CD40 stimulation improves osteogenesis from mesenchymal stem cells via the activation of TGF-β

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1. Supplementary Methods:

1.1. Cell Lines and Primary Cells:

Bone marrow-derived MSCs (BM-MSCs) were obtained from mouse BM using the established protocol. While NIH-3T3 (ATCC #TIB-71) is a mouse fibroblast cell line, RAW 264.7 (ATCC #CRL-1658) is a mouse macrophage-like cell line. MC3T3-E1 (ATCC #CRL-2593) is a mouse osteoblast progenitor cell line and HDPSC (HiMedia #CL008) is a human dental pulp stem cell line. MG-63 is osteosarcoma cell line (ATCC#CRL-1427).

1.2. Flow cytometry Quantification:

(CD44-Pe-Cy5, BD Pharmingen; CD73-APC, Biolegend; CD90-APC, BD Pharmingen; CD105-PE, eBioscience; CD40-APC, eBioscience)

1.3. Treatment of cells with agonistic anti-CD40 antibody, CpG, SB431542:

(anti-CD40 # 553787, BD Pharmingen; CpG:tlrl-1826, Invivogen; SB431542, 616461, Merck)

1.4. Osteogenic differentiation:

osteo-induction medium (A1007201, Invitrogen), 1-Amino-2-methyl-1 propanol (A9199, Sigma Chemicals, St Louis, MO), PnPP (p-Nitrophenyl phosphate Disodium Salt Hexahydrate # 88485, SRL), microscope (Nikon, Eclipse, TE2000-U)

1.5. Adipogenic differentiation:

BM-MSCs were seeded in 24-well plates and cultured. After 72 h, adipogenic medium (A1007001, Invitrogen) was added with partial changes every 3 days; controls received DMEM only. On days 15, 18, and 21, cells were stained with Oil Red O to visualize lipid droplets. Stain was quantified by dissolving in isopropanol for 30 min and measuring absorbance at 510 nm.

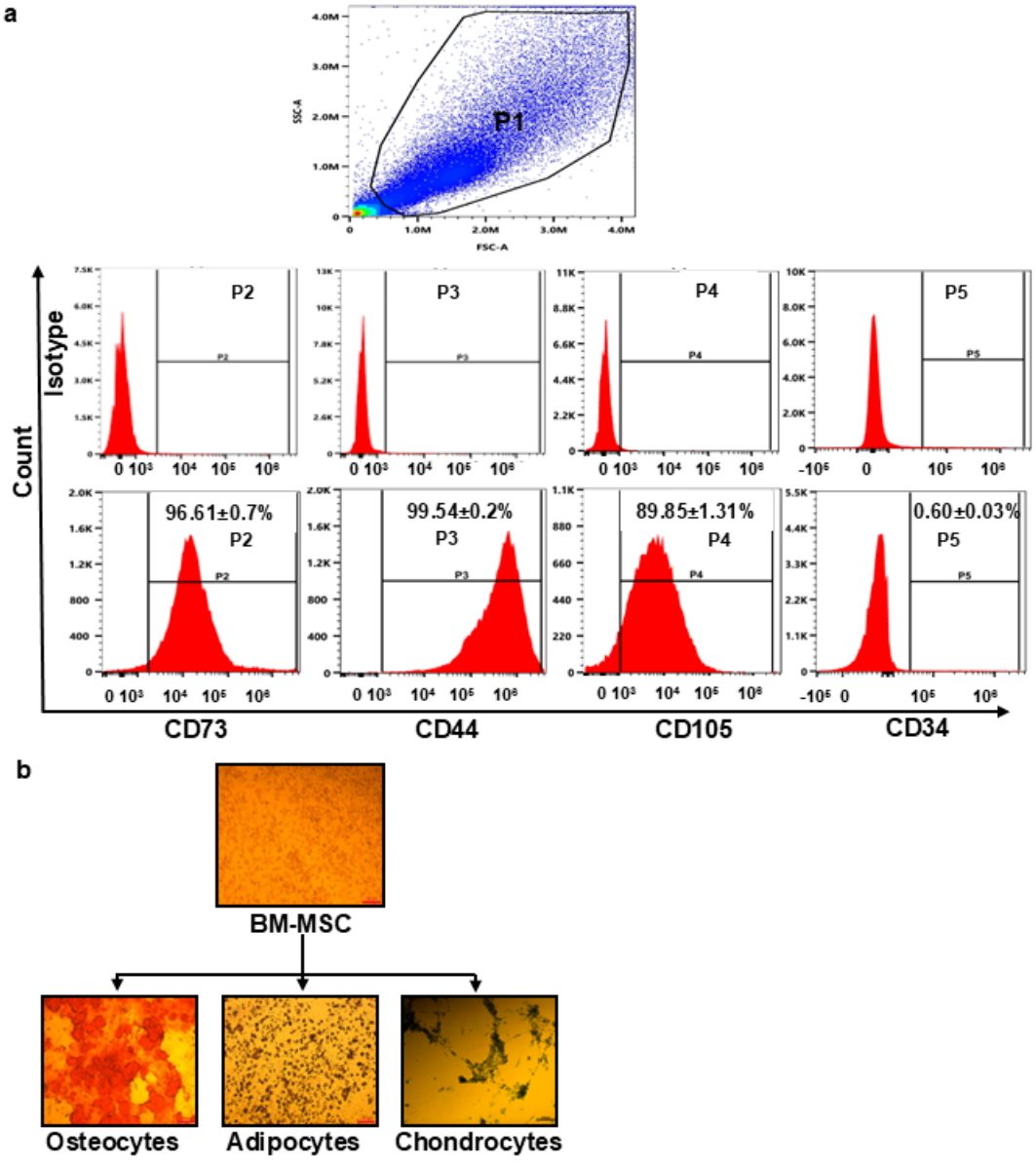
1.6. Chondrogenic differentiation:

BM-MSCs were seeded in 24-well plates and cultured. After 72 h, chondrogenic medium (A1007101, Invitrogen) was added with partial medium changes every 3 days; controls received DMEM only. On day 21, cells were stained with Alcian Blue to detect glycosaminoglycans (GAGs) and proteoglycan clusters under the microscope. For quantification, the stain was dissolved in 8M guanidine HCl at room temperature for 30 min on a rocker, and absorbance was measured at 620 nm.

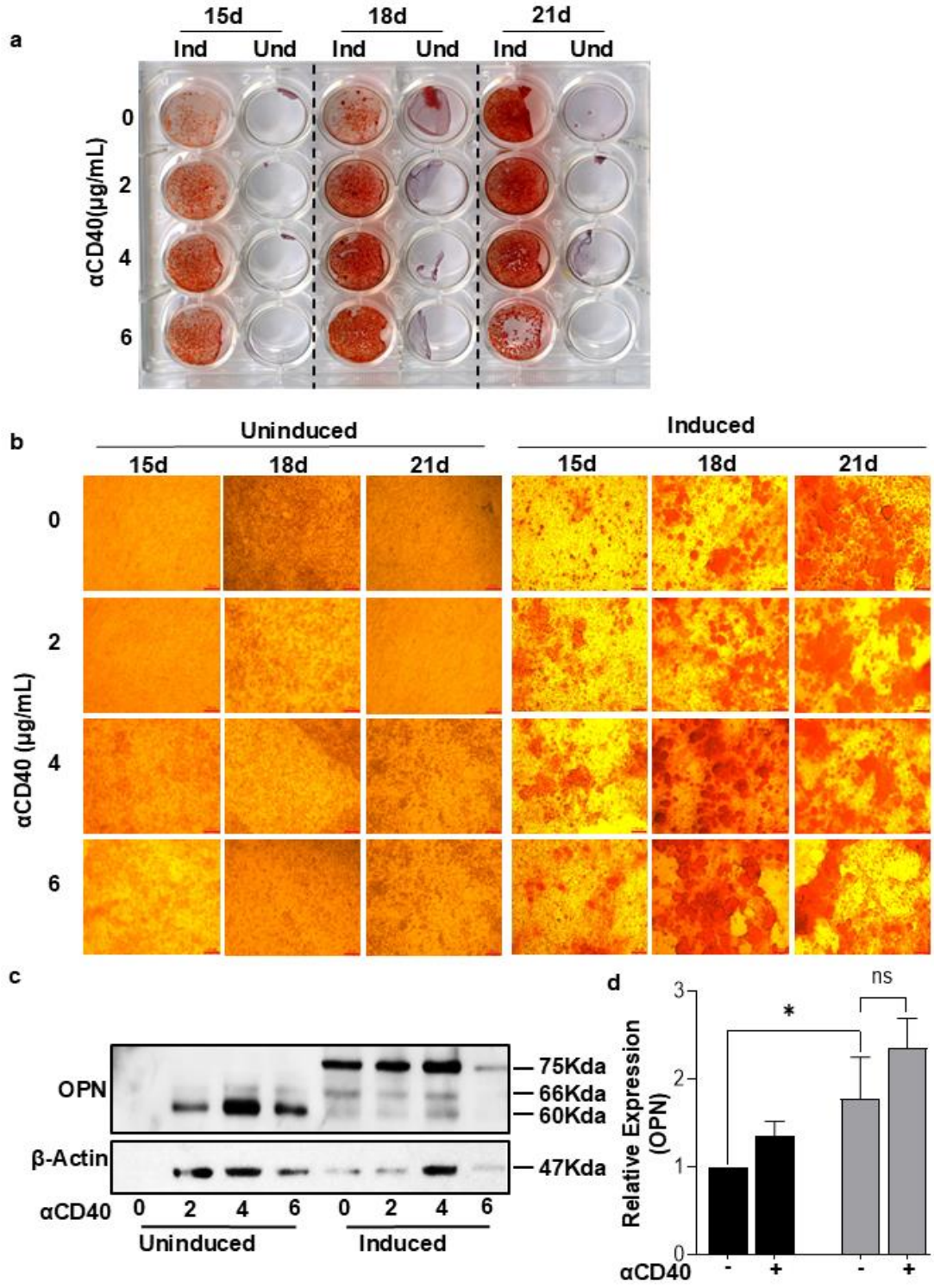
1.7. Quantitative Real time PCR:

RNAiso Plus (Takara #9109); Thermoscript RT-PCR system (Invitrogen #11146-016) 2× SYBR Premix Ex Taq II (5 μl; Takara #RR420).

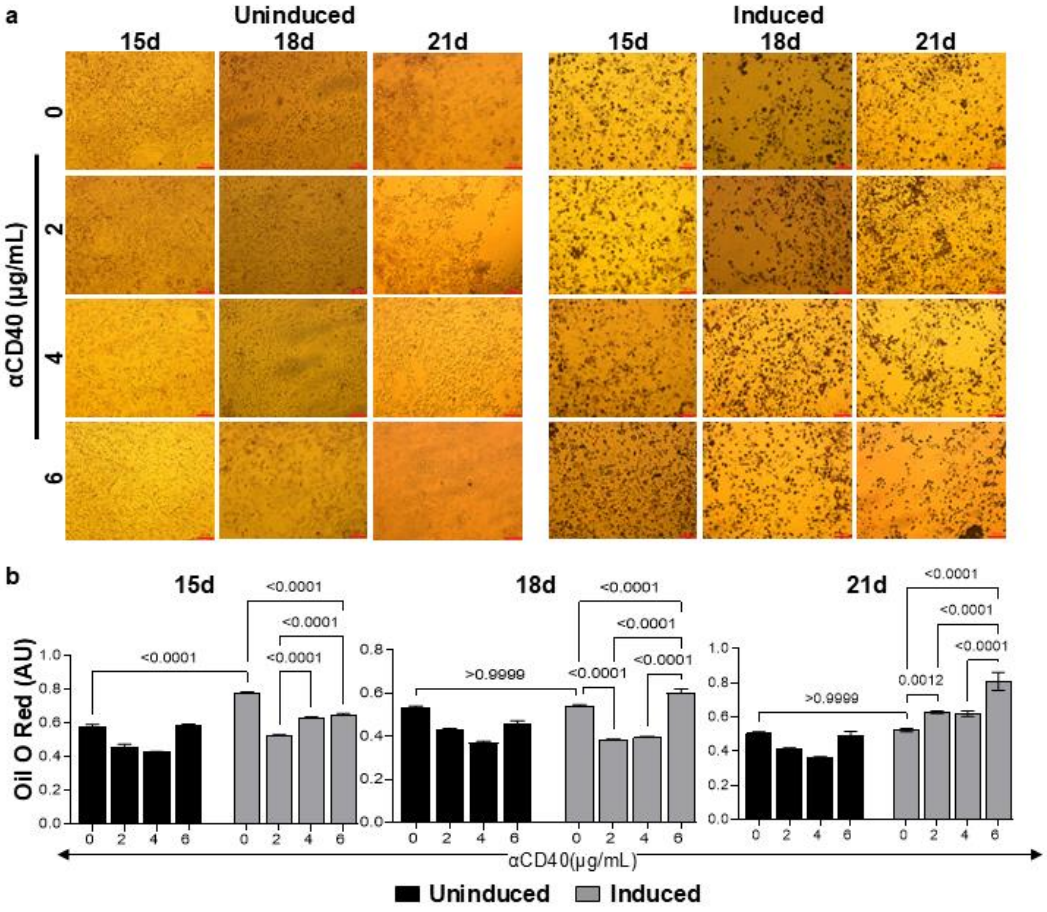
2. **Supplementary Figures:**



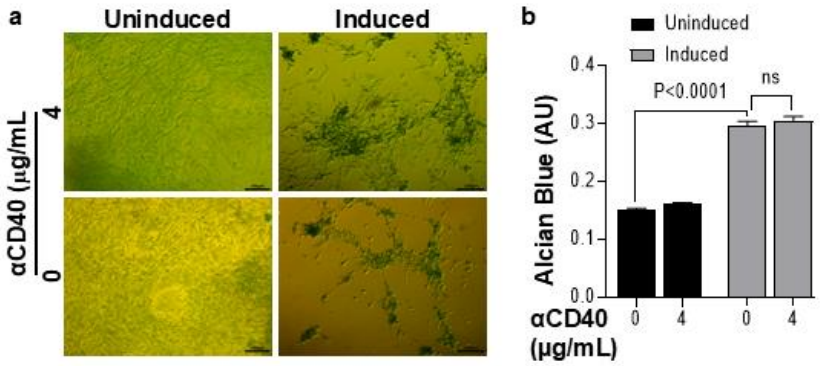
Supplementary Figure 1: Bone marrow mesenchymal stem cell characterization. (a) MSC-specific positive markers expression in passage-3 BM-MSCs revealed 96.61±0.70%, 99.54±0.2% and 89.85±1.31% cells to be positive for CD73, CD44 and CD105, respectively, while the $CD34^{+}$ cells, a negative marker, were negligible (0.60±0.03%). (b) Induced differentiation of BM-MSCs into Osteocytes, Adipocytes, and Chondrocytes confirmed the tri-lineage differentiation capability. Data represented as mean ± SEM; n = 3.



