Stem Cell Reports, Volume *6*

Supplemental Information

Inhibition of IKK/NF-κB Signaling Enhances Differentiation of Mesenchymal Stromal Cells from Human Embryonic Stem Cells

Peng Deng, Chenchen Zhou, Ruth Alvarez, Christine Hong, and Cun-Yu Wang

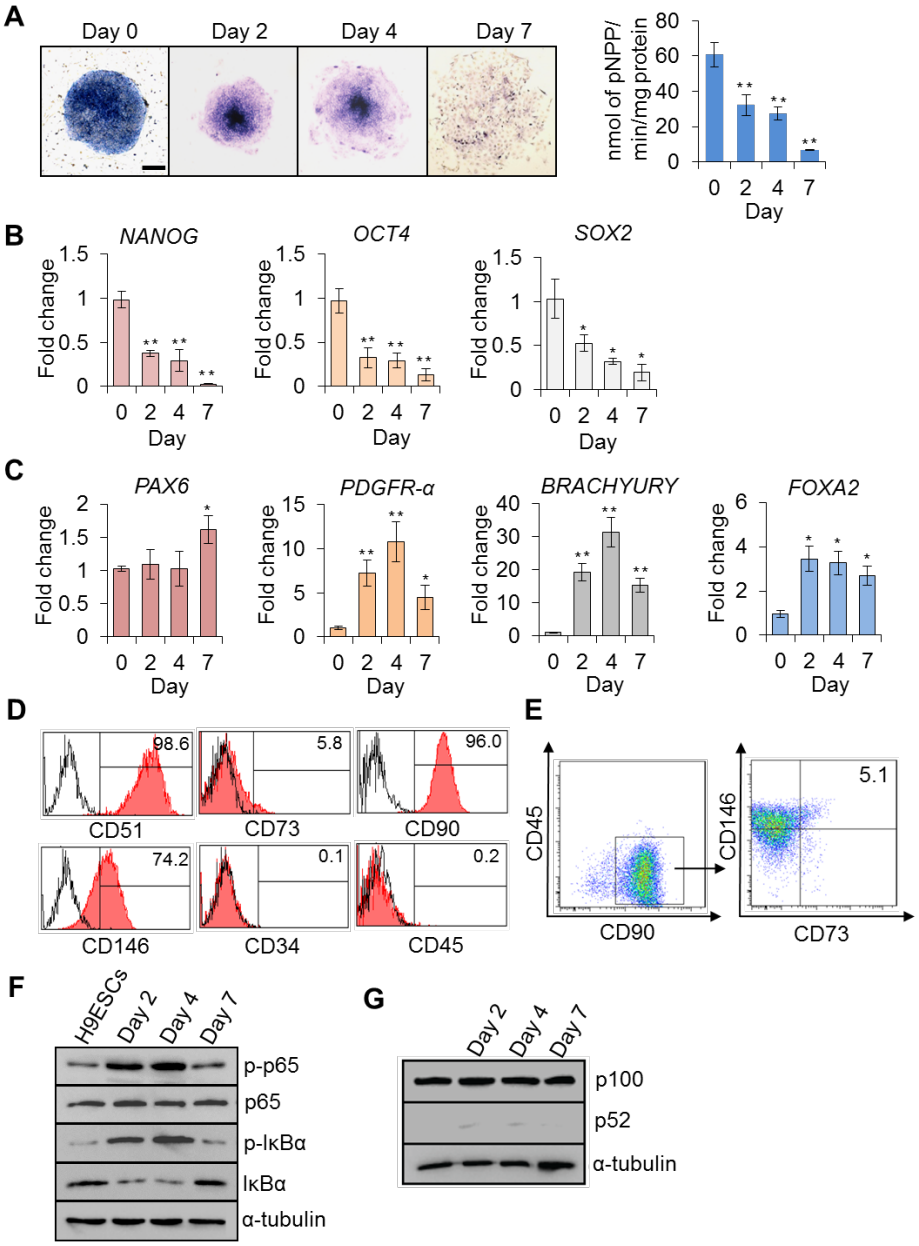

Figure S1. Spontaneous differentiation of H9 hESCs. Related to Figure 1. (A) ALP staining and quantitative ALP activity assay at 0, 2, 4, and 7 days. Bar indicates 200 μm. (B) qRT-PCR results of pluripotent markers (NANOG, OCT4, POU5F1) at 0, 2, 4, and 7 days. (C) qRT-PCR results of an ectodermal gene (PAX6), mesodermal genes (PDGFR-α and BRACHYURY), and an endodermal gene (FOXA2) at 0, 2, 4, and 7 days. (D) Flow cytometry analysis of cells expressing MSC surface markers. (E) Four-color flow cytometry analysis for CD45, CD90, CD73, CD146 expression was used to isolate $CD73^{+}CD90^{+}CD146^{+}CD45^{-}$ MSCs. (F) Western blot of both phosphorylated p65 and IκBα at 0, 2, 4, and 7 days of H9 hESC differentiation. (G) Western blot analysis of p100 and p52 at 0, 2, 4, and 7 days of H1 hESC differentiation. Three independent experiments were performed. * $p < 0.05$, ** $p < 0.001$.

