SUPPLEMENTAL MATERIAL

for

Aged Human Multipotent Mesenchymal Stromal Cells Can Be Rejuvenated by Neuron-Derived Neurotrophic Factor and Improve Heart Function after Injury

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Supplemental Methods

Culture of human bone marrow multipotent mesenchymal stromal cells (hBM-MSCs)

A two-step procedure reported by Bartmann et al. (1) was adopted for culturing of hBM-MSCs. In brief, 1 ml of BM was harvested in a heparin sodium anticoagulation tube and gently mixed with 44 ml of 10% FBS-IMDM culture medium, composed of IMDM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). The mixture was then distributed into three T-75-flask. The medium was first changed 48h later and then every week until obvious clones formed and then changed every three days until 80% confluence was attained. The cells were then detached with 0.25% trypsin plus 0.02% EDTA and sub-cultured at a density of 250 cells/cm^2. Cells were used at 80% confluence at passage 3 for subsequent experiments. For the anti-apoptosis analysis, cells were cultured in hypoxia conditions (0.1% oxygen) for 48h and then TUNEL staining was performed or harvested for total protein for Western blot analysis.

hBM-MSC proliferation analyses

To examine hBM-MSC proliferation, cells were incubated with a medium containing bromodeoxyuridine (BrdU, 10 μM) for 24h, and then fixed in PBS with 4% paraformaldehyde at room temperature for 20 min. After DNA denaturation in 2 N HCl, cells were permeabilized with Triton X-100 (0.1%) in PBS, and treated with serum to block nonspecific binding sites. The cells were incubated with rat anti-BrdU (ab6326, Abcam,1:100) overnight at 4°C, stained with Alexa Fluor 546 conjugated goat anti-rat antibody (A11081, Invitrogen,1:400) for 1h at 37°C , and then mounted in mounting medium with 4',6-diamidino-2-phenylindole (DAPI).

To evaluate cell growth, hBM-MSCs from different groups were seeded in T-75-flasks at a density of 250 cells/cm^{2}with complete culture medium and allowed to adhere to the flasks overnight. Cell numbers from triplicate flasks were counted daily for 8 days and cell growth recorded.

hBM-MSC migration analyses

hBM-MSCs from different groups grown to confluence in 35 mm plates were scratched with a sterile pipette tip, washed twice with PBS and incubated in serum-free medium for 24h at 37°C. Pictures were taken at 40X magnification using a phase-contrast microscope (Nikon) and the migration rate was calculated using ImageJ software (NIH).

Over-expression of NDNF in old hBM-MSCs and human dermal fibroblasts

Cell transduction was carried out using a lentiviral expression vector carrying the NDNF gene (Lenti-Puro-EF1α- NDNF-Homo- IRES-eGFP, Cyagen Biosciences Inc., Santa Clara, CA) according to the manufacturer's instructions. NDNF mRNA and protein levels were detected by reverse transcription-polymerase chain reaction(RT-PCR) and Western blot to ensure NDNF mRNA and protein over-expression in old hBM-MSCs and human dermal fibroblasts (Fibro). Human dermal fibroblasts were purchased from Invitrogen (C-013-5C) and cultured according to the manufacturer's instruction.

Immunofluorescence staining

Cells grown on coverslips in 24-well plates at a density of 8000 cells/well were washed

with PBS and then fixed with 4% paraformaldehyde in PBS. The fixed cells were permeabilizated with 0.2% Triton X-100 in PBS and blocked for 1 h in PBS containing 10% bovine serum albumin. They were then incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies and then mounted in mounting medium with DAPI. Antibodies used included: NDNF (ab175602, Abcam, 1:100); mitochondria (MAB1273, Millipore, 1:100); α-SMA (A2547, Sigma, 1:400).Isolectin B4 (121412, Thermo Fisher Scientific,1:50) staining was carried out for 30min at RT to assess capillary density.

