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Supplemental Information

Craniometaphyseal Dysplasia Mutations in ANKH Negatively Affect Human Induced Pluripotent Stem Cell Differentiation into Osteoclasts

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Supplemental Data

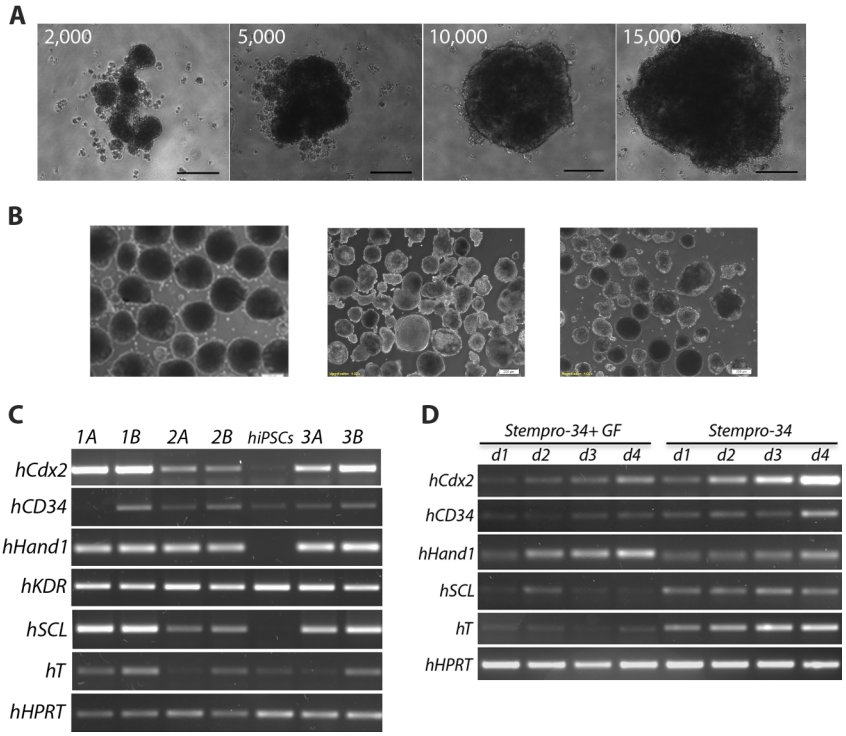

Figure S1. Promotion of mesoderm differentiation. Related to Figure 1 A) To generate relatively uniformly sized EBs, 2,000, 5,000, 10,000 and 15,000 cells/well were cultured on 96-well Nunclon Sphera microplates. Sizes of EBs are corresponding to the initial seeding densities. Scale bar=100µm. **B**) Sizes of EBs generated using our protocol (left panel, re-use the image of Figure 1B) are more homogenous compared to other published methods (middle and right panels) (1, 2). Scale bar=200µm. **C)** Optimization of culture conditions to promote EB mesoderm differentiation. Gene expression of mesoderm markers from day 4 EBs maintained under various culture conditions 1A, 1B, 2A, 2B, 3A and 3C (described in Table S1). **D**) Gene expression of mesoderm markers of EBs at day 1, 2, 3 and 4 cultured in Stempro-34 medium with or without supplementary growth factors (GF) provided with the Stempro kit. (n=3 technical replicates for each hiPSC line, 2 wild type hiPSCs used, 2 clones of each hiPSC line tested)