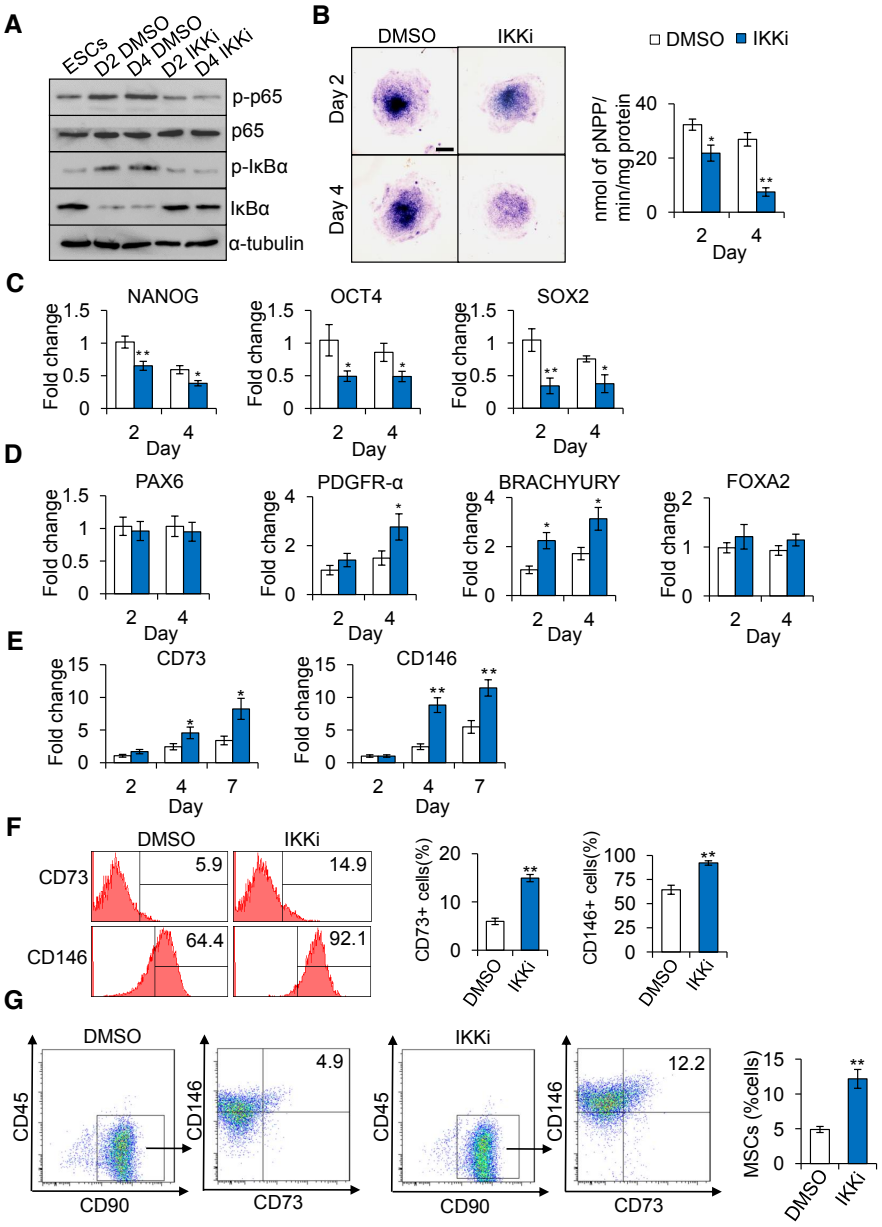

Figure S2. Effect of IKKi treatment on mesenchymal lineage specification of H9 hESCs. Related to Figure 2. (A) Western blot of p65, phosphorylated p65, IκBα and phosphorylated IκBα at 0, 2, and 4 days of H9 hESC differentiation. (B) ALP staining and quantitative ALP activity assay at 2 and 4 days. Bar indicates 200 μm. (C) qRT-PCR results of *NANOG, OCT4, POU5F1* at 2 and 4 days. (D) qRT-PCR results of *PAX6*, *PDGFR-α, BRACHYURY*, and *FOXA2* at 2 and 4 days. (E) qRT-PCR results of *CD73* and *CD146* at 2, 4, and 7 days. (F) Flow cytometry analysis of cells with or without IKKi treatment examining MSC marker expression. (G) Four-color flow cytometry analysis for CD45, CD90, CD73, CD146 expression to isolate $CD73^{+}CD90^{+}CD146^{+}CD45^{-}$ MSCs following 7 days of H9 hESC differentiation with or without IKKi. Proportions of $CD73^{+}CD90^{+}CD146^{+}CD45^{-}$ MSCs generated. Three independent experiments were performed. * $p < 0.05$, ** $p<0.001$.

