&D Systems, Minneapolis, MN, USA
TUBB3	Mouse	1:1000	mab1195	R&D Systems, Minneapolis, MN, USA
ISLET1	Rabbit	1:300	ab178400	Abcam, Cambridge, UK
HB9	Rabbit	1:100	81.5C10	DSHB, Iowa, USA
HOXC6	Rabbit	1:100	NBP2-54948	Novus Biologicals, Littleton, Colorado,USA
HRGFP	Rabbit	1:50	240142	Agilent Technologies,Cedar Creek, USA
OCN	Rabbit	1:100	MAB1419	R&D Systems, Minneapolis, MN, USA
OPG	Mouse	1:500	ABC463	Millipore，Massachusetts，USA
CD45	Mouse	1:50	12-0451-81	eBioscience，San Diego，USA

Supplementary Table 3. Primers for qPCR

Gene		Primers
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAAGATGGTGATGGGATTTC
T	Forward	TATGAGCCTCGAATCCACATAGT
	Reverse	CCTCGTTCTGATAAGCAGTCAC
SOX2	Forward	GGGAAATGGGAGGGGTGCAAAAGAGG
	Reverse	TTGCGTGAGTGTGGATGGGATTGGTG
CDX1	Forward	GGTGGCAGCGGTAAGACTC
	Reverse	TGTAACGGCTGTAATGAAACTCC
FGF17	Forward	CTGCTGATTCTCTGCTGTCAA
	Reverse	GTAGAGTTGGTACTCGCGGAT
NKX1-2	Forward	CCCTCCCACCACAAGATTTCT
	Reverse	GACCTCCGCCAAACTTTTCCT
SP5	Forward	GAAACAGTGCTCGGGTTTTC
	Reverse	TCAACGGGGTCTTCCTGTAG
WNT8A	Forward	GAACTGCCCTGAAAATGCTCT
	Reverse	TCGAAGTCACCCATGCTACAG
OCT4	Forward	GACAGGGGGAGGGGAGGAGCTAGG
	Reverse	CTTCCCTCCAACCAGTTGCCCCAAAC
NANOG	Forward	CAGCCCAGATTCTTCCACCAGTCCC
	Reverse	CGGAAGCGTTCCCAGTCGGGTTCACC

SOX1	Forward	CAGTACAGCCCCATCTCCAAC
	Reverse	GCGGGCAAGTACATGCTGA
SOX10	Forward	CCTCACAGATCGCCTACACC
	Reverse	CATATAGGAGAAGGCCGAGTAGA
SOX17	Forward	GTGGACCGCACGGAATTTG
	Reverse	GGAGATTCACACCGGAGTCA
ALP	Forward	AGCACTCCCACTTCATCTGGAA
	Reverse	GAGACCCAATAGGTAGTCCACATTG
COL1	Forward	CAGCCGCTTCACCTACAGC
	Reverse	TTTTGTATTCAATCACTGTCTTGCC
OC	Forward	CAGCGAGGTAGTGAAGAGA
	Reverse	GAAAGCCGATGTGGTCAG
RUNX2	Forward	AGAAGGCACAGACAGAAGCTTGA
	Reverse	AGGAATGCGCCCTAAATCACT
PPAR-γ	Forward	AGGAGCAGAGCAAAGAGG
	Reverse	TTGGTCGTTCAAGTCAAGAT
ADIPOQ	Forward	AACATGCCCATTCGCTTTACC
	Reverse	TAGGCAAAGTAGTACAGCCCA
FABP4	Forward	ACTGGGCCAGGAATTTGACG
	Reverse	CTCGTGGAAGTGACGCCTT
LPL	Forward	ACAAGAGAGAACCAGACTCCAA
	Reverse	AGGGTAGTTAAACTCCTCCTCC

SOX9	Forward	AGCGAACGCACATCAAGAC
	Reverse	GCTGTAGTGTGGGAGGTTGAA
AGN	Forward	TGCATTCCACGAAGCTAACCTT
	Reverse	GACGCCTCGCCTTCTTGAA
COL2	Forward	GGCAATAGCAGGTTCACGTACA
	Reverse	CGATAACAGTCTTGCCCCACTT
GAPDH mouse	Forward	TCAATGAAGGGGTCGTTGAT
	Reverse	CGTCCCGTAGACAAAATGGT
IL-4 mouse	Forward	GGTGTTCTTCGTTGCTGTGA
	Reverse	TCTCGAATGTACCAGGAGCC
IL-6 mouse	Forward	TGGTACTCCAGAAGACCAGAGG
	Reverse	AACGATGATGCACTTGCAGA
IL-10 mouse	Forward	AGACACCTTGGTCTTGGAGC
	Reverse	TTTGAATTCCCTGGGTGAGA
IL-17 mouse	Forward	AGAATTCATGTGGTGGTCCAG
	Reverse	ACTACCTCAACCGTTCCACG
TNF-α mouse	Forward	GGTCTGGGCCATAGAACTGA
	Reverse	CAGCCTCTTCTCATTCCTGC
IFN-γ mouse	Forward	TGAGCTCATTGAATGCTTGG
	Reverse	AGGCCATCAGCAACAACATA
HOXA10	Forward	CTCGCCCATAGACCTGTGG
	Reverse	GTTCTGCGCGAAAGAGCAC

HOXA11	Forward	TGCCAAGTTGTACTTACTACGTC
	Reverse	GTTGGAGGAGTAGGAGTATGTCA
HOXB2	Forward	CCTAGCCTACAGGGTTCTCTC
	Reverse	CACAGAGCGTACTGGTGAAAAA
HOXB9	Forward	ACAAAGAGAGGCCGGATCAAA
	Reverse	TGCCCTGCTCCTTATTCATTTTC
HOXC10	Forward	ACATGCCCTCGCAATGTAACT
	Reverse	GAGAGGTAGGACGGATAGGTG
HOXC11	Forward	ATGTTTAACTCGGTCAACCTGG
	Reverse	GCATGTAGTAAGTGCAACTGGG
HOXC12	Forward	ATGGGCGAGCATAATCTCCTG
	Reverse	CGTGGGTAGGACAGCGAAG
HOXD9	Forward	GGACTCGCTTATAGGCCATGA
	Reverse	GCAAAACTACACGAGGCGAA
HOXD10	Forward	AGACAGTTGGACAGATCCGAA
	Reverse	CGAAATGAGTTTGTTGCGCTTAT
HOXD11	Forward	TCGACCAGTTCTACGAGGCA
	Reverse	AAAAACTCGCGTTCCAGTTCG
ANGPT1	Forward	GCCATCTCCGACTTCATGTT
	Reverse	CTGCAGAGAGATGCTCCACA
SCF	Forward	AATCCTCTCGTCAAAACTGAAGG
	Reverse	CCATCTCGCTTATCCAACAATGA

CXCL12	Forward	TGGGCTCCTACTGTAAGGGTT
	Reverse	TTGACCCGAAGCTAAAGTGG
TPO	Forward	ATGTCCTGTGCCTTGGTCTC
	Reverse	CTTCGTGACTCCCATGTCCT
FLT3L	Forward	CTATGCATCCTCTGGCTGGT
	Reverse	CTGCTGCTTGTGGAGCACT
OPN	Forward	AGATGGGTCAGGGTTTAGCC
	Reverse	CATCACCTGTGCCATACCAG
VCAM-1	Forward	GTCTCCAATCTGAGCAGCAA
	Reverse	TGAGGATGGAAGATTCTGGA

Supplementary Figure legends

Figure S1. Treatment with TGFβ1, Chir99021, and bFGF could efficiently induced hESC lines including H1 and H9 to differentiate to SOX2+/T+ NMP. Scale bar: 100 μm.

Figure S2. The NMP differentiation efficiency (the percentage of SOX2+/T+ cells) of hiPSC was monitored by FACS from day 0 to day 3. The data represent mean ± SEM of three independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001, and n.s. is non-significant.

Figure S3. mRNA expression of specific genes of hiPSC-NMP was analyzed by qRT-PCR.

A. The expression of pluripotency genes and neuromesoderm-specific genes were detected during NMP differentiation from day 0 to day 5.

B. The mRNA levels of SOX1 (neural epithelium), SOX10 (neural crest), and SOX17 (endoderm) were detected during NMP differentiation from day 0 to day 5.

C. Lumbosacral HOX gene expression of undifferentiated hPSC and NMP (day 2) were detected when treated with lower (2 ng/ml) or higher (5-10 ng/ml) concentration of TGFβ1. The data represent mean ± SEM of three independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001, and n.s. is non-significant.

Figure S4. Characterization of NMP-MSC derived from H1 and H9

A. The sample gating strategy during FACS was provided. Cell debris (SSC-A vs. FSC-A), clumps or doublets (FSC-H vs FSC-A; SSC-H vs SSC-A), and dead cells (PI positive cells) were excluded, and the live cell gate is further analyzed for their CD

antigens expression.

B. FACS analysis for detection of typical MSC surface markers.

C. The osteogenic, adipogenic, and chondrogenic differentiation potential of NMP-MSC were verified by Alizarin Red S staining, oil red O staining, and toluidine blue staining, respectively. Scale bar: 100 μm.

Figure S5. Tumor formation assay of NMP-MSC. No tumor was observed in NMP-MSC group. However, control hiPSC generated tumors in NOG mice with a high efficiency (n=5).

Figure S6. Pearson correlations of pair-wise comparisons were calculated for all expressed genes between NMP-MSC and BMSC.

Figure S7. The enriched mRNA expression in hPSC, hPSC-NMP and NMP-MSC were analyzed in RNA-Seq results.

Figure S8. The HOX gene expression pattern was analyzed by RNA-Seq (A) and qRT-PCR (B). The data represent mean ± SEM of three independent experiments.

Figure S9. Immunoregulatory effect of NMP-MSC derived from H1 and H9 on CD3+ T cells *in vitro* were detected. The data represent mean ± SEM of four independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001, and n.s. is non-significant.

Figure S10. Comparision of immunoregulatory effect between NMP-MSC at P20 and BMSC at P5. The data represent mean ± SEM of four independent experiments. *p ＜0.05, **p＜0.01, ***p＜0.001, and n.s. is non-significant.