Western blotting

Cells or tissues were harvested on ice using RIPA buffer with protease inhibitor and phosphatase inhibitors. Cells were harvested at 80% confluence. Equal amounts of protein was loaded onto SDS-polyacrylamide gel and transferred to PVDF membranes. After blocking, membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies. An ECL Western blotting detection reagent was used to detect signals. Antibodies used include: β-actin (TA-09, ZSGB-BIO,1:2000); NDNF (ab175602, Abcam,1:1000); regucalcin(ab67336, Abcam,1:500); p16 (sc-1661, Santa Cruz Biotechnology,1:100); Bax (sc-20067, Santa Cruz Biotechnology,1:200); GAPDH (MAB374, Millipore,1:5000); p-Akt (4060, Cell Signaling,1:2000); Akt (4691, Cell Signaling,1:1000); p-ATM(5883, Cell Signaling,1:1000); ATM(sc-377293, Santa Cruz Biotechnology,1:100); p-chk1(2348, Cell Signaling,1:1000); chk1 (sc-8408, Santa Cruz Biotechnology,1:100); p-p53(sc-377567, Santa Cruz Biotechnology,1:100); p53(sc-126, Santa Cruz Biotechnology,1:100); p27(3686s, Cell Signaling,1:1000); p21(2947s, Cell Signaling,1:1000).The grey level of bands was obtained by ImageJ software(NIH),and protein expression level was calculated using the

ratio to the loading control (β-actin or GAPDH).

Total RNA extraction and RT-PCR

Total RNA was extracted from cells with trizol reagent and converted to cDNA. PCR reaction mixture contained *Taq* 2X master mix, 1ul primer (forward and reverse mixture) and 50ng cDNA. Reaction products were analyzed by 1% agarose gel electrophoresis. ACTB (β-actin) served as the endogenous control for normalization. For quantification, densitometry of the NDNF bands was divided by the corresponding densitometry of the β-actin band using ImageJ software. To compare from assay to assay (gel to gel), the average of the infant group (Fig. 1) or the average of the Old MSCs group (Fig. 3) on the same gel was set at a value of 1 as a control and all the other groups were compared with the corresponding control on the same gel. The primers used were as follows: NDNF–forward primer: 5'-CCTTTGGAGTGGAAGCTGAG-3'; reverse primer: 5'-GTAGACATGACGCCCCAGTT-3'; ACTB–forward primer: 5'-AGAAAATCTGGCACCACACC-3'; reverse primer: 5'-AGGAAGGAAGGCTGGAAGAG-3'.Collagen, type I, alpha 1 (COL1A1)–forward primer: 5'-CCCTGGAAAGAATGGAGATG-3'; reverse primer: 5'-CCATCCAAACCACTGAAACC-3';Lipoprotein lipase (LPL)–forward primer: 5'-GGGCATGTTGACATTTACCC-3';reverse primer: 5'-AGCCCTTTCTCAAAGGCTTC-3'.

SA-β-gal staining

Cells grown in 6-well plates were washed with PBS and then stained with the senescence β-galactosidase staining kit (C0609,Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instruction. Briefly, the cells were fixed with the fixing solution for 15 min, then

washed with PBS and incubated with the SA-ß-gal staining solution(SA-ß-gal staining solution A 10μl, SA-ß-gal staining solution B 10μl, SA-ß-gal staining solution C 930μl and X-gal 50μl) overnight at 37 °C. Pictures were taken using a Nikon microscope.

Colony formation assay

hBM-MSCsfrom different groups were seeded in 10 cm dish(200 cells/dish) and let to grow for 3 weeks. Then the cells were washed twice with PBS, allowed to air dry for 5 min and subsequently fixed with methanol for 3 min at room temperature. Giemsa staining was carried out with a Giemsa staining solution (G1015, SolarbioScience&Technology Ltd., Beijing, China) at room temperature for 30min.The images were captured and colonies were counted.

Adipogenic and Osteogenic Differentiation

hBM-MSCs from different groups were plated into12-well platesand grown to confluence.The medium was changed to adipogenic or osteogenic differentiation medium and cultured according to the manufacturer's instructions(HUXMA-90021 or HUXMA-90021, Cyagen Biosciences Inc., Jiangsu, China). The differentiation characteristics were examined with Oil Red O staining or Alizarin red staining according to the manufacturer's instructions.

Knockdown of NDNF in young hBM-MSCs

Transfection of NDNF siRNA or a scrambled control siRNA was carried out (GenePharma Co.,Ltd., Shanghai, China) according to the manufacturer's instructions. NDNF mRNA and protein levels were evaluated by reverse transcription-polymerase chain reaction(RT-PCR) and

Western blot to ensure the NDNF mRNA and protein was successfully knocked down in young hBM-MSCs.