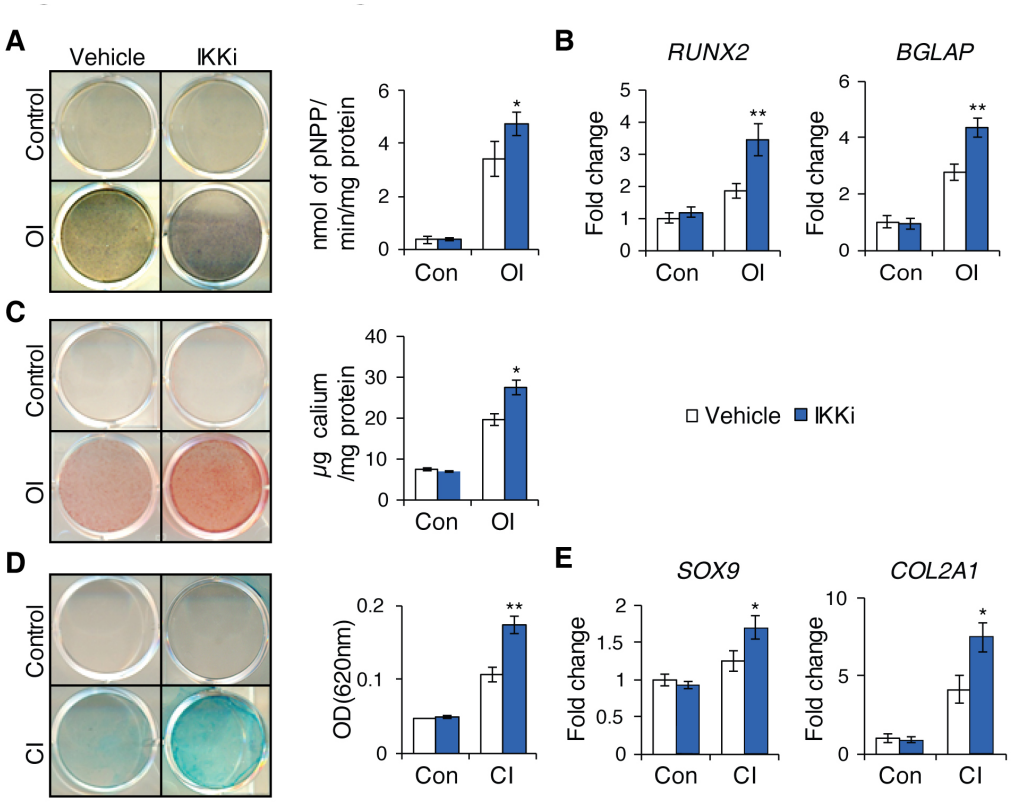

Figure S3. Osteogenic and Chondrogenic differentiation potential of differentiated hESCs with or without IKKi treatment. Related to Figure 2. After 7 days of differentiation, hESCs with or without IKKi treatment were digested by trypsin to generate a single-cell suspension. To test the differentiation potential of these cells, they then were seeded into plates, and cultured in induction medium as indicated. (A) ALP staining and quantitative ALP activity assay after 14 days of osteogenic induction (OI). (B) qRT-PCR results of osteogenic markers (*RUNX2* and *BGLAP*) after 7 days of OI. (C) ARS staining and quantification after 14 days of OI. (D) Alcian blue staining and quantification of derived cells with DMSO or IKKi treatment after 21 days of chondrogenic induction (CI). (E) qRT-PCR results of chondrogenic markers (*SOX9* and *COL2A1*) after 14 days of CI. For all *in-vitro* experiments, three independent experiments were performed. * $p < 0.05$, ** $p < 0.001$.

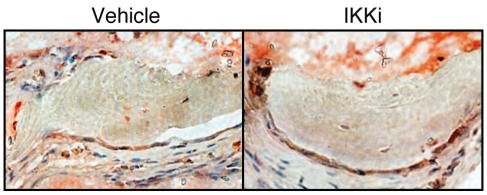

Figure S4. Osteocalcin staining of ectopic bone *in vivo* by sorted MSCs derived from IKKi-treated H1 hESCs. Related to Figure 4.

Table S1. Primers for RT-PCR

Name	Forward	Reverse
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