Figure S11. IDO blockage assay was performed and the results revealed that the immunoregulatory ability of NMP-MSC was greatly compromised after treating with

with 1-MT. The data represent mean ± SEM of four independent experiments. *p＜0.05, **p＜0.01, ***p＜0.001, and n.s. is non-significant.

Figure S12. qRT-PCR and ELISA were applied to examine the mRNA and protein levels of pro-inflammatory mediators (IFN-γ, TNF-α, IL-17, and IL-6) in the inflamed ears respectively.

A. The mRNA levels of TNF-α, IFN-γ, IL-17, and IL-6 were examined by qRT-PCR. mRNA samples were extracted from the inflamed ears of each group at 24 hours post-injection (n=3). The data are expressed as mean±SEM. *p＜0.05, **p＜0.01, ***p＜0.001, and n.s. is non-significant.

B. The concentrations of TNF-α, IFN-γ, IL-17, and IL-6 in homogenates of inflamed ears obtained from each group 24 hours post-injection were determined by ELISA (n=4). The data are expressed as mean±SEM. *p＜0.05, **p＜0.01, ***p＜0.001, and n.s. is non-significant.

Figure S1

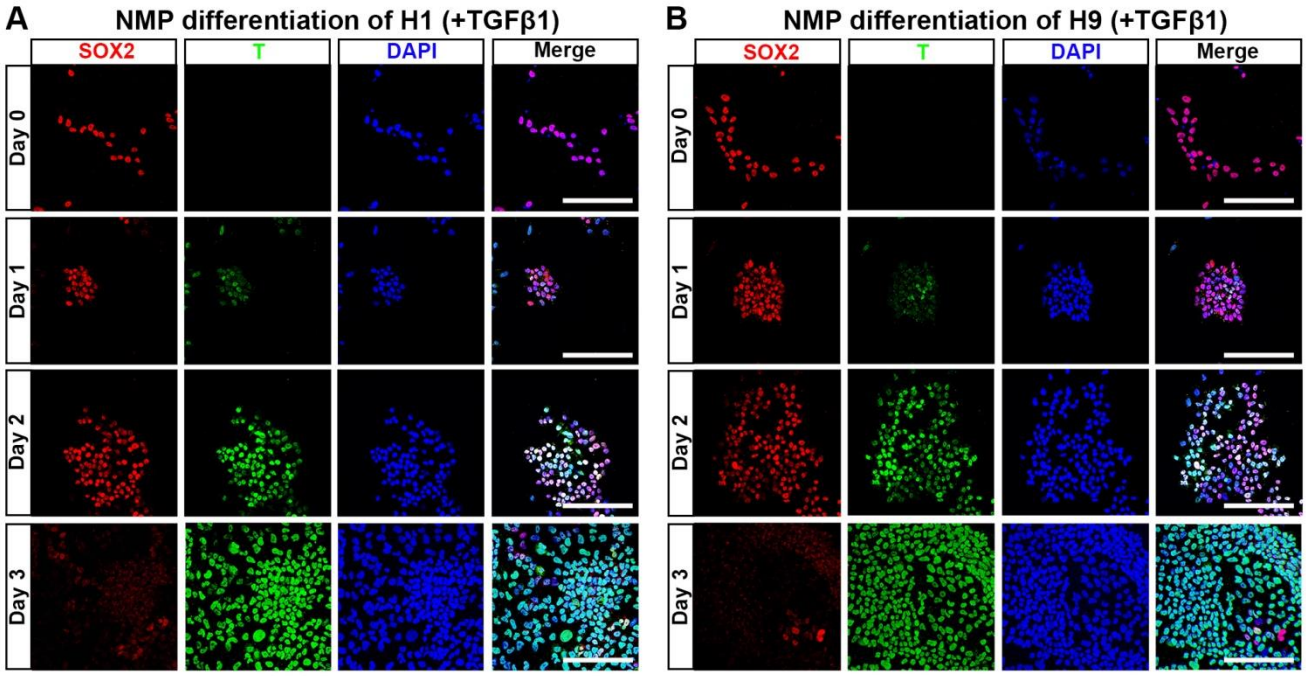

A
NMP differentiation of H1 (+TGFβ1)
SOX2
T
DAPI
Merge
Day 0
Day 1
Day 2
Day 3
B
NMP differentiation of H9 (+TGFβ1)
SOX2
T
DAPI
Merge
Day 0
Day 1
Day 2
Day 3

Figure S2

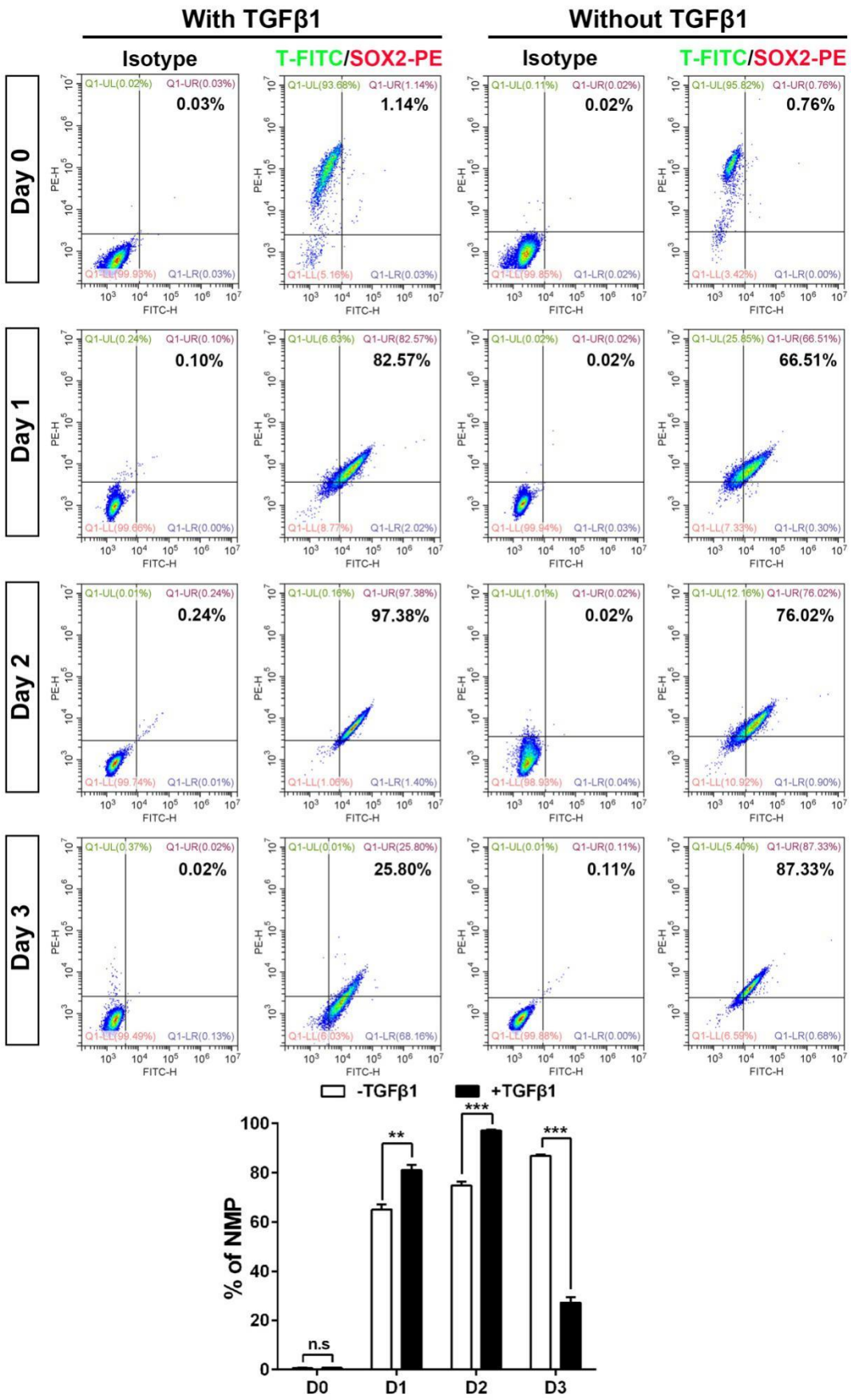

With TGFβ1
Without TGFβ1
Isotype
T-FITC/SOX2-PE
Day 0
Day 1
Day 2
Day 3
0.03%
1.14%
0.02%
0.76%
0.10%
82.57%
0.02%
66.51%
0.24%
97.38%
0.02%
76.02%
0.02%
25.80%
0.11%
87.33%
PE-H
FITC-H
-TGFβ1
+TGFβ1
% of NMP
n.s
**
***
***
D0
D1
D2
D3