ELISA and in vitro *tube formation assay*

NDNF-transduced hBM-MSCs (Old+NDNF) or empty vector-transduced hBM-MSCs (Old) were normally cultured to 70-80% confluence, after which the culture medium was replaced with 2% FBS-IMDM and cultured for 4 more days. The conditioned medium was then harvested for the following Enzyme-Linked Immunosorbent Assay (ELISA) analysis and tube formation assay. The harvested conditioned medium was concentrated using an Amicon ultra-4 centrifugal filter unit (Millipore) and the NDNF level was detected in the conditioned medium with the NDNF ELISA kit (SEU433Hu, USCN Business Co., Wuhan, China) following the manufacturer's instructions.

For the endothelial tube formation assay, human umbilical vein endothelial cells (HUVECs) were used. Briefly, 100ul/well matrigel was coated in the 96-well plate and HUVECs were suspended with the conditioned medium. 2.5×10^4 cells/well were dispensed and incubated for 3 h. Pictures were taken using a Nikon microscope and the numbers of tubes counted to measure tube-forming ability.

1. Bartmann C, Rohde E, Schallmoser K, Pürstner P, Lanzer G, Linkesch W, Strunk D. Two steps to functional mesenchymal stromal cells for clinical application. Transfusion. 2007;47(8):1426-35.

Supplemental Figure Legends

Supplemental Figure 1: NDNF transduction rejuvenated old hBM-MSCs by suppressing the senescence-associated phenotype.

(A) Western blot showed that the expression of phospho-ATM and total ATM, the senescence-associated DNA damage protein, was significantly higher in empty vector-transduced (Old) compared to NDNF-transduced (NDNF+Old) hBM-MSCs. **(B)** Western blot showed that the expression of phospho-chk1, phospho-p53 and total p53, the senescence-associated DNA damage proteins, was significantly higher in empty vector-transduced (Old) compared to NDNF-transduced (NDNF+Old) hBM-MSCs. **(C)** Western blot showed that the expression of p-27 and p-21, the senescence-associated CDK inhibitors, was significantly higher in empty vector-transduced (Old) compared to NDNF-transduced (NDNF+Old) hBM-MSCs. n=5/group. ATM: Ataxia telangiectasia mutated kinase; p-ATM: Phospho-ATM (Ser1981), p-chk1: phospho-chk1(Ser345); p-53: phospho-p53 (Thr155); p27: p27(Kip1); p21: p21(Waf1); CDK inhibitors: cyclin-dependent kinase inhibitors.

Supplemental Figure 2: NDNF transduction enhanced the self-renewal ability and the differentiation potential to form adipocytic and osteocytic lineage of old hBM-MSCs.

(A) Digital image showing colonies produced by old hBM-MSCs.The number of colonies was significantly lower in empty vector-transduced (Old) compared to NDNF-transduced (NDNF+Old) hBM-MSCs. n=5/group. **(B)** Representative Oil Red O staining and quantification of Oil Red O stained area indicated that there were more MSCs undergoing adipogenic differentiation in NDNF+Old than in Old group. **(C)** Higher mRNA level of

adipogenic-related gene LPL in NDNF-transduced (NDNF+Old) compared to empty vector-transduced (Old) hBM-MSCs.n=5/group. **(D)** Osteoblast differentiation of hBM-MSCs was determined by Alizarin Red S staining.There were more Alizarin Red S stained area in NDNF+Old than in Old group. **(E)** Higher mRNA level of osteogenic-related gene COL1A1 in NDNF-transduced (NDNF+Old) compared to empty vector-transduced (Old) hBM-MSCs. n=5/group. LPL:Lipoprotein lipase; COL1A1: Collagen, type I, alpha 1.

Supplemental Figure 3: Knock-down NDNF in young hBM-MSCs increased cell senescence and decreased cell proliferative capacity