NANOG	CTGGCTGAATCCTTCCTCTC	CATGAGATTGACTGGATGGG
SOX2	GCGAACCATCTCTGTGGTCT	GGAAAGTTGGGATCGAACAA
OCT4	GAAGGATGTGGTCCGAGTGT	GTGAAGTGAGGGCTCCCATA
PAX6	TGGGCAGGTATTACGAGACTG	ACTCCCGCTTATACTGGGCTA
PDGFR-α	TATGTGCCAGACCCAGATGT	GGAGTCTCGGGATCAGTTGT
BRACHYURY	TATGAGCCTCGAATCCACATAGT	CCTCGTTCTGATAAGCAGTCAC
FOXA2	GGAGCAGCTACTATGCAGAGC	CGTGTTCATGCCGTTCATCC
CD73	TTACACAGGCAATCCACCTTC	TTACACAGGCAATCCACCTTC
CD146	CTGCTGAGTGAACCACAGGA	CACCTGGCCTGTCTCTTCTC
cIAP2	CCTAGCTGCAGATTCGTTCA	GAGCCACGGAAATATCCACT
IL-6	GGCACCTCAGATTGTTGTTG	TAAGTTCTGTGCCCAGTGGA
IL-8	ATGACTTCCAAGCTGGCCGTG	TCTCAGCCCTCTTCAAAAACTTCTC

Supplemental Experimental Procedures

Induction of Osteogenic, Chondrogenic, and Adipogenic Differentiation

To induce osteogenic differentiation, cells were grown in osteogenic induction (OI) medium containing 1nM dexamethasone, 100μM ascorbic acid, and 5mM beta-Glycerophosphate. To induce chondrogenic differentiation, cells were cultured in chondrogenic induction (CI) medium containing 100mM sodium pyruvate, 40μg/mL proline, 100nM dexamethasone, 200μM ascorbic acid, and 10ng/mL TGF-β3. To induce adipogenic differentiation, the cells were cultured in adipogenic induction (AI) medium containing 1μM dexamethasone, 10μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.2mM indomethacin. Media was changed every 2 days.