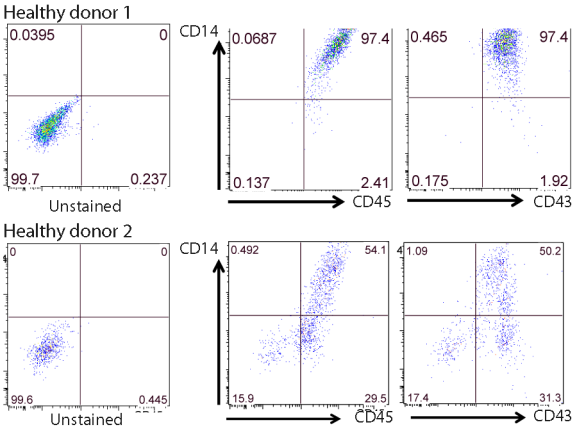

Figure S2. Related to Figure 1: Expansion analysis of myelomonocytic population collected at day 17 during Stage 2 from healthy donor 1 and 2 cultures. FACS analysis using cell surface markers CD14, CD43 and CD45. Negative staining controls are shown on left panel. (n=3 technical replicates for each hiPSC line)

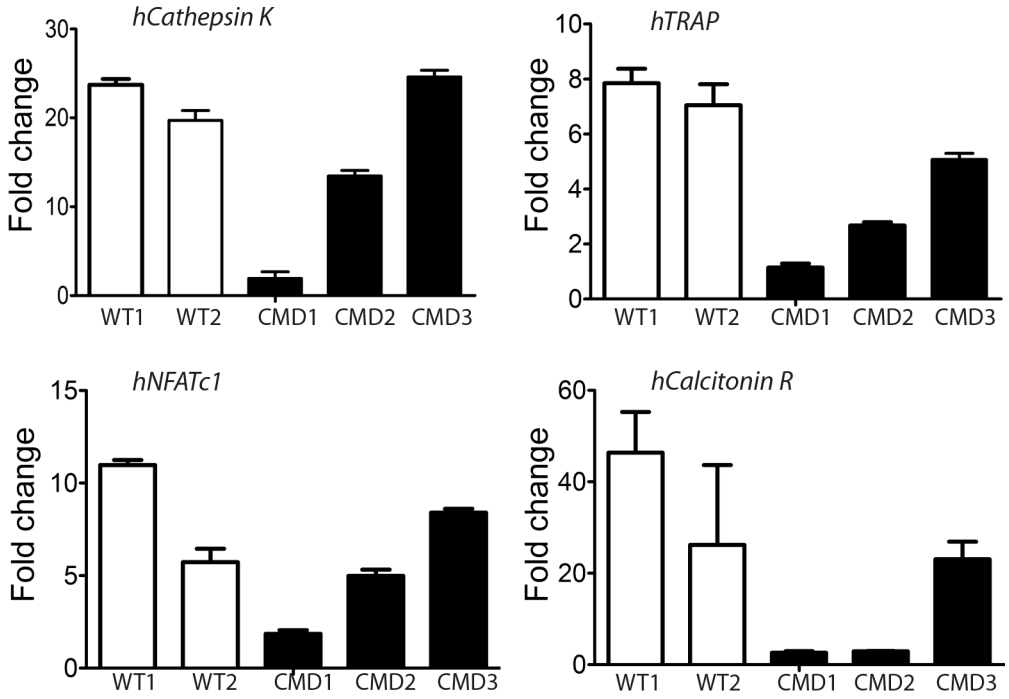

Figure S3. Related to Figure 2: Relative expression of osteoclast marker genes in individual hiPSC line from healthy donors and CMD patients shown by qPCR. Data presented are mean ± SD. (n=3 technical replicates for each hiPSC line)

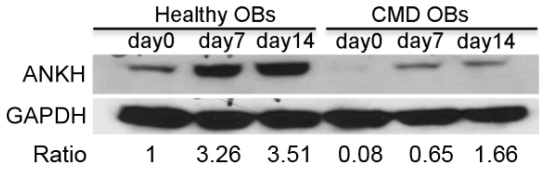

Figure S4. Related to Figure 3: A representative ANKH immunoblot of bone explant cultures from one healthy donor and one CMD patient differentiating for 0, 7, and 14 days. Numbers below indicate ratio of ANKH to GAPDH. (n=3 technical replicates)

Table S1: Culture conditions to maintain EBs derived from hiPSCs. Related to Figure 1.