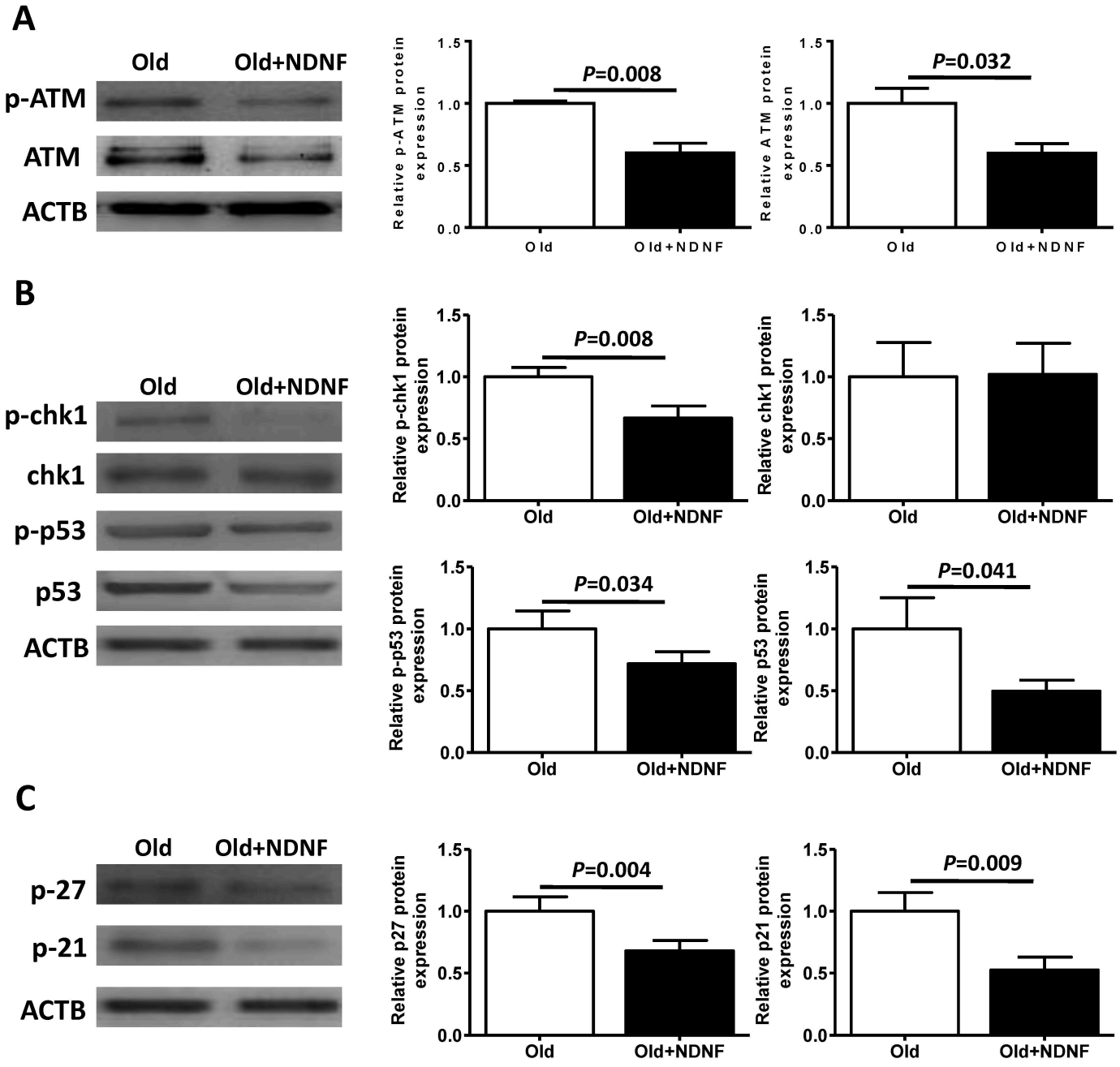
(A) Representative micrographs of senescence-associated β galactosidase staining and quantification of β-gal^{+} cells in the scrambled siRNA (Young) and NDNF siRNA (Young+siNDNF) transfected hBM-MSCs. **(B)** Cell growth curves showed that the growth rate was significantly higher in the scrambled siRNA (Young) compared to NDNF siRNA (Young+siNDNF) transfected hBM-MSCs. **(C)** Western blot showed that the expression of phospho-ATM and total ATM, the senescence-associated DNA damage protein, was significantly lower in the scrambled siRNA (Young) compared to NDNF siRNA (Young+siNDNF) transfected hBM-MSCs. **(D)** Western blot showed that the expression of phospho-chk1, phospho-p53 and total p53, the senescence-associated DNA damage proteins, was significantly lower in the scrambled siRNA (Young) compared to NDNF siRNA (Young+siNDNF) transfected hBM-MSCs. **(E)** Western blot showed that the expression of p-27 and p-21, the senescence-associated CDK inhibitors, was significantly lower in the scrambled siRNA (Young) compared to NDNF siRNA (Young+siNDNF) transfected hBM-MSCs. n=5/group. ATM: Ataxia telangiectasia mutated kinase; p-ATM: Phospho-ATM (Ser1981), p-chk1: phospho-chk1(Ser345); p-53:

phospho-p53 (Thr155); p27: p27(Kip1); p21: p21(Waf1); CDK inhibitors: cyclin-dependent kinase inhibitors.