Figure S3

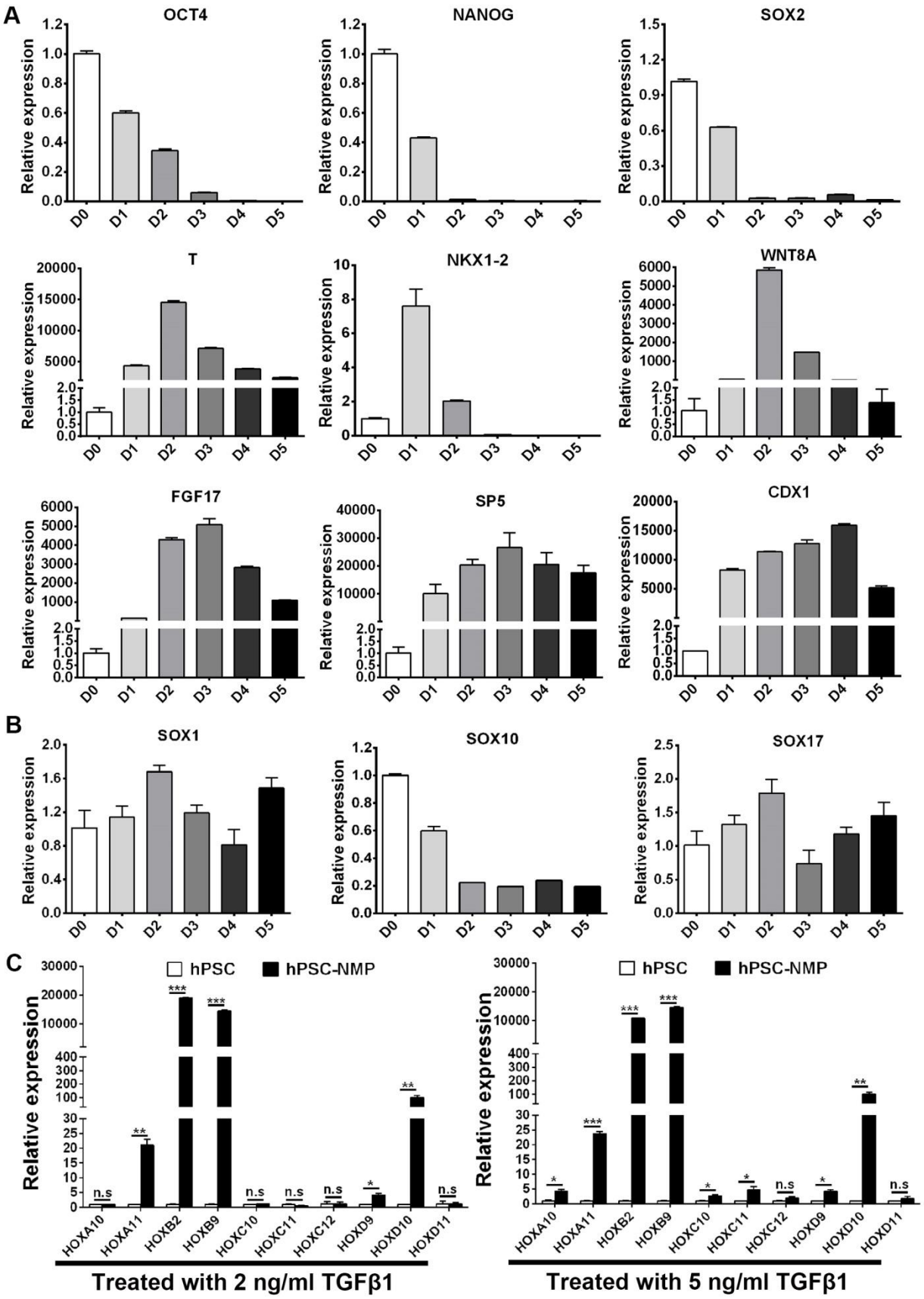

A
OCT4
NANOG
SOX2
T
NKX1-2
WNT8A
FGF17
SP5
CDX1
Relative expression
D0
D1
D2
D3
D4
D5
B
SOX1
SOX10
SOX17
C
hPSC
hPSC-NMP
n.s
HOXA10
HOXA11
HOXB2
HOXB9
HOXC10
HOXC11
HOXC12
HOXD9
HOXD10
HOXD11
Treated with 2 ng/ml TGFβ1
Treated with 5 ng/ml TGFβ1

Figure S4

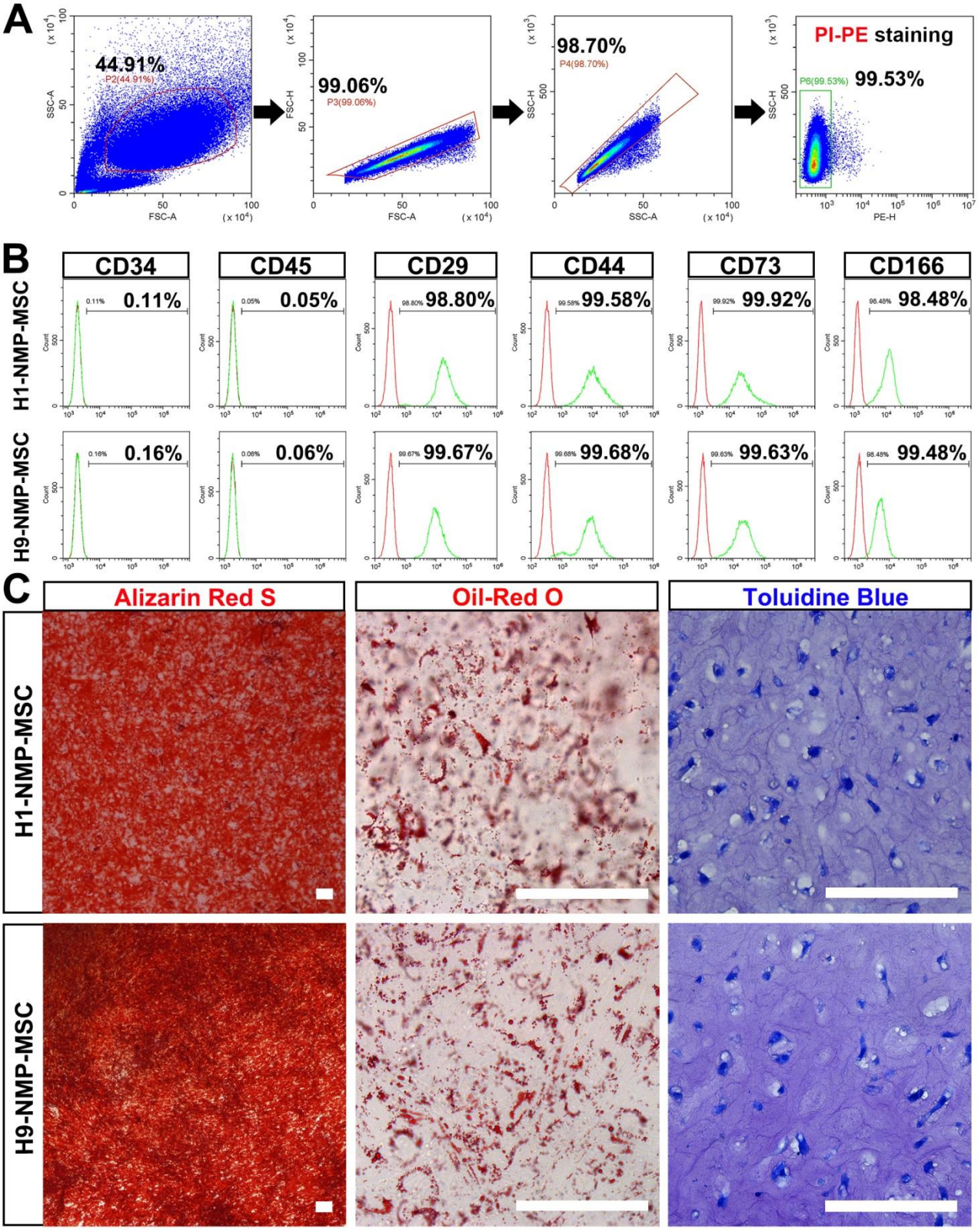
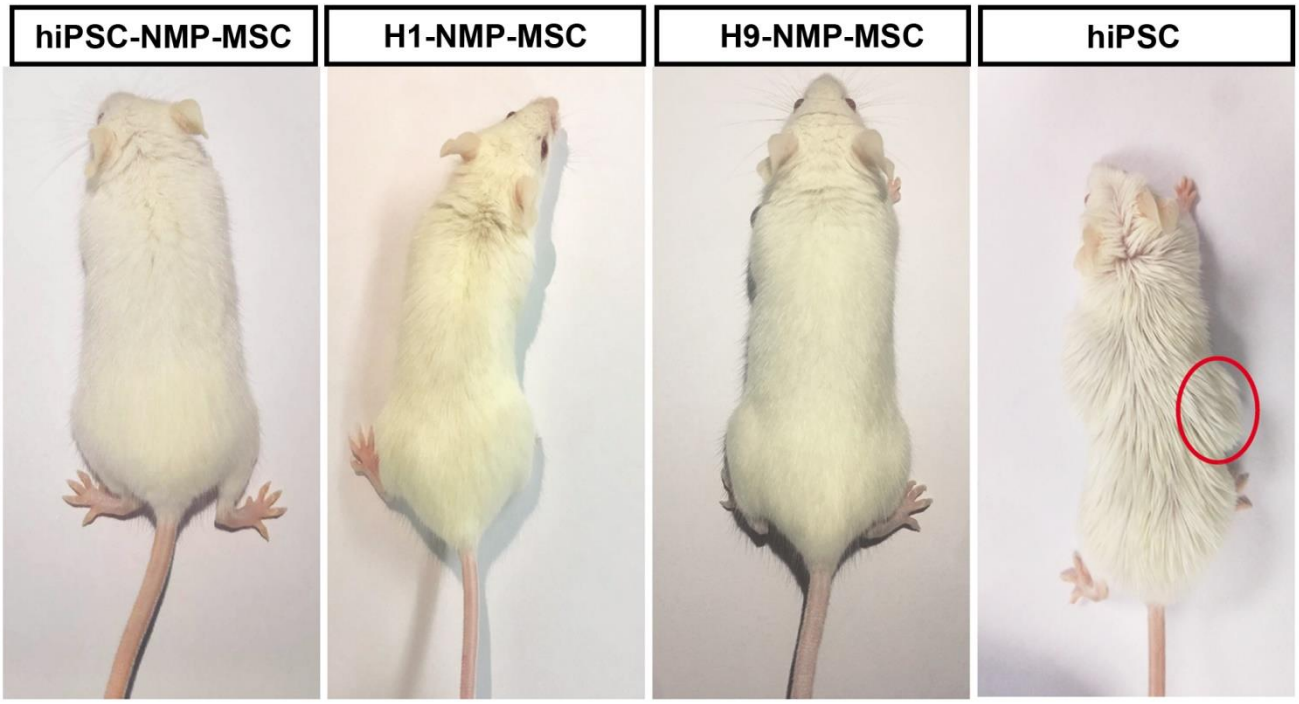

A
44.91%
P2(44.91%)
SSC-A
FSC-A
99.06%
P3(99.06%)
FSC-H
FSC-A
98.70%
P4(98.70%)
SSC-H
SSC-A
PI-PE staining
P6(99.53%)
99.53%
SSC-H
PE-H
B
CD34
CD45
CD29
CD44
CD73
CD166
H1-NMP-MSC
0.11%
0.05%
98.80%
99.58%
99.92%
98.48%
H9-NMP-MSC
0.16%
0.06%
99.67%
99.68%
99.63%
99.48%
C
Alizarin Red S
Oil-Red O
Toluidine Blue
H1-NMP-MSC
H9-NMP-MSC

