Down-regulation of Dicer1 promotes cellular senescence and decreases the differentiation and stem cell-supporting capacities of mesenchymal stromal cells in patients with myelodysplastic syndrome

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SUPPLEMENTARY INFORMATION

SUPPLEMENTAL MATERIALS AND METHODS

Isolation and Culture of BM-MSCs

BM samples from patients and controls were harvested, and bone marrow mononuclear cells (BMNCs) were isolated using a Ficoll solution (GE Healthcare, Uppsala, Sweden). The cells were plated at a density of $1x10^6$ cells/ml in Human Mesenchymal Stem Cell Growth Medium (Cyagen Biosciences Inc., Guangzhou, China) supplemented with 10% fetal bovine serum (FBS), glutamine and 100 U/mL penicillin-streptomycin at 37ºC with 5% CO_2 in fully humidified atmosphere. After 72 h, the culture medium was replaced, and non-adherent cells were removed;thereafter,the medium was replaced every 3-4 days. When the MSCs were confluent, the cells were recovered by the addition of 0.25% trypsin-EDTA and cryopreserved for future experiments.

Immunophenotype analysis of MSCs

MSCs were washed with PBS and incubated with primary antibodies (10-20 ng/mL) for 15 min in the dark at room temperature. Primary antibodies included CD34-fluorescein isothiocyanate (FITC), CD45-FITC, CD73-FITC, CD90-FITC, CD105-FITC, CD166-phycoerythrin (PE) (BD Biosciences Pharmingen, USA). Cell analysis was performed with a FACSCalibur system using CellQuest

software.

Clonogenic and proliferative potential of MSCs

A colony-forming unit fibroblast (CFU-F) assay was used for the evaluation of the clonogenic potential of MSCs at P1, P3 and P5. Cells were seeded at 100 cells/well in 6-well plates. They were then cultured in Human Mesenchymal Stem Cell Growth Medium for 14 days. Colonies were stained with Giemsa and counted under a microscope. The proliferative potential of the MSCs was evaluated using a Cell-Counting Kit-8 (CCK8) proliferation assay at P3 according to the manufacturer´s directions and by estimating the population doubling time through P1–P5.

SA-β-Gal assay

Approximately $5x10^4$ MSCs were seeded in 6-well plates. After 72 h of culture, the cells were stained using a β-Galactosidase (β-Gal) kit (Beyotime, China) according to the manufacturer´s directions. The number of β-Gal-positive cells was counted using an inverted microscope.

MSC differentiation

For osteogenic differentiation, 3×10^4 MSCs were replated in 6-well plates pre-coated with a gelatin solution of Human Mesenchymal Stem Cell Osteogenic Differentiation Medium (Cyagen Biosciences Inc., Guangzhou, China), and the medium was replaced every 3-4 days. Calcium mineralization and Alkaline phosphatase activity（ALP） activity

were assessed, and ALP and Runx2 mRNA expression levels were analyzed by quantitative real-time PCR (qPCR)of MSCs during osteogenic induction.

For adipogenesis differentiation, 2×10^5 MSCs were replated in 6-well plates in Mesenchymal Stem Cell Growth Medium. Four days after the cells were confluent, the medium was changed to adipogenesis-inducing medium (AIM) (Cyagen Biosciences Inc., Guangzhou, China). After 72 h, the AIM was changed to adipogenesis maintenance medium (AMM) (Cyagen Biosciences Inc., Guangzhou, China) for 24 h and then switched to AIM again. Alternating incubations with the two different media were repeated three times, and the cells were then maintained in AMM for 1 week.Oil Red-O staining and the expression of fatty acid binding protein 4 (FABP4) and C/EBPα were used to evaluate adipogenic differentiation.

Western blotting (WB)

Cells were homogenized in a cocktail of lysis bufferand protease inhibitor, and protein concentrations were determined using the bicinchoninic acid (BCA) assay (Tiangen, Beijing, China). A total of 30 μg of proteins in the cell lysate was separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Primary antibodies used were: rabbit-Dicer1, rabbit-p21, rabbit-RB and rabbit-p16, with rabbit-GAPDH as the control (ABclonal Biotechnology Co., Ltd,USA). Immunoreactive bands were visualized using an ECL Plus kit (Tiangen, Beijing, China),

followed by exposure to x-ray film (RX-U; Fujifilm) for visualization.