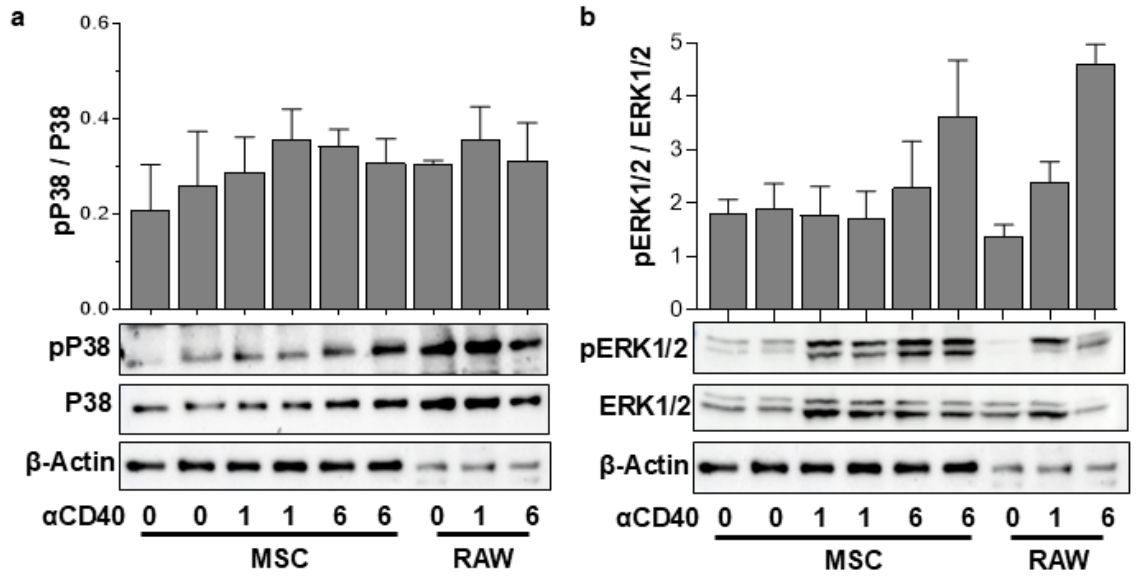
Supplementary Figure 2: CD40 stimulation promotes osteogenesis in BM-MSCs. (a) Microscopy images of the ARS-stained cells (Scale Bar: 100μm) reflected a significantly higher osteocalcin (OCN) expression upon CD40 stimulation in osteoinduced MSCs. (b) Quantification of OCN by recording the absorbance at 415 nm revealed a dose-dependent CD40 stimulation-induced osteoinduction in induced MSCs (Scale Bar: 100μm). (c) OPN expression detected by immunoblotting on day 14 of induction following αCD40 stimulation at 2, 4, and 6 μg/mL doses (n = 3). (d) Densitometric quantification of OPN expression for samples stimulated with 4 μg/mL αCD40 (n = 4).