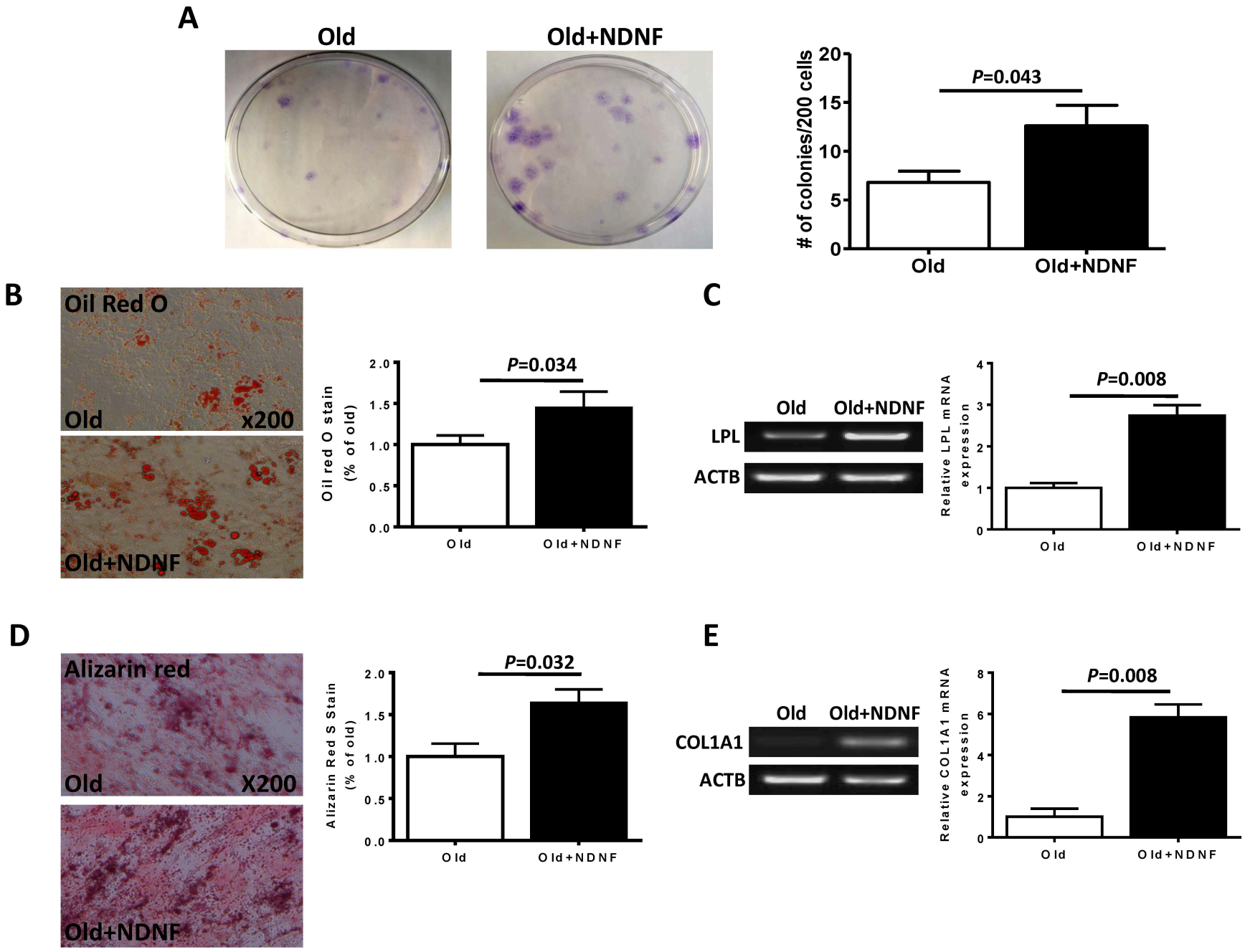
Supplemental Figure 4: Knock-down of NDNF decreased the self-renewal ability but did not alter the differentiation potential to form adipocytic and osteocytic lineage of young hBM-MSCs.