Long-term culture

Briefly, 1×10^6 MSCs were plated in 96-well plates and grown until confluent and then irradiated with 30 Gray using Gulmay RS225 X-ray equipment. Approximately 1×10^4 CD34+cells were added to the MSC feeder layer and were cultivated in 5 ml MyeloCult H5100 Medium (Stem Cell Technologies, Vancouver, Canada) for 5 weeks. Every week, half of the medium was replaced. After 5 weeks, the medium was replaced with clonogenic methylcellulose medium H4435 (Stem Cell Technologies, Vancouver, Canada). Cultures were kept at 37ºC for another two weeks, and then colonies (CFU–granulocyte-macrophage progenitors [CFU-GM], burst-forming units–erythroid [BFU-E] and CFU–granulocyte, erythrocyte, monocyte, megakaryocyte [CFU-GEMM]) of >50 cells were counted using an inverted microscope.

Dicer1 shRNA transductions

MSCs (P2) derived from a 42-year-old volunteer were transfected. The MSCs were seeded in 6-well plates. When the density of the cellsreached 80%, they were transfected in three groups as follows: (i) the control group; (ii) the negative group; and (iii) the shRNA group. Each group was composed of 4 wells. The transfection was performed according to the manufacturer's protocol. Briefly, the MSCs were seeded in 6-well plates at a density of $2x10^5$cells/well and cultured for 24 h to reach 80%

confluence. Twenty microliters of shRNA expression vectors were added into the 6-well plates, which were then cultured for 6 h. After 6 h of incubation, the medium was replaced. The transfection efficiency was determined to be >90% by direct visualization of green fluorescent protein (GFP) expression 48 h after transfection. The transfected cells were selected by adding 2μg/ml of puromycin to the media for 1 week.

Cell cycle and apoptosis analysis

The cell cycle analysis was performed using ethanol-fixed cells stained with propidium iodide in buffer containing RNase A. The DNA content was assessed by flow cytometry（FCM）(Becton–Dickinson, Sunnyvale, USA), and cell cycle analysis was performed using the MultiCycle AV software package (Phoenix Flow Systems, San Diego). The quantification of apoptotic cells was performed using the AnnexinV-FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The analyses were performed on the FACScan flow cytometer. The experiments were repeated three times.

SUPPLEMENTAL TABLES

Supplementary table S1. Clinical characteristics of MDS patients

Parameter		
Sex (median value)	Male,47	
	Female,30	
Age (median value)	59 (range25-84)	
WHO classification	RA	4
	RARS	6
	RCMD	36
	RCMD-RS	2
	RAEBI	15
	RAEBII	11
	MDS-U	3
IPSS	<=1	51
	>1	26

Supplementary table S2.　Primer sets for RT-PCR

Name	Primer sequence	Size
GAPDH	F. GCACCGTCAAGGCTGAGAAC	138
	R.GTGGTGAAGACGCCAGTGGA	
VEGFA	F.AGGGCAGAATCATCACGAAGT	75
	R.AGGGTCTCGATTGGATGGCA	
G-CSF	F.GCTGCTTGAGCCAACTCCATA	285
	R.GAACGCGGTACGACACCTC	
CXCL12	F.ATTCTCAACACTCCAAACTGTGC	88
	R.ACTTTAGCTTCGGGTCAATGC	
P21	F.TGTCCGTCAGAACCCATGC	139
	R.AAAGTCGAAGTTCCATCGCTC	
IL-6	F.CCTGAACCTTCCAAAGATGGC	75
	R.TTCACCAGGCAAGTCTCCTCA	
TPO	F.CTGTCACGCTGGTTATGGC	101
	R.GCTAGAGACACGAGACTCCTCA	
VCAM1	F.GGGAAGATGGTCGTGATCCTT	89
	R.TCTGGGGTGGTCTCGATTTTA	
SCF	F.CCATCTCGCTTATCCAACAATGA	163
	R.AATCCTCTCGTCAAAACTGAAGG	
Dicer1	F.TGCTATGTCGCCTTGAATGTT	114
	R.AATTTCTCGATAGGGGTGGTCTA	
P16	F.ATGGAGCCTTCGGCTGACT	108
	R.GTAACTATTCGGTGCGTTGGG	
RB	F.TTGGATCACAGCGATACAAACTT	149
	R.AGCGCACGCCAATAAAGACAT	
TGF-β	F.CAATTCCTGGCGATACCTCAG	86
	R.GCACAACTCCGGTGACATCAA	
Collagen 1	F.GAGGGCCAAGACGAAGACATC	140
	R.CAGATCACGTCATCGCACAAC	