ALP, Alizarin Red Staining, Alcian Blue Staining and Oil-Red O Staining

1x 10^5 cells were seeded into each well in 12-well plates for further differentiation and staining. ALP activity assay and ARS were performed as described previously after 7 days and 14 days of osteogenic differentiation respectively (Chang et al., 2009). After 3 weeks of chondrogenic differentiation, Alcian blue staining was performed. Cells were fixed with 10% neutral buffered formalin for 15 min at room temperature, and then incubated in Alcian blue staining solution (1% Alcian blue in 3% Acetic acid) for 30 min. For quantification, stained Alcian blue was eluted with 6 M guanidine HCl for 6 hrs at room temperature. The optical absorbance was measured at 650 nm using a microplate reader. After 21 days of adipogenic induction, Oil-Red-O staining was performed using an OIL-RED-O STAIN KIT according to the manufacturer's instructions (Diagnostic BioSystems, Pleasanton, CA, USA). For quantification, stained Oil-Red-O was eluted with 100% isopropanol and the optical absorbance was measured at 450nM using a microplate reader.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from ES-MSCs using Trizol reagents (Invitrogen). Two-μg aliquots of RNA were synthesized using random hexamers and reverse transcriptase according to the manufacturer's protocol (Invitrogen). qRT-PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) and the Icycler iQ Multi-color Real-time PCR Detection System (BioRad) and normalized to GAPDH. Data shown are representative of three independent experiments. Primer sequences are listed in Table

S1.

Western Blot Analysis

Protein was isolated from cells using CelLytic MT solution (Sigma), supplemented with protease inhibitor cocktail (PIC, Promega, Southampton, USA). 40 µg aliquots of protein were separated by a 7.5% SDS-polyacrylamide (PAGE) gel. The following primary antibodies and reagents were used: rabbit anti-human phosphorylated p65-S536 (#3033S, Cell Signaling, Danvers, MA, USA), rabbit anti-human p65 (SC-109, Santa Cruz Biotech, Dallas, Texas, USA), rabbit anti-human phosphorylated IκBα (2859, Cell Signaling), rabbit anti-human phosphorylated IκBα-S32 (2859, Santa Cruz Biotech), rabbit anti-human p100 and p52 (4882, Cell Signaling), mouse anti-human α-tubulin (SC-8035, Santa Cruz Biotech, Dallas, Texas, USA). Detection was performed with using Luminal/Enhancer Solution and Super Signal West Stable Peroxide Solution (Thermo). Blots were then exposed to HyBlot CL autoradiography films (Denville Scientific, South Plainfield, NJ, USA).

Immunohistochemistry (IHC)

Paraffin-embedded sections were performed by the UCLA Translational Pathological Core Laboratory. Samples were deparaffinized with xylene and rehydrated with distilled water through an ethanol series. Tissue antigen retrieval was carried out and immunohistochemistry were performed by using EnVision system (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions. Rabbit anti-human osteocalcin antibody (1:100; SC-30044) was purchased from Santa Cruz Biotechnology. The section counterstained with hematoxylin QS (Vector Laboratories, Burlingame, CA, USA), and at least three pictures of each sample were taken randomly (Olympus).

Flow Cytometry and Fluorescence-Activated Cell Sorting (FACS)

Cells were digested with trypsin (Invitrogen) for 2 min at 37°C, neutralized and passed through a 40 µm cell strainer. Then, cells were washed twice with FACS buffer (PBS, 10 mM EDTA, and 2% FBS) and resuspended at a maximum concentration of 2 x 10^5 cells per 100 µl. Cells were stained with indicated antibodies for 30 min on ice in dark, washed, and resuspended in PBS. Samples were analyzed on a BD LSR II analyzer or sorted on a BD FACS Aria III. Cell gating was based on comparison with isotype negative controls and single stained controls. Cells were sorted into serum-free DMEM

media for gene expression analysis or into complete media for cell culture. Antibodies used include CD34 (PE; 316407), CD45 (PerCP-Cy5.5; 103234), CD51 (PE; 327910), CD73 (APC; 344006), CD90 (FITC; 328108), CD146 (PE; 342004) (Biolegend, London, UK).

Transplantation in Immunocompromised Mice

All procedures were performed in accordance with the approved protocol by the University of California, Los Angeles (UCLA), and were oversight by UCLA Animal Research Committee (ARC). Briefly, sorted MSCs (1×10^6) were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) scaffolds and then transplanted subcutaneously in 8-week-old nude mice (n=6). Sorted ES-MSCs derived from vehicle-treated hESCs were transplanted into the left dorsal subcutaneous sites and sorted MSCs derived from IKKi-treated H1 hESCs were transplanted into the left dorsal subcutaneous sites. Eight weeks after transplantation, the transplants were collected. H&E staining was performed and at least three pictures of each sample were taken randomly (Olympus). For quantification of mineralized tissue, SPOT 4.0 software (Diagnostic Instruments) was used to measure the area of mineralized tissue versus total area.