Figure S5


hiPSC-NMP-MSC
H1-NMP-MSC
H9-NMP-MSC
hiPSC

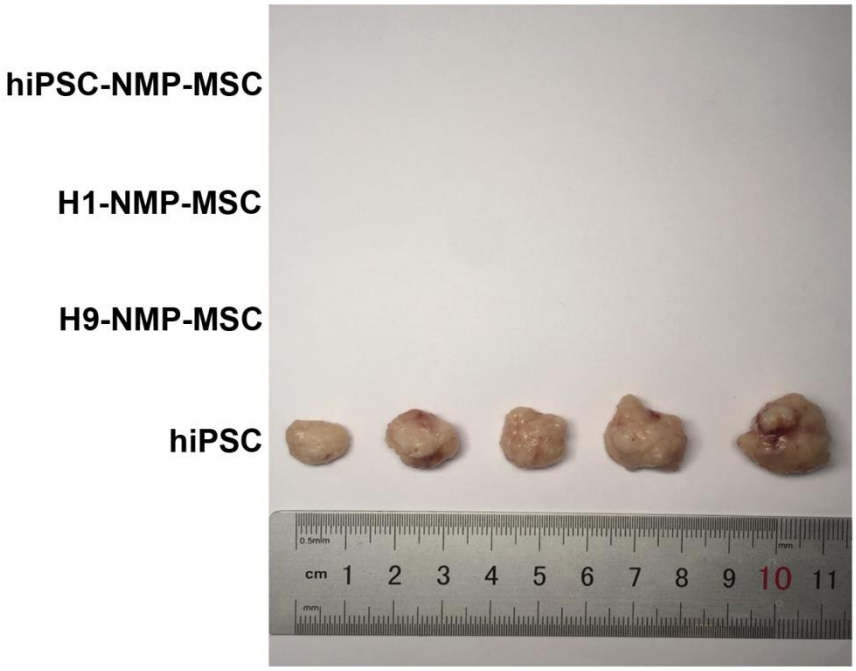

hiPSC-NMP-MSC
H1-NMP-MSC
H9-NMP-MSC
hiPSC
cm 1 2 3 4 5 6 7 8 9 10 11

Figure S6

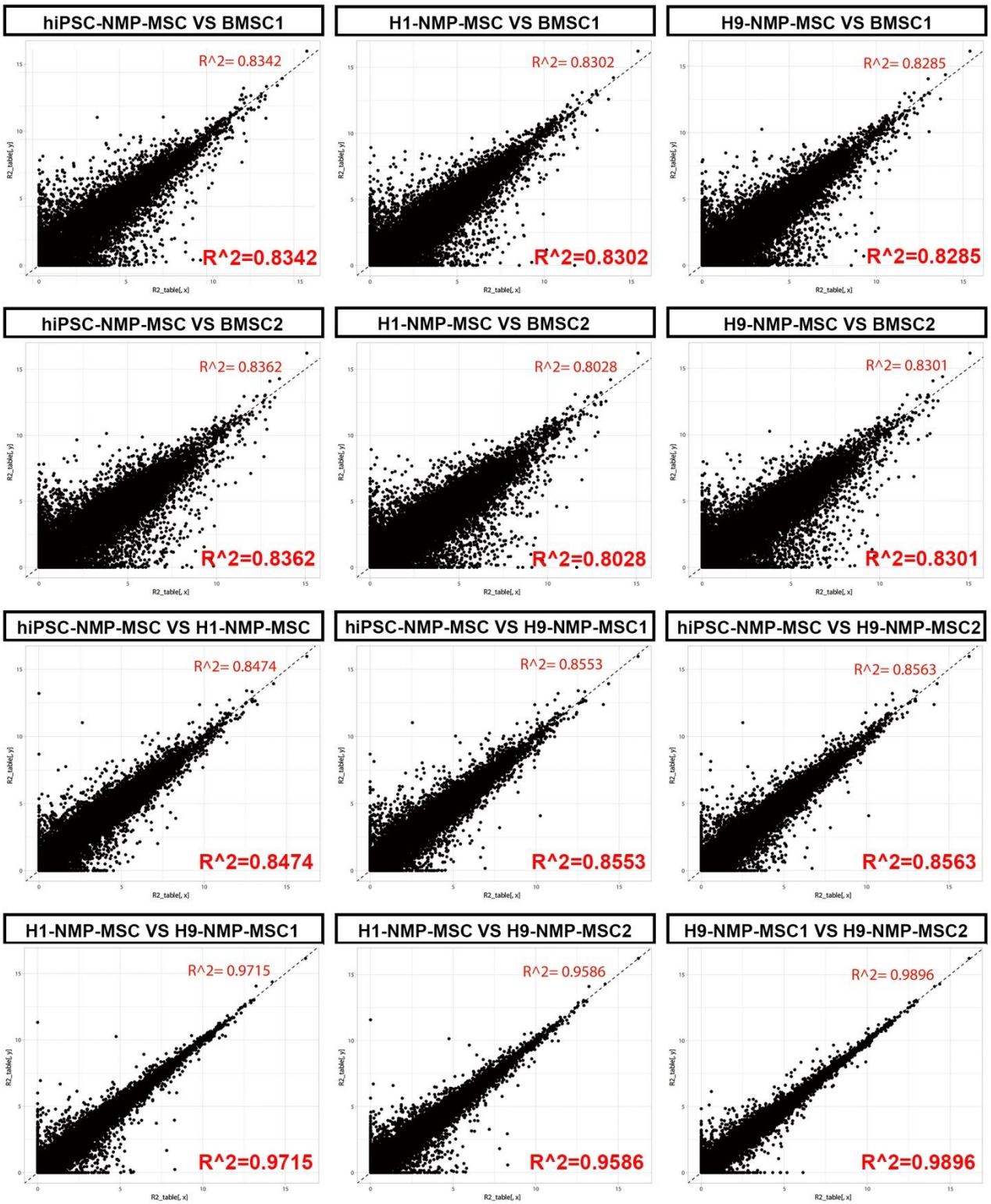

hiPSC-NMP-MSC VS BMSC1
R^2= 0.8342
R^2=0.8342
H1-NMP-MSC VS BMSC1
R^2= 0.8302
R^2=0.8302
H9-NMP-MSC VS BMSC1
R^2= 0.8285
R^2=0.8285
hiPSC-NMP-MSC VS BMSC2
R^2= 0.8362
R^2=0.8362
H1-NMP-MSC VS BMSC2
R^2= 0.8028
R^2=0.8028
H9-NMP-MSC VS BMSC2
R^2= 0.8301
R^2=0.8301
hiPSC-NMP-MSC VS H1-NMP-MSC
R^2= 0.8474
R^2=0.8474
hiPSC-NMP-MSC VS H9-NMP-MSC1
R^2= 0.8553
R^2=0.8553
hiPSC-NMP-MSC VS H9-NMP-MSC2
R^2= 0.8563
R^2=0.8563
H1-NMP-MSC VS H9-NMP-MSC1
R^2= 0.9715
R^2=0.9715
H1-NMP-MSC VS H9-NMP-MSC2
R^2= 0.9586
R^2=0.9586
H9-NMP-MSC1 VS H9-NMP-MSC2
R^2= 0.9896
R^2=0.9896
R2_table[, y]
R2_table[, x]