SUPPLEMENTARY FIGURES LEGENDS

Supplementary Fig. S1

Cellular senescence in expanded (A) and primary (CD271+) MSCs (B, C, D) from MDS patients. (A) The distribution of percentages of senescent cells in expanded MSCs. (B) SA-β-gal stain (100 × magnification). (C) The mean number of SA-β-gal-positive cells. (D) Expression of senescence-related molecular p21. The results are expressed as means ± SD. Compared with HC-MSCs, the significance was set as * $p \leq 0.05$.

Supplementary Fig. S2

S-MDS-MSC hematopoietic support capacity. S-MDS-MSCs (n=14) or HC-MSCs (n=12) were cultured in LTBMC medium with CD34+ cells from 4 healthy controls for 5 weeks, followed by methylcellulose progenitor culture for an additional 2 weeks. (A) LTC-IC frequency. (B) The numbers of CFU-GM, CFU-E and CFU-GEMM. The results are expressed as means ± SD. Compared with HC-MSCs, the significance was set as * $p \leq 0.05$; ** $p \leq 0.001$.

Supplementary Fig. S3

Dicer1-KD inhibited osteogenic differentiation of MSCs. The same approaches were used to assess the capacities of Dicer1-KD MSCs (shRNA), negative MSCs and control MSCs to differentiate into osteogenic lineages. (A) Representative images of mineralization on day 21 of osteogenic differentiation. (B) ALP activity. (C) Quantitation of mineralization using Image-Pro Plus 6.0 software. (D, E) Relative RUNX2 and ALP mRNA expression levels. The average of three replicates is displayed. Compared with controls, the significance was set as * $p \leq 0.05$; ** $p \leq 0.001$.