(A) Digital image showing colonies produced by young hBM-MSCs.The number of colonies was significantly higher in young compared to young+siNDNF transfected hBM-MSCs. n=5/group. **(B)** Representative Oil Red O staining and quantification of Oil Red O stained area indicated that adipogenic differentiation in young was the same as the young+siNDNF transfectedhBM-MSCs. **(C)** The mRNA level of adipogenic-related gene LPL in young compared to young+siNDNF group.n=5/group. **(D)** Osteoblast differentiation of hBM-MSCs was determined by Alizarin Red S staining.The Alizarin Red S stained area in young was the same as in young+siNDNF group. **(E)** The mRNA level of osteogenic-related gene COL1A1 in young compared to siNDNF-transfected (young) hBM-MSCs. n=5/group. LPL:Lipoprotein lipase; COL1A1: Collagen, type I, alpha 1.

Supplemental Figure 1



Supplemental Figure 2



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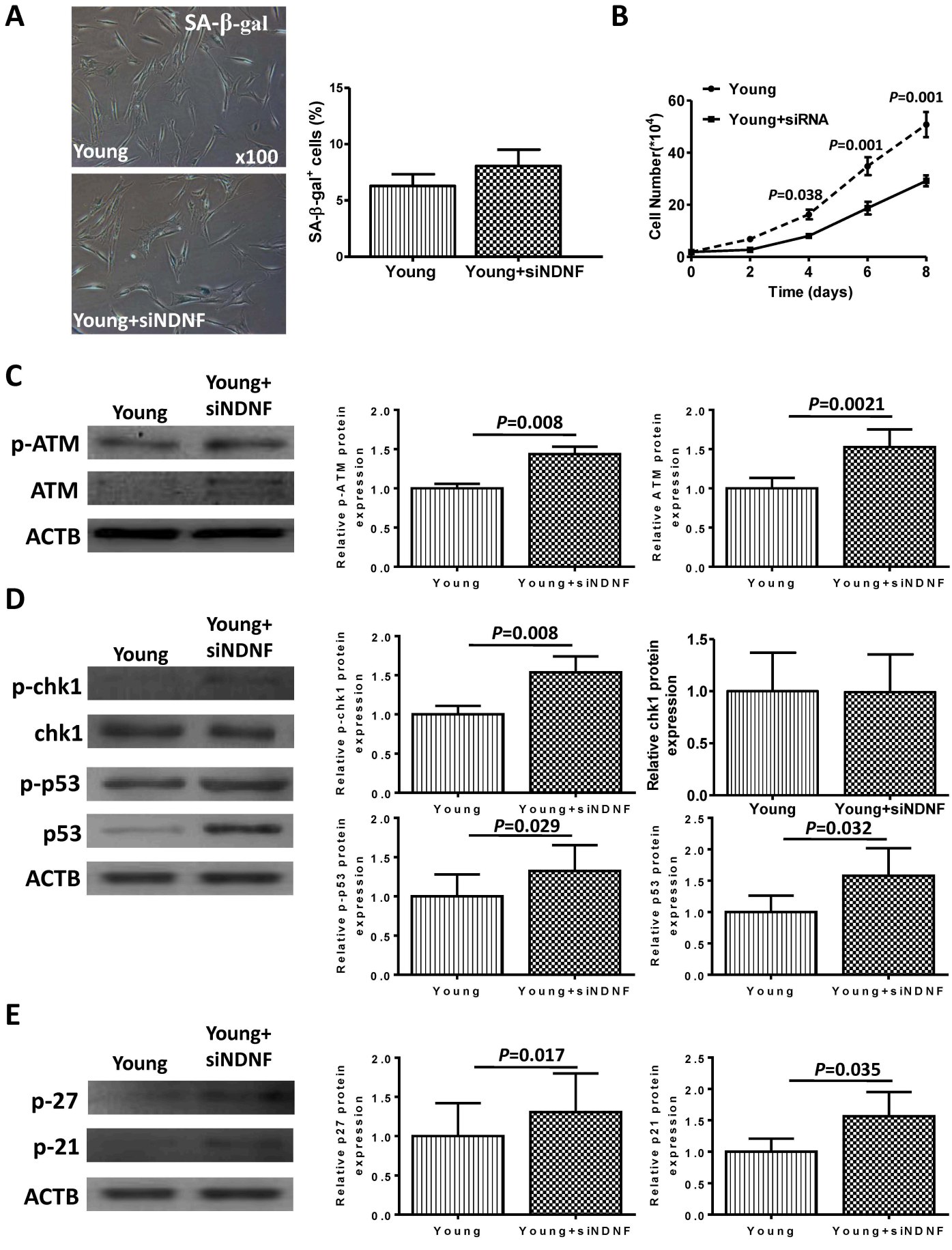

Supplemental Figure 4