Figure S7

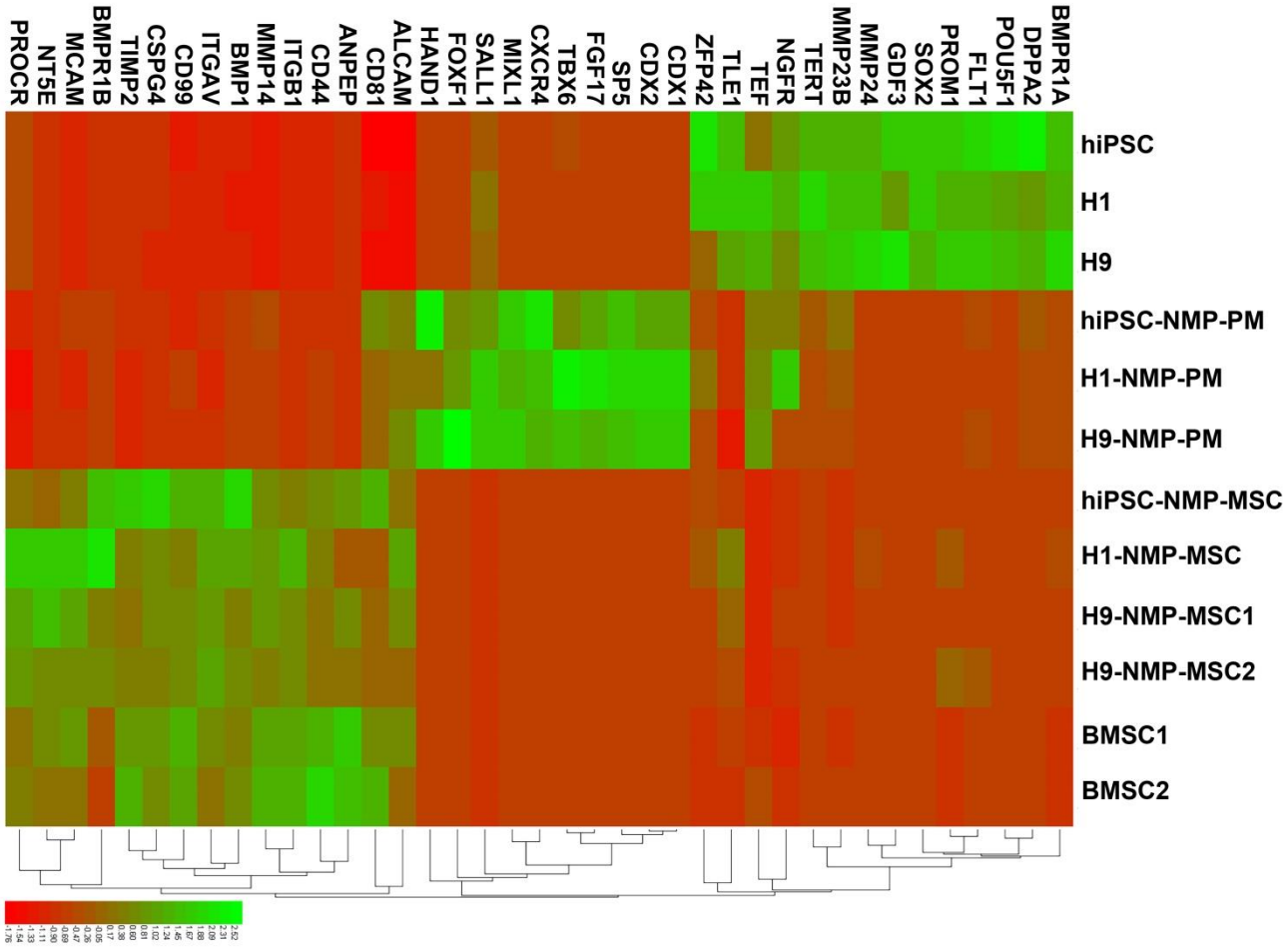
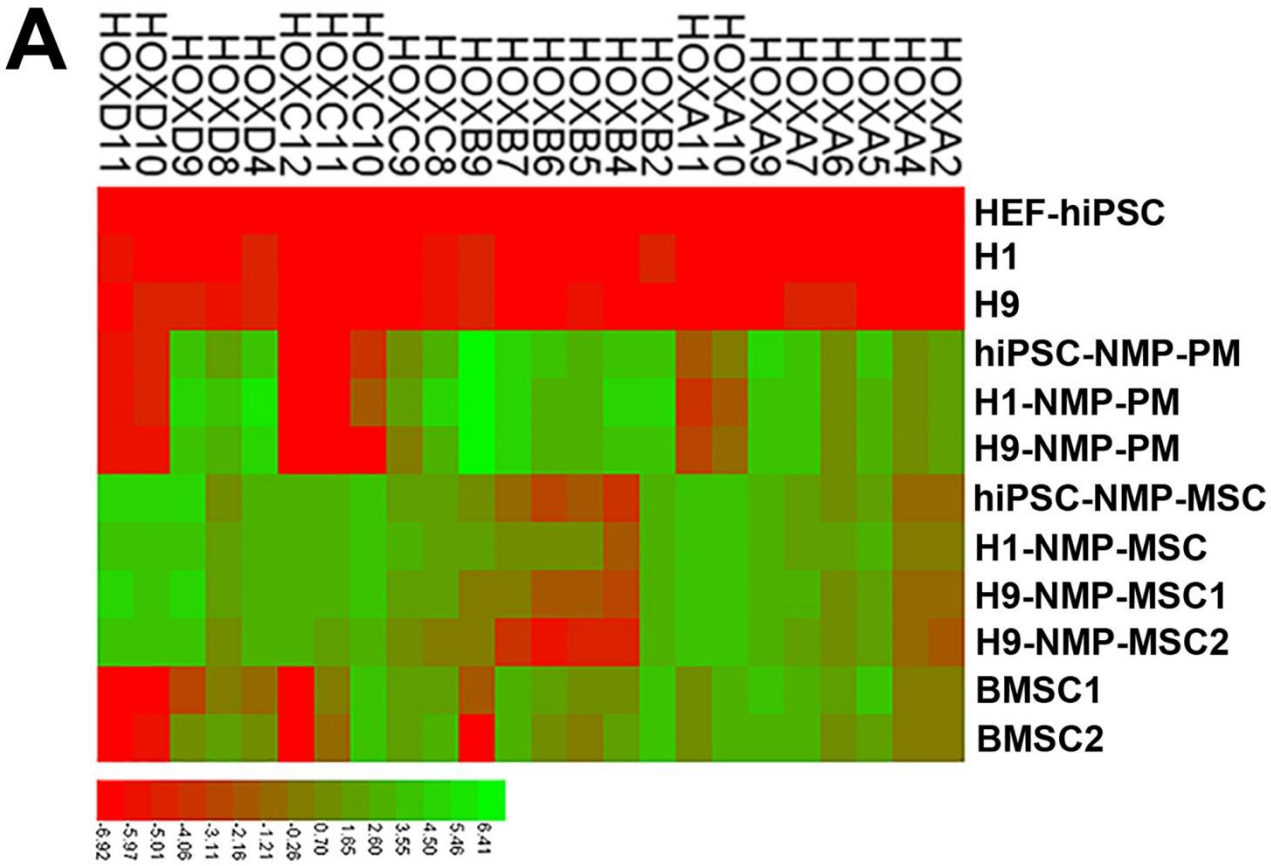

BMPR1A
DPPA2
POU5F1
FLT1
PROM1
SOX2
GDF3
MMP24
MMP23B
TERT
NGFR
TEF
TLE1
ZFP42
CDX1
CDX2
SP5
FGF17
TBX6
CXCR4
MIXL1
SALL1
FOXF1
HAND1
ALCAM
CD81
ANPEP
CD44
ITGB1
MMP14
BMP1
ITGAV
CD99
CSPG4
TIMP2
BMPR1B
MCAM
NT5E
PROCR
hiPSC
H1
H9
hiPSC-NMP-PM
H1-NMP-PM
H9-NMP-PM
hiPSC-NMP-MSC
H1-NMP-MSC
H9-NMP-MSC1
H9-NMP-MSC2
BMSC1
BMSC2

Figure S8


A
HOXD11
HOXD10
HOXD9
HOXD8
HOXD4
HOXC12
HOXC11
HOXC10
HOXC9
HOXC8
HOXB9
HOXB7
HOXB6
HOXB5
HOXB4
HOXB2
HOXA11
HOXA10
HOXA9
HOXA7
HOXA6
HOXA5
HOXA4
HOXA2
HEF-hiPSC
H1
H9
hiPSC-NMP-PM
H1-NMP-PM
H9-NMP-PM
hiPSC-NMP-MSC
H1-NMP-MSC
H9-NMP-MSC1
H9-NMP-MSC2
BMSC1
BMSC2
-6.92
-5.97
-5.01
-4.06
-3.11
-2.16
-1.21
-0.26
0.70
1.65
2.60
3.55
4.50
5.46
6.41

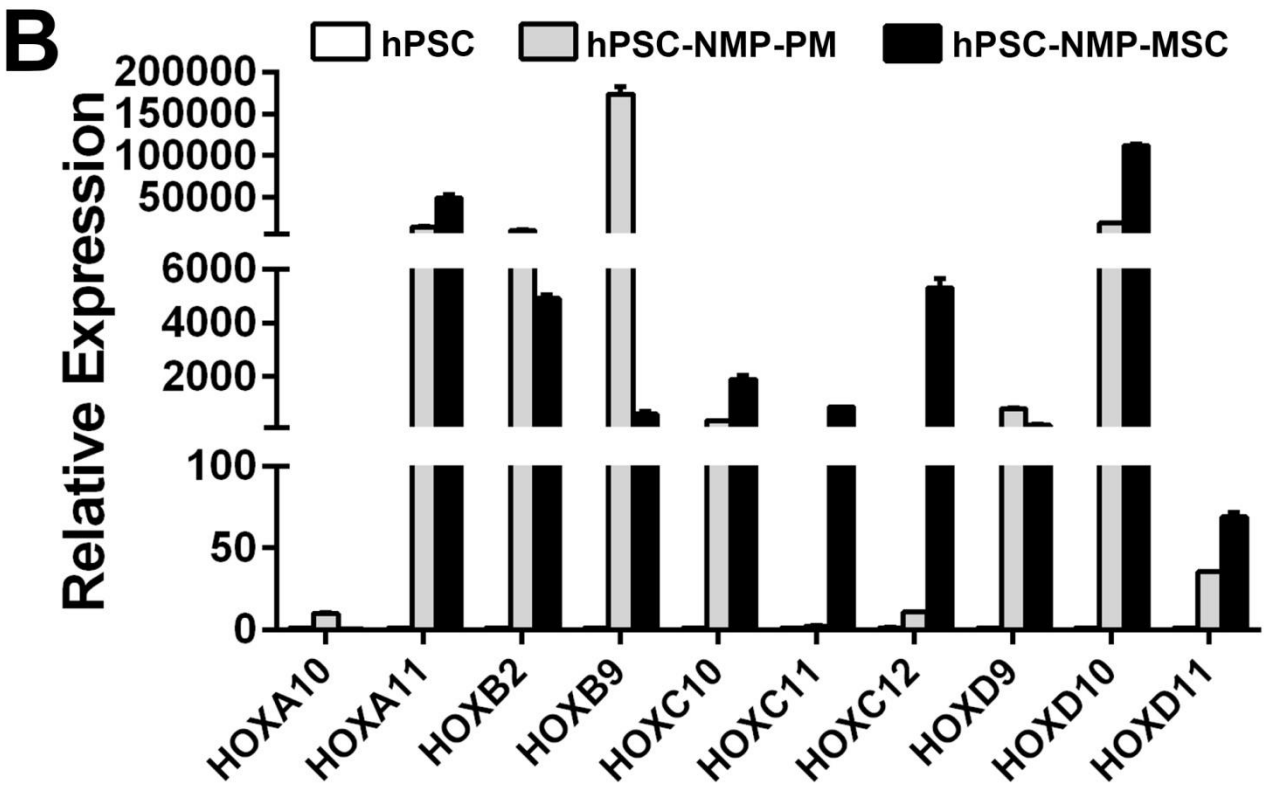

B
hPSC
hPSC-NMP-PM
hPSC-NMP-MSC
Relative Expression
200000
150000
100000
50000
6000
4000
2000
100
50
0
HOXA10
HOXA11
HOXB2
HOXB9
HOXC10
HOXC11
HOXC12
HOXD9
HOXD10
HOXD11