Supplementary Fig. S4

Dicer1-KD inhibited adipogenic differentiation of MSCs. (A) Oil Red-O staining on day 21 of adipogenic differentiation. (B,C) Relative FABP4 and C/EBPαmRNA expression levels during adipogenic differentiation. The average of three replicates is displayed. Compared with controls, the significance was set as * $p \leq 0.05$; ** $p \leq 0.001$.
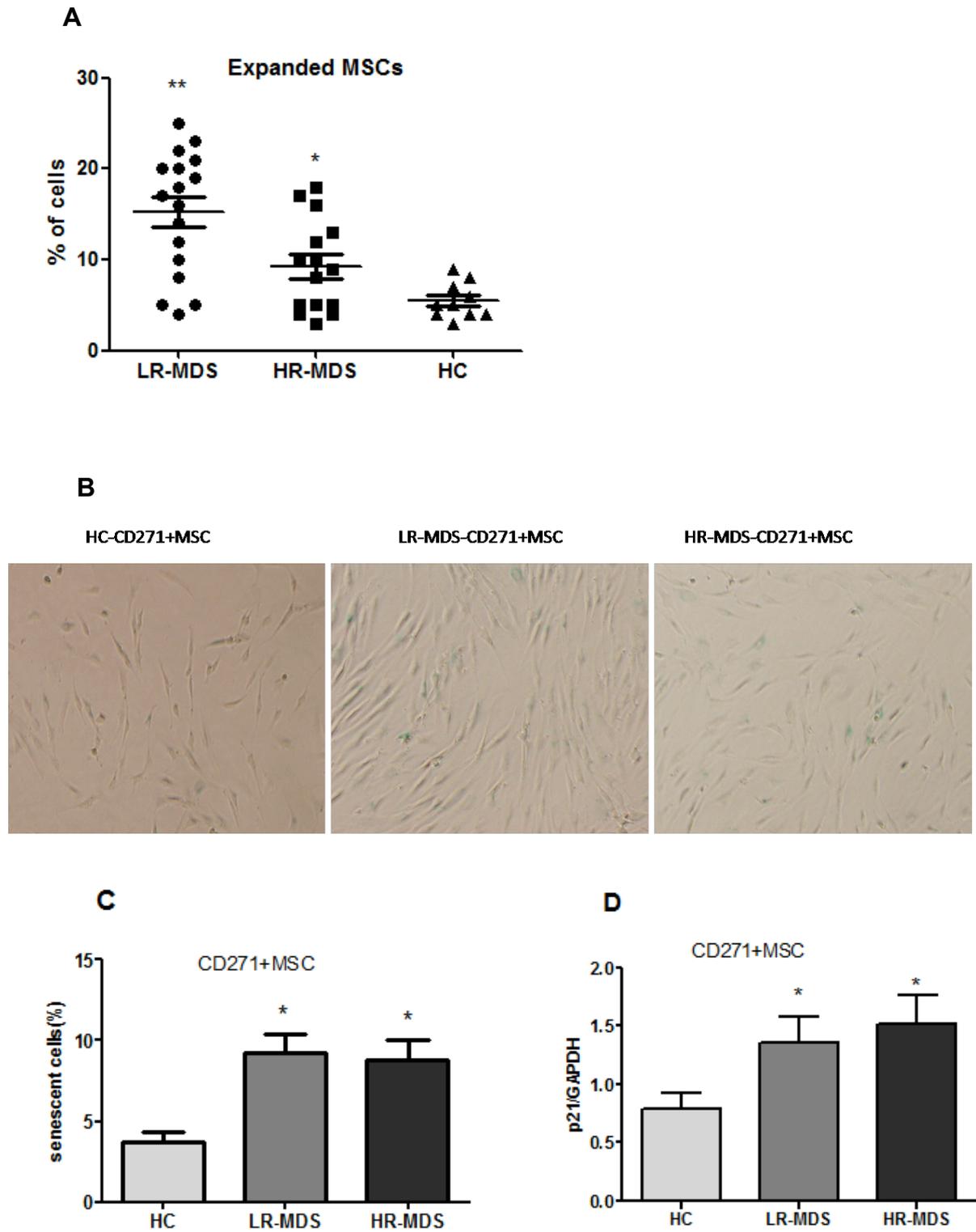
Supplementary Fig. S5

Overexpression of Dicer1 enhanced stem cell properties in MDS-MSCs. (A) Representative