Conditions	Culture medium	Cytokines
1A	Stempro-34, 2mM glutamine, 0.4mM MTG, A.A. 50µg/ml	Bmp4 (10) for 1 day, Bmp4 (10) + bFGF (5) for another 3 days
1 B	Stempro-34, 2mM glutamine, 0.4mM MTG, A.A. 50µg/ml	Bmp4 (25) + VEGF (50) for 2 days, day 2-4 Bmp4 (25), VEGF (50), SCF (50), TPO (50), FLT3 (50), bFGF (20)
2A	Stemline II, 20% FBS, 2mM glutamine, 0.4mM MTG, A.A. 50 µg/ml	Bmp4 (10) for 1 day, Bmp4 (10) + bFGF (5) for another 3 days
2B	Stemline II, 20% FBS, 2mM glutamine, 0.4mM MTG, A.A. 50 µg/ml	Bmp4 (25) + VEGF (50) for 2 days, day 2-4 Bmp4 (25), VEGF (50), SCF (50), TPO (50), FLT3 (50), bFGF (20)
3A	KO-DMEM, 20% FBS, 2mM L-glutamine, 0.1mM ME, 0.1mM NEAA	Bmp4 (10) for 1 day, Bmp4 (10) + bFGF (5) for another 3 days
3B	KO-DMEM, 20% FBS, 2mM L-glutamine, 0.1mM ME, 0.1mM NEAA	Bmp4 (25) + VEGF (50) for 2 days, day 2-4 Bmp4 (25), VEGF (50), SCF (50), TPO (50), FLT3 (50), bFGF (20)

MTG: monothioglycerol; A.A.: ascorbic acid; KO-DMEM: Knockout Dulbecco modificed Eagle medium; FBS: fetal bovine serum; NEAA: nonessential amino acids; ME: β-mercaptoethanol; cytokine concentrations in parentheses are ng/ml. Condition 1B was used in the described hiPSC osteoclast differentiation protocol.

Table S2: Primer sequences for qPCR. Related to Figure 2 and 3.

Gene	Forward primer	Reverse primer
CDX2	5'-CCCTAGGAAGCCAAGTGAAAACC-3'	5'-CTCCTTGGCTCTGCGGTTCTG-3'
CD34	5′-AAATCCTCTTCCTCTGAGGCTGGA-3′	5'-AAGAGGCAGCTGGTGATAAGGGTT-3'
Brachyury (T)	5′-TGTCCCAGGTGGCTTACAGATGAA-3′	5′-GGTGTGCCAAAGTTGCCAATACAC-3′
HAND1	5'-GAAAGCAAGCGGAAAAGGGAG-3'	5'-GGTGCGCCCTTTAATCCTCTT-3'
SCL	5'-AAGGGCACAGCATCTGTAGTCA-3'	5'-AAGTCTTCAGCAGAGGGTCACGTA-3'
CTSK	5'- CAGTGAAGAGGTGGTTCAGA -3'	5'-AGAGTCTTGGGGCTCTACCTT -3'
Calcitonin Receptor	5′-TCTCAGGAGTGAAAGCATTGCACATA -3′	5′-AATGCTATGACCGAATGCAGCAGTTA-3′
NFATc1	5'- AGAATTCGGCTTGCACAGG -3'	5'- CTCTGGTGGAGAAGCAGAGC -3'
TRAP (APC5)	5'-ACCTAGTTTGTTCTCTGATCGCCT-3'	5'-GGGATCTGTAATCTGACTCTGTCCTT-3'
HPRT	5'-ACTTGTCGCAGAAGCATC-3'	5'-GTGGGCGAACAGTGTAGAA-3'

Supplemental Experimental Procedures

Maintenance of human induced pluripotent stem cells (hiPSCs)

We maintained hiPSC cultures on plates coated with Matrigel (BD Biosciences) in PeproGrow hESC embryonic stem cell medium (Peprotech) which was changed every other day. Undifferentiated hiPSCs were passaged every 5 days by "cut and paste" technique.