Figure S9

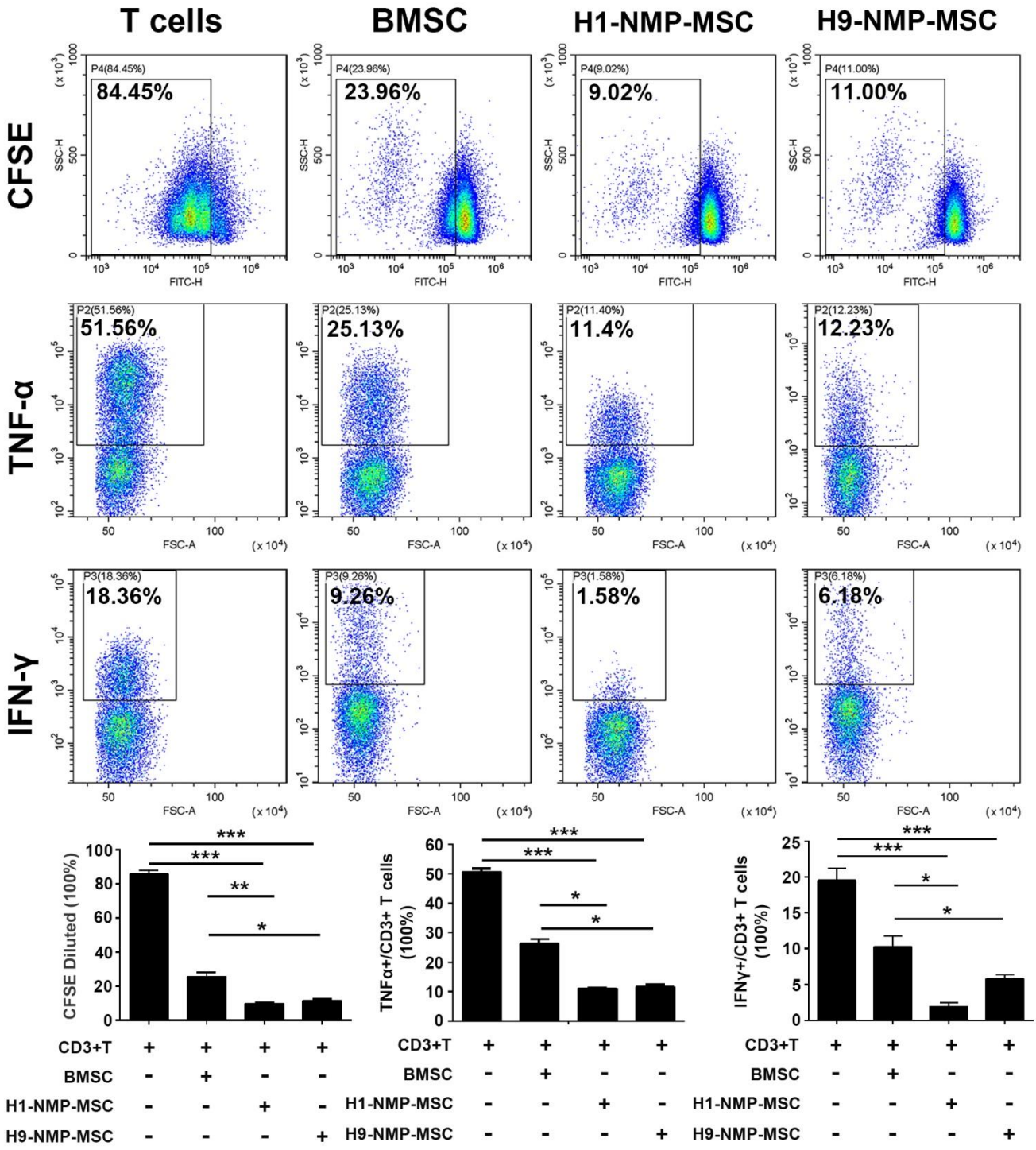

T cells
BMSC
H1-NMP-MSC
H9-NMP-MSC
CFSE
TNF-α
IFN-γ
P4(84.45%)
84.45%
P4(23.96%)
23.96%
P4(9.02%)
9.02%
P4(11.00%)
11.00%
P2(51.56%)
51.56%
P2(25.13%)
25.13%
P2(11.40%)
11.4%
P2(12.23%)
12.23%
P3(18.36%)
18.36%
P3(9.26%)
9.26%
P3(1.58%)
1.58%
P3(6.18%)
6.18%
SSC-H
FITC-H
FSC-A
CFSE Diluted (100%)
TNFα+/CD3+ T cells (100%)
IFNγ+/CD3+ T cells (100%)
CD3+T
BMSC
H1-NMP-MSC
H9-NMP-MSC

Figure S10

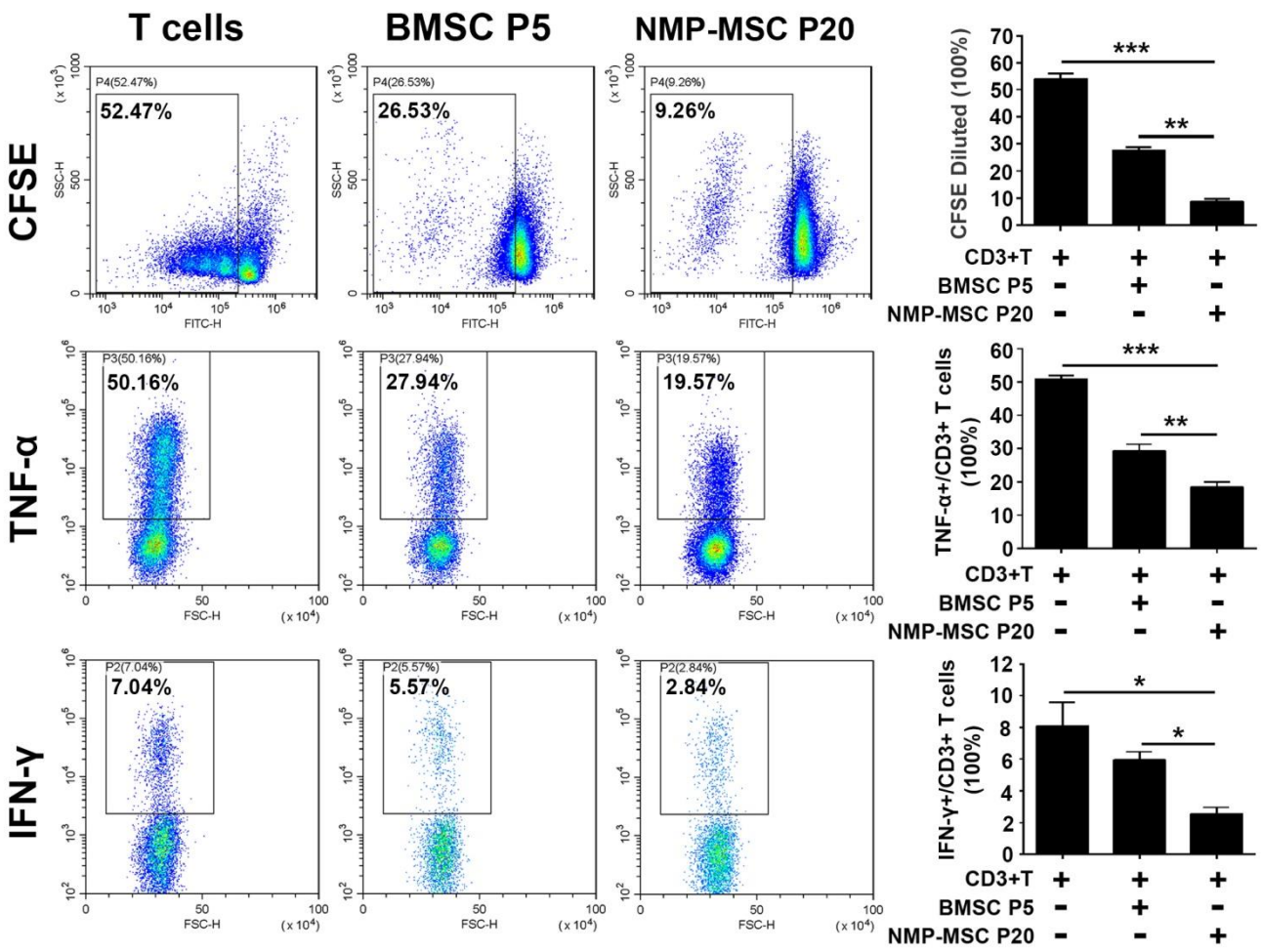

T cells
BMSC P5
NMP-MSC P20
CFSE
TNF-α
IFN-γ
P4(52.47%)
52.47%
P4(26.53%)
26.53%
P4(9.26%)
9.26%
P3(50.16%)
50.16%
P3(27.94%)
27.94%
P3(19.57%)
19.57%
P2(7.04%)
7.04%
P2(5.57%)
5.57%
P2(2.84%)
2.84%
SSC-H
FITC-H
FSC-H
CFSE Diluted (100%)
TNF-α+/CD3+ T cells (100%)
IFN-γ+/CD3+ T cells (100%)
***
**
*
CD3+T
BMSC P5
NMP-MSC P20