images of mineralization on day 21 of osteogenic differentiation.(B) Supports proliferation of CD34+cells. CD34+ cells were stained with CFSE and co-cultured for 5 days with MSCs.
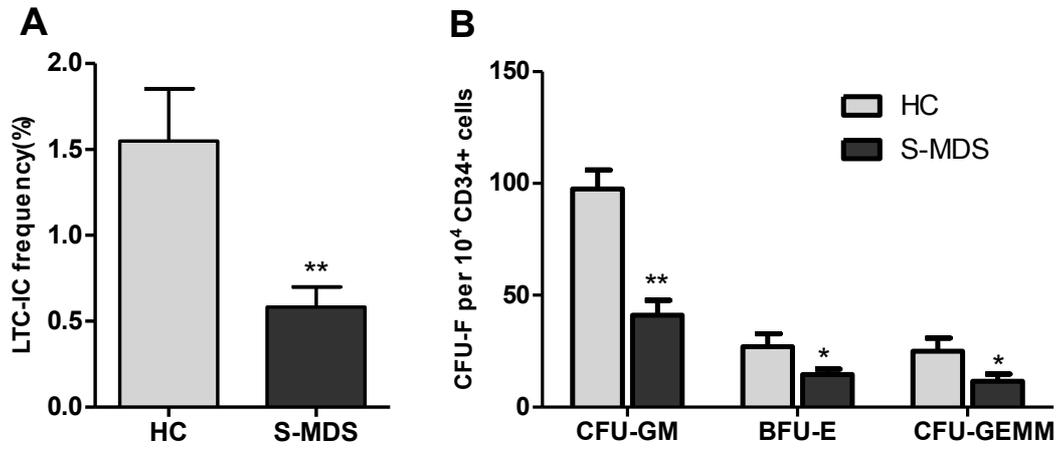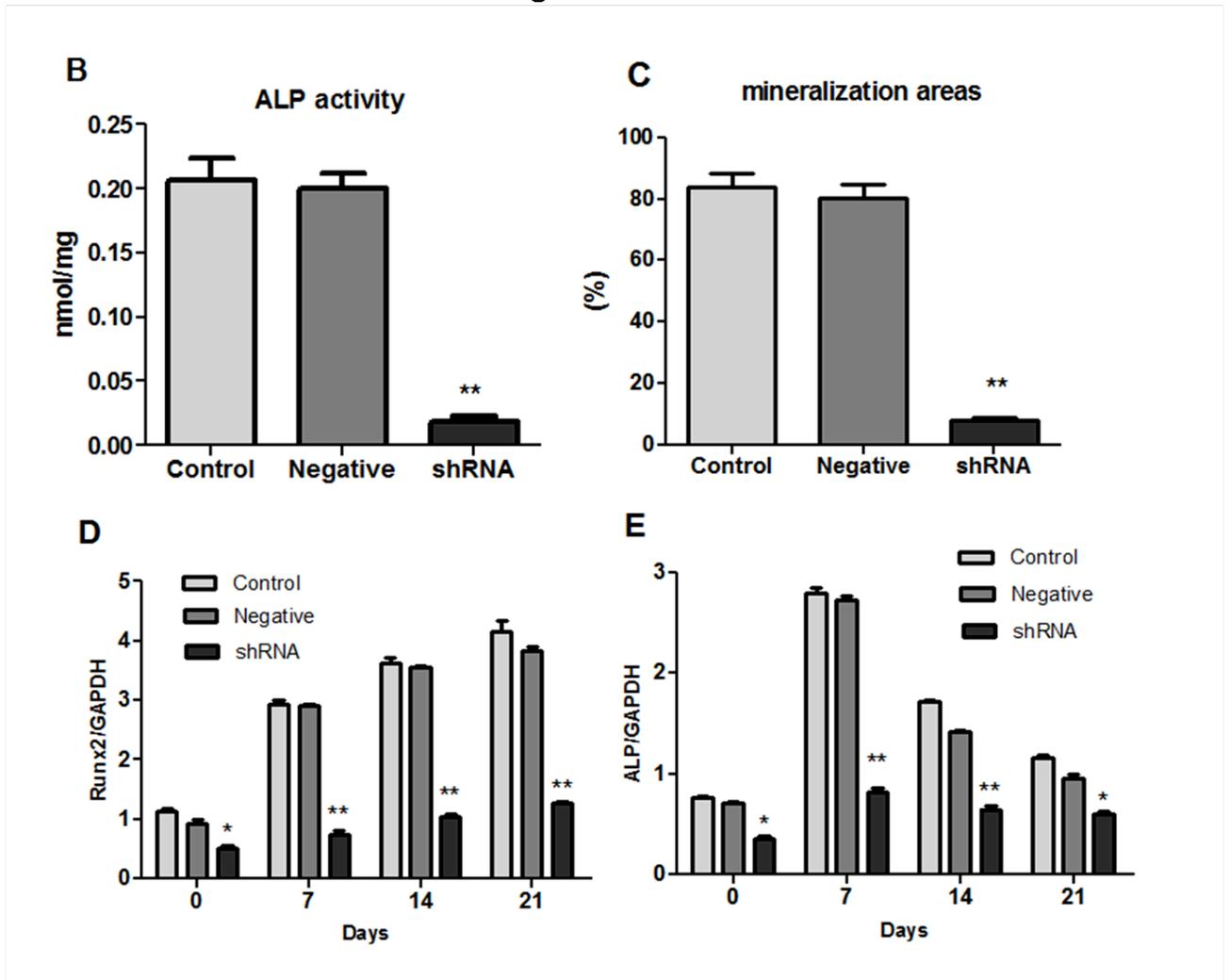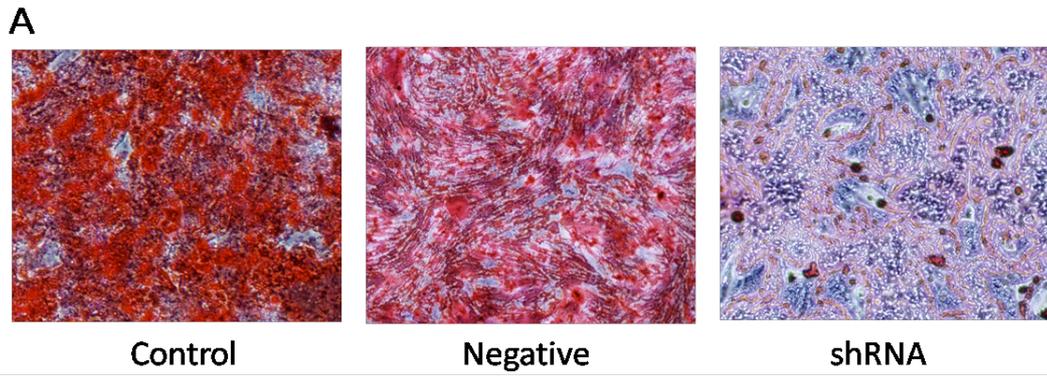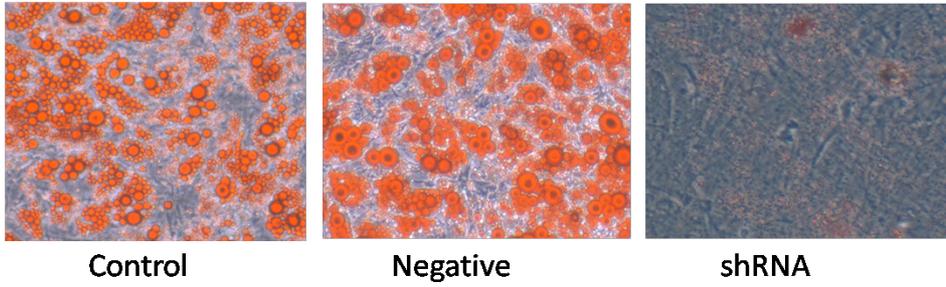
Supplementary Fig. S6