Supplementary Figure 3: Effect of CD40 stimulation on BM-MSC adipogenesis. (a) BM-MSCs were either induced with adipogenic media (Induced) or maintained using expansion media (DMEM with 10% FCS & Glutamine) (Uninduced) along with anti-CD40 antibody (2µg/ml, 4 µg/ml, and 6 µg/ml) for 15, 18 and 21 days with partial media replenishment every 72h. Oil O red assay was performed on respective days (Scale Bar: 100µm). (b) The Oil O red stain was dissolved in 100% isopropanol and absorbance was taken at 510nm and values were plotted. Data represent mean ± SEM; n = 3.



Supplementary Figure 4: αCD40 stimulation does not significantly affect Chondrogenesis on day 21 of Induction. (a) Representative microscopy images showing spindle-shaped morphology of uninduced MSCs and Alcian Blue-stained extracellular matrix rich in polysaccharides in induced chondrogenic samples. (b) Quantitative analysis of Alcian Blue staining (absorbance at 600nm) in αCD40-stimulated and unstimulated cultures. Data represented as mean ± SEM; n = 3.



Supplementary Figure 5: CD40 doesn’t act through ERK and P38 MAPK in BM-MSCs. BM-MSCs derived from BALB/c BM passage 3 cells treated with anti-CD40 antibody (1μg/ml or 6μg/ml) for 15 min, followed by lysis with RIPA lysis buffer. (a)Activation of P38 MAPK (b) and ERK1/2 in response to αCD40 stimulation at indicated doses in BM-MSCs. RAW cells served as the control. β-ACTIN was taken as the loading control for normalization. Data represented as mean ± SEM; n = 3.

Supplementary Tables:

Table-1: Sequences of the qPCR primers used in the study.

Gene	Primer	Sequence
CD40	Forward	5’-AAGGAAGGACAACACTGC -3’
	Reverse	5’-GACAGACGGTATCAGTGGT-3’
β-Actin	Forward	5’-ATGGTGGGAATGGGTCAGAA-3’
	Reverse	5’-TTGTAGAAGGTGTGGTGCC-3’
TLR9	Forward	5′-ACTGAGCACCCCTGCTTCTA-3′
	Reverse	5′-AGATTAGTCAGCGGCAGGAA-3′
TGF-βR1	Forward	5’-CGGTTTGGAGAAGTTTGGCG-3’
	Reverse	5’-TCTCTGCCTCTCGGAACCAT-3’

Table-2: The antibodies used for immune-blot for the study:

Primary antibodies	Dilutions	Secondary antibodies	Dilutions
CD40:CD40(C-20); sc-975	1:2000	Anti-Rabbit IgG HRP (sc-2004)	1:5000
pP38: p-p38 MAPK (D-8); sc-7973	1:1000	Anti-Mouse IgM HRP (sc-2064)	1:3000
P38: p38 MAPK (A-12); sc-7972	1:2000	Anti-Mouse IgG HRP(sc-2005)	1:3000
pERK: p-ERK Antibody (E-4); sc-7383	1:3000	Anti-Mouse IgG HRP(sc-2005)	1:3000
ERK: ERK 2 Antibody (C-14); sc-154)	1:3000	Anti-Rabbit IgG HRP(sc-2004)	1:3000
p-Smad2/3 (Ser 423/425); sc-11769	1:2000	Anti-Goat IgG HRP(sc-2354)	1:3000
Smad2/3 Antibody (C-8); sc-133098	1:2000	Anti-Mouse IgG HRP(sc-2005)	1:3000
RunX2 antibody (F-2); SC-390351	1:3000	Anti-Mouse IgG HRP(sc-2005)	1:5000
RunX2 antibody (D1L7F); CST-12556S	1:3000	Anti-Rabbit IgG HRP(sc-2004)	1:5000
OPN antibody(AKm2A1); SC-21742	1:3000	Anti-Mouse IgG HRP(sc-2005)	1:5000