Figure S11

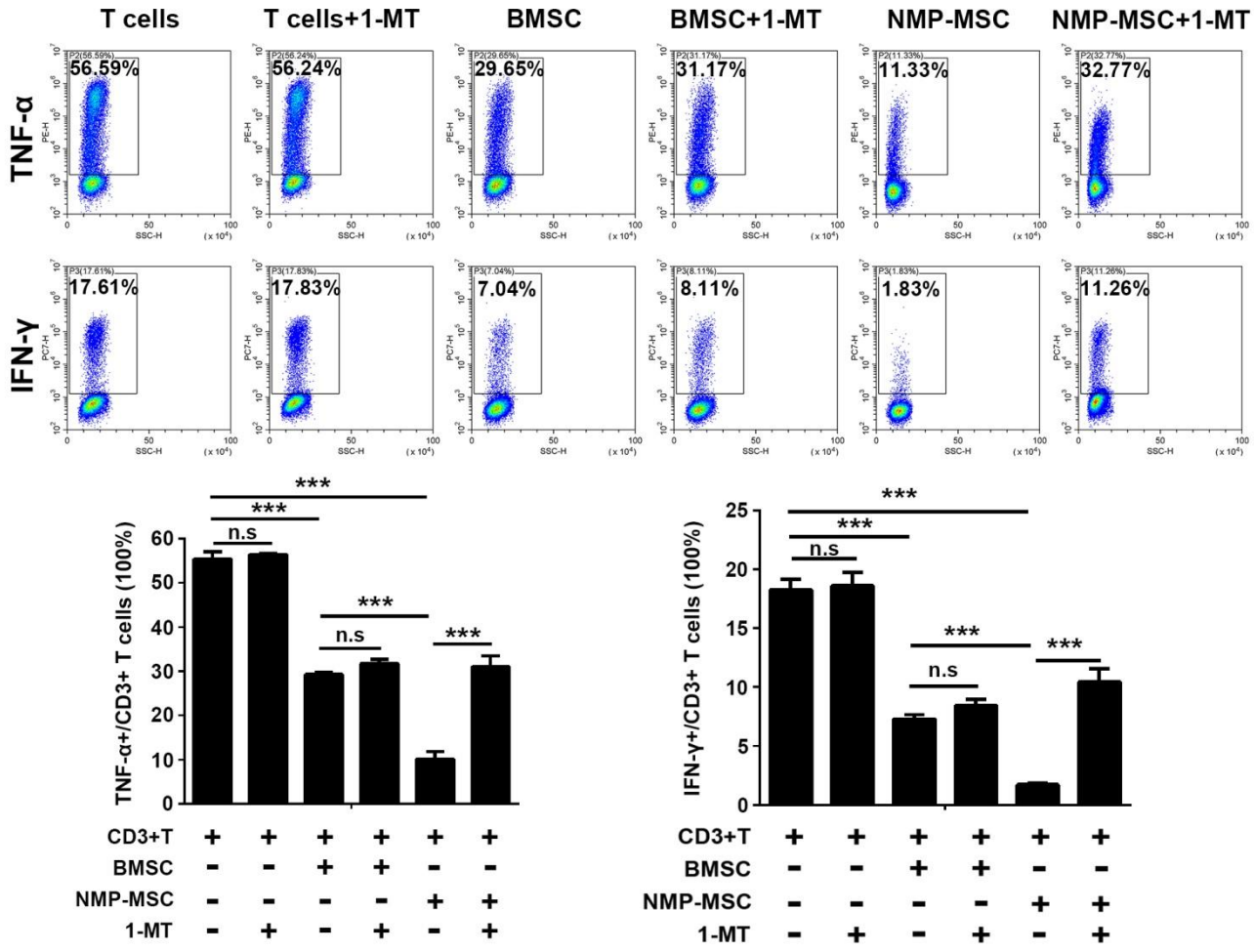
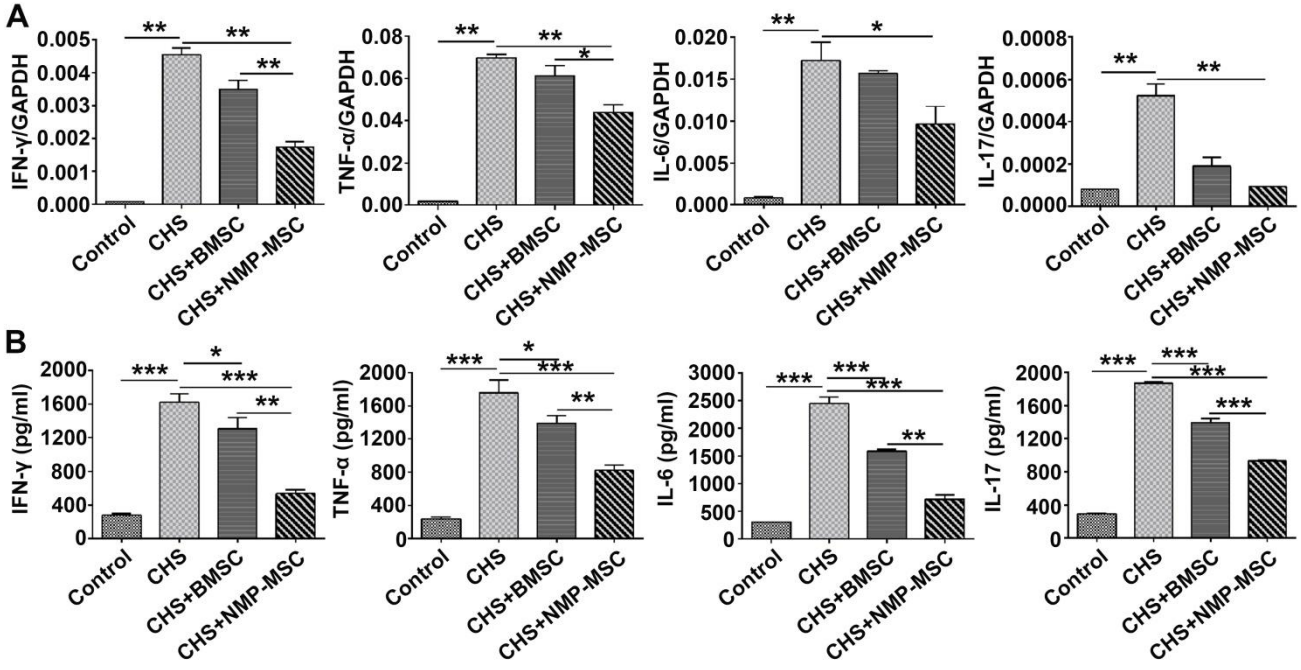

T cells
T cells+1-MT
BMSC
BMSC+1-MT
NMP-MSC
NMP-MSC+1-MT
TNF-α
IFN-γ
56.59%
56.24%
29.65%
31.17%
11.33%
32.77%
17.61%
17.83%
7.04%
8.11%
1.83%
11.26%
TNF-α+/CD3+ T cells (100%)
IFN-γ+/CD3+ T cells (100%)
n.s
***
CD3+T
BMSC
NMP-MSC
1-MT

Figure S12


A
IFN-γ/GAPDH
TNF-α/GAPDH
IL-6/GAPDH
IL-17/GAPDH
B
IFN-γ (pg/ml)
TNF-α (pg/ml)
IL-6 (pg/ml)
IL-17 (pg/ml)
Control
CHS
CHS+BMSC
CHS+NMP-MSC

Supplementary Materials and Methods

Neuromesoderm differentiation of hPSC

To differentiate hPSC into NMP, confluent hPSC (hiPSC, H1, and H9) were washed twice with phosphate-buffered saline (PBS) and dissociated to single cells by incubation with Accutase at 37°C for 2-3 minutes. These cells ($1\text{-}2\times10^5$ cells/cm^2) were seeded to Matrigel-coated plates and cultured in mTeSR medium containing 10 μM Y27632 (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. Neuromesoderm differentiation was initiated by culturing cells in E6 medium supplemented with 20 ng/ml basic fibroblast growth factor (bFGF), with or without 2-5 ng/ml TGFβ1 (both from Peprotech, Rocky Hill, NJ, USA), and 10 μM Chir99021 (Stemgent, Cambridge, MA, USA) for 2-5 days (Fig. 1A).

To test whether NMP could generate posterior neural cells, hPSC-NMP were cultured in N2B27 serum-free medium containing 100 nM retinoic acid (RA) (Sigma-Aldrich), 50 ng/ml noggin (Peprotech), and 10 μM SB431542 (Stemgent) for 7 days to generate posterior neural stem/progenitor cells. To differentiate the obtained cells to posterior neurons, the cells were cultured in neural differentiation medium supplemented with brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT3) (all from Peprotech), ascorbic acid (AA), and dibutyryl-cAMP (db-cAMP) (both from Sigma-Aldrich) for 2-3 weeks, as previously described [1]. The expression of NMP or posterior neural markers in differentiated cells was detected at different time points by immunofluorescence and quantitative reverse transcription polymerase chain reaction

(qRT-PCR).

MSC differentiation of hPSC-NMP

For MSC differentiation, paraxial mesoderm cells that had been differentiated in medium with bFGF, TGFβ1, and Chir99021 for 4-5 days were cultured for 2-3 weeks in animal component-free (ACF) and serum-free medium (MesenCult™-ACF Plus Medium; Stemcell Technologies), which is suitable for the isolation and expansion of tissue-derived MSC. The resulting cells were maintained in MesenCult™-ACF Plus Medium. The phenotype and multipotency of NMP-derived MSC (NMP-MSC; including hiPSC-NMP-MSC, H1-NMP-MSC, H9-NMP-MSC1, and H9-NMP-MSC2) were assessed by FACS analysis and tested for their ability to differentiate into mesenchymal-lineage cells (osteoblasts, adipocytes, and chondrocytes), as previously described [1]. BMSC from three healthy donors (BMSC1, BMSC2, and BMSC3) [2] were cultured in MesenCult™-ACF Plus Medium and used as controls.

NMP-MSC proliferation assay

The proliferation ability of NMP-MSC was determined using the fluorescence-based Cell Counting Kit-8 (CCK8) Cell Proliferation Assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. In brief, 100 μl of cell suspension was plated to a 96-well plate at 1,500 cells/well and cultured for 24 hours, after which 10 μl of CCK-8 solution was added to each well and the plate was incubated for 1-4 hours. Cell proliferation was assessed by measuring absorbance at 450 nm using a microplate reader (Tecan Trading AG, Switzerland) daily; until cells reached confluence. BMSC of the same passage served as controls.

Lymphocyte proliferation assay

CD3+ T lymphocytes were obtained from healthy donors who provided fully informed consent. The lymphocytes were isolated by fluorescence activated cell sorting (FACS) and stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE) using a CellTrace™ CFSE Cell Proliferation kit (Life Technologies) according to the manufacturer's instructions. The proliferation of labeled CD3+ T cells that cultured with or without MSC was stimulated with anti-CD3 mAb and anti-CD28 mAb (both from BD-Pharmingen; Palo Alto, CA, USA) for 96 hours and evaluated by flow cytometric analysis.