Overexpression of miR-93/miR-20a decreased cellular senescence in MDS-MSCs. MDS-MSCs with miR-93/miR-20a transfection displayed upregulated miRNA expression (A), increased proliferation (B), decreased number of cells in the G1 phase (C) and reduced number of SA-β-gal-positive cells (D). The average of three replicates is displayed. Compared with controls, the significance was set as * $p \leq 0.05$; ** $p \leq 0.001$.
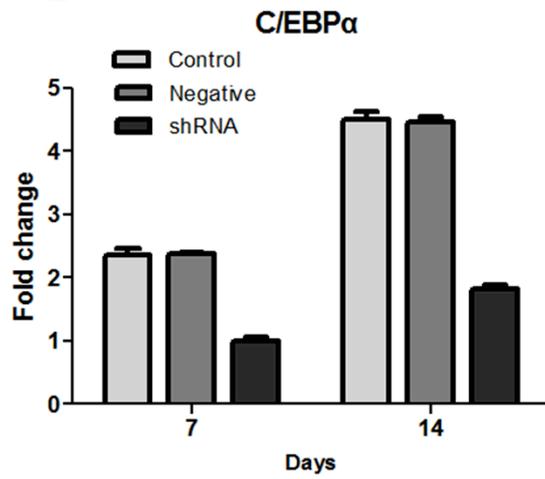
Supplementary Fig.S1

A

B

HC-CD271+MSC   LR-MDS-CD271+MSC   HR-MDS-CD271+MSC

C

D

Supplementary Fig.S2

Supplementary Fig.S3

A

Control   Negative   shRNA

B **ALP activity**

C **mineralization areas**

D

E

Supplementary Fig.S4

A

Control   Negative   shRNA

Supplementary Fig.S5

Supplementary Fig.S6