Intracellular cytokine staining

Isolated CD3+ T cells were resuspended in RPMI-1640 (Life Technologies) and cultured with or without MSC for 72 hours. In IDO blockage assay, 1mM 1-MT (IDO inhibitor; Sigma-Aldrich) was added. Thereafter, 10 μg/ml brefeldin A (BFA), 50 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1 μg/ml ionomycin (all from Sigma-Aldrich) were added to the culture medium, and cells were cultured for an additional 6 hours. The CD3+ cells were fixed, permeabilized, stained for cell-surface CD3, cytoplasmic TNF-α, and IFN-γ, and analyzed by flow cytometry.

Exposure of NMP-MSC to pro-inflammatory cytokines

NMP-MSC and control BMSC were plated in 6-well plates at 4×10^5 cells/well. After cells reached 60-70% confluence, 20 ng/ml IFN-γ was added to the medium and culture was continued for 24 or 48 hours. The cells were then harvested, and the mRNA expression levels of anti-inflammatory mediators (IL6, IL8, and CCL2) and

pro-inflammatory mediators (IDO, TSG6, and PGE2) were detected by qRT-PCR.

***In vivo* bone formation assay**

The scaffolds, hydroxyl-apatite/tricalcium phosphate ceramic powder (HA/TCP; Zimmer Scandinavia, Denmark), were washed twice with PBS and immersed in MSC culture medium for 12 hours. NMP-MSC or BMSC were suspended at 2×10^7 cells/ml in osteogenic differentiation medium, and 50 μl of the cell suspension (10^6 cells) was poured onto each scaffold. NMP were used as control cells. The cells were allowed to adhere to the scaffolds for 2 hours at 37℃, the cell-scaffold complexes were covered with 2 ml of osteogenic differentiation medium, and cells were allowed to differentiate for 3 days. The assembled cell-scaffolds were transplanted subcutaneously to the dorsal surfaces of 2-month-old female nude mice (Vital River, Beijing, China). Transplants were recovered 8 weeks after transplantation, and transplant sections were analyzed by hematoxylin and eosin (H&E) staining, and immunostaining with anti-OPG, anti-OCN, or anti-CD45 antibodies.

Tumor formation assay

To evaluate the tumorigenicity of hPSC-NMP-MSC derived from different pluripotent stem cell lines, $5x10^6$ NMP-MSC in PBS containing 30% matrigel were injected subcutaneously into 8-week-old NOG mice (n=5 for each cell line; from Vital River). Undifferentiated human induced pluripotent stem cells were used as a control. Tumor occurrence was evaluated 8 weeks after cell injection.

Migration assays

The migration of hPSC-NMP-MSC was detected using three different assays.

Time-lapse analysis was performed with a Zeiss Axio Observer Z1 microscope. Cells were cultured at 37°C in an enclosed chamber with CO2 controlled. Cell migration was quantified at 10-minute intervals for 12 hours using computer-assisted tracking. The migration distances of individual cells were determined and analyzed.

Transwell assays were performed using inserts with 8-μm-pore membrane filters (Chemicon, Temecula, CA, USA), as described by the manufacturer. The extracellular matrix (ECM) layer of the inserts was rehydrated with serum-free medium for 1-2 hours, 300 μl of cell suspension (1×10^5 cells/ml) in serum-free medium was loaded into each upper chamber, and 500 μl of culture medium was loaded into the lower chamber. Incubation was performed for 6 or 24 hours, the upper sides of the filters were carefully washed with cold PBS, and any remaining non-migrating cells were gently removed with a cotton-tipped swab. The cells on the lower surface (migrated cells) were stained with crystal violet solution.

Migration was also assessed using a wound-healing assay. Cells (2×10^6) were grown to confluence on 60-mm culture dishes and treated with 0.5 μM mitomycin C (Sigma-Aldrich, Chicago, IL, USA) for 2 hours prior to wounding. Monolayers were scratched with a P-20 pipette tip and washed repeatedly with PBS. Representative images were taken of the scratched areas at various time points. To estimate the relative migration of the cells, average gap measurements (AG; percentage) were used to quantify the data. The condition at 0 h was taken as 100%.

Transplantation of NMP-MSC in a mouse model of contact hypersensitivity (CHS)

NMP-MSC and BMSC were transfected with an EGFP-encoding construct for *in vivo* cell tracing, as previously described [3]. The mouse CHS model was established according to a previously reported protocol [3]. In brief, the contact-sensitizing agent, 2, 4-dinitrofluorobenzene (DNFB; dissolved in 4:1 acetone/olive oil; 0.5%) (Sigma-Aldrich) was applied to the shaved back of each mouse (sensitization) (BALB/c; from Vital River). Five days later, 0.2% DNFB was applied to the right ear of each mouse. Age- and sex-matched syngeneic naive mice of the control group received ear DNFB challenge without prior sensitization. To compare the immunomodulatory activity and therapeutic effect of NMP-MSC and BMSC on the CHS model, the mice were subjected to intravenous (i.v.) injection of one million NMP-MSC or BMSC on day 1 post-challenge. The degree of swelling was calculated as the thickness of the right ear (challenged ear) minus the baseline thickness of the left ear (unchallenged ear); this was determined daily for 3 days post-challenge [3] using a dial thickness gauge (Mitutoyo, Kawasaki, Japan). Mice were then sacrificed and ear samples were harvested for further analysis.

All experimental procedures involving animals were approved by the Animal Ethics Committee of Sun Yat-sen University.

Measurement of MPO Activity

The MPO Activity was analyzed as described [3]. In brief, 24 hr after MSC injection, ear tissues were homogenized, passed through a nylon mesh, centrifuged at 3000 rpm for 20 min at 4℃, and collected. MPO activity was assessed with the MPO kit (R&D Systems) according to the manufacturer’s instructions.

RNA sequencing

Total mRNA was isolated from undifferentiated hPSC (hiPSC, H1, and H9), hPSC-NMP-derived paraxial mesoderm (hPSC-NMP-PM; including hiPSC-NMP-PM, H1-NMP-PM, and H9-NMP-PM), hPSC-NMP-MSC (Passage 5; including hiPSC-NMP-MSC, H1-NMP-MSC, H9-NMP-MSC1, and H9-NMP-MSC2), BMSC1 (Passage 5), and BMSC2 (Passage 5). RNA sequencing libraries were constructed using an Illumina mRNA-seq Prep Kit (Illumina, San Diego, CA, USA) as recommended by the manufacturer. The fragmented and randomly primed 150 bp paired-end libraries were sequenced using Illumina HiSeq X Ten. Sequencing data were processed using Consensus Assessment of Sequence and Variation (CASAVA, version 1.8.2; Illumina) under the default settings. The transcripts per million (TPM) values were used to evaluate the expression levels of genes, and Pearson correlations were calculated (R2) to measure the similarities between different lines. Finally, the RNA-Seq data were analyzed using Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems Inc., Redwood City, CA, USA) to categorize the differentially regulated genes.

Fluorescence activated cell sorting (FACS) analyses

FACS was used to examine the marker expression of NMP and NMP-MSC. The cells were trypsinized, filtered, and incubated with monoclonal antibodies against various human antigens, including CD24, CD29, CD34, CD44, CD45, CD73, CD90, and CD166 (all from BD-Pharmingen; Table S1). An irrelevant isotype-identical antibody (BD Biosciences) was used as a negative control. The data were analyzed

with the FlowJo software (BD Biosciences).

Immunocytochemistry and histochemistry

Differentiated hPSC and hPSC-NMP-PM were fixed with 4% paraformaldehyde for 20 minutes, blocked in blocking buffer (0.2% Triton X-100 with 10% goat serum or 2% bovine serum albumin) for 1 hour, and incubated with the appropriate primary antibody at 4°C overnight. The utilized primary antibodies are listed in Table S2. The cells were washed three times with PBS, and then incubated with secondary antibody, Alexa Fluor 488, or Alexa Fluor 594 (Life Technologies) at room temperature for 1 hour in the dark. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and the results were analyzed by confocal fluorescence microscopy.

The differentiation potential of NMP-MSC was detected as described [1]. For osteogenic differentiation, cells were fixed and incubated with Alizarin Red S (Sigma-Aldrich) for 30 minutes for detection of calcium deposits. For adipogenic differentiation, cells were fixed and incubated with Oil Red O (Sigma-Aldrich) for 20 minutes for detection of lipid droplets. For chondrogenic differentiation, cells were fixed and incubated with 0.1% toluidine blue for 10-15 minutes. The results were analyzed by direct observation under a phase-contrast microscope.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses

Total RNA was prepared using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and then reverse transcribed into cDNA using a

Quantitect Reverse Transcription kit (Qiagen, Valencia, CA, USA) in a 20-μL reaction system. qPCR was performed using a DyNAmo ColorFlash SYBR Green qPCR kit (ThermoFisher Scientific, Rutherford, NJ, USA) and a LightCycler 480 Detection System (Roche Diagnostics, Mannheim, Germany). The thermocycling conditions comprised 40 cycles of denaturation at 94℃ for 15 seconds, annealing at 60℃ for 15 seconds, and extension at 72℃ for 40 seconds. GAPDH was amplified as an internal control, and gene expression levels were calculated as fold changes. Primer details are provided in Table S3.

ELISA

The levels of TNF-α, IFN-γ, IL-6, and IL-17 were detected by commercially available ELISA kits (all from R&D Systems) in the inflamed ears of each group according to the manufacturer's recommended procedures.

Statistics

All data are presented as the mean ± SEM obtained from at least three independent experiments. Comparisons between groups were performed using a one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. All statistical analyses were performed with the aid of SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA).

References

1. Li W, Huang L, Zeng J, Lin W, Li K, Sun J, et al. Characterization and transplantation of enteric neural crest cells from human induced pluripotent stem cells. Molecular psychiatry. 2018; 23: 499-508.

2. Peng Y, Chen X, Liu Q, Zhang X, Huang K, Liu L, et al. Mesenchymal stromal cells infusions improve refractory chronic graft versus host disease through an increase of CD5+ regulatory B cells producing interleukin 10. Leukemia. 2015; 29: 636-46.

3. Zhang X, Huang W, Chen X, Lian Y, Wang J, Cai C, et al. CXCR5-Overexpressing Mesenchymal Stromal Cells Exhibit Enhanced Homing and Can Decrease Contact Hypersensitivity. Molecular therapy: the journal of the American Society of Gene Therapy. 2017; 25: 1434-47.