Differentiation of hiPSCs into osteoclasts

We differentiated hiPSCs into mature and functional osteoclasts following a three-stage protocol. These steps were Stage 1) to induce mesoderm differentiation through EBs; Stage 2) to expand myelomonocytic populations released from attached EBs; Stage 3) to promote osteoclast maturation.

Mesoderm differentiation: For *in vitro* EB formation, day 5 undifferentiated hiPSCs were dissociated with Accutase cell detachment solution (EMD Millipore) for 10 minutes at 37°C. Single cells were washed in DMEM/F12 medium (Thermo Fisher Scientific) and centrifuged at 1300 rpm for 7 minutes. To induce mesoderm differentiation, EBs were generated by culturing 15,000 cells/well in 96-V Lipidure plates (NOF Corporation) or 96-U Nunclon plates (Thermo Fisher Scientific) in Stempro-34 (Thermo Fisher Scientific) medium and 5% PeproGrow hESC embryonic stem cell medium (Peprotech) supplemented with 2mM glutamine and $4x10^{-4}$ M monothioglycerol. Ascorbic acid (50 μg/ml), hBMP4 (25 ng/ml), hVEGF (50 ng/ml) and Rock inhibitor Y-27632 (10 μM) were freshly added into EB medium and cells were cultured in 5% CO_2 and 5% O_2. After culturing for 2 days, half of the medium was changed by adding Stempro-34 medium supplemented with ascorbic acid (50 μg/ml), hBMP4 (25 ng/ml), hVEGF (50 ng/ml), hTPO (50 ng/ml), hFLT3 (50 ng/ml), hSCF (50 ng/ml) and bFGF (20 ng/ml) for another 2 days in 5% CO_2 and 5% O_2. All cytokines were purchased from Peprotech Inc.

Myelomonocytic expansion: At day four, 40-50 EBs were transferred to one well of a 6-well plate coated with gelatin (EMD Millipore). To promote hematopoietic cell expansion, EBs were maintained in advanced DMEM medium (Thermo Fisher Scientific) supplemented with hIL-3 (25 ng/ml) and hMCSF (50 ng/ml) for 4 days in a cell culture incubator with 5% CO_2 and 5% O_2. Medium was changed every 4 days. hMCSF concentration was increased to 100 ng/ml after culturing EBs for 4 days and continued for another 13 days.

Osteoclast maturation: To generate mature and functional osteoclasts, single cells released from EBs attached on gelatin plate were collected and centrifuged at 1300 rpm for 7 minutes. Osteoclast progenitors were cultured in alpha MEM medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco), $2x10^{-7}$ M vitamin D (Sigma-Aldrich), hTGFβ (5 ng/ml), hMCSF (30 ng/ml) and hRANKL (50 ng/ml).

Human osteoblast explant cultures

Bone specimens were obtained after medically indicated surgery from one healthy donor and one CMD patient. Bone was cut into small pieces and maintained in DMEM (Gibco) until explant culture was confluent. Cells were then transferred to alpha-MEM (Gibco) osteoblast differentiating medium containing 10% fetal bovine serum (Gibco), 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco), 50 μg/ml ascorbic acid and 8 mM β-glycerophosphate (Sigma-Aldrich). The medium was changed every 2-3 days.

Flow cytometry analysis

To examine the progress of hematopoietic cell development, floating cells released from day 10, 13, 17 and 21 EBs (in Stage 2) were collected and stained with primary antibodies against CD14 (catalogue number 325604, FITC-conjugated, Biolegend), CD43 (catalogue number 560198, APC-conjugated, Miltenyi Biotec) and CD45 (catalogue number 304012, APC-conjugated, Biolegend) in 2% FBS/2mM EDTA/PBS. Cells were stained for 30 minutes in the dark and analyzed on a MACSQuant flow cytometer (Miltenyi Biotec). Samples without antibody staining were served as negative control.

RNA analysis

Total RNA from EBs and hiPSC-OCs was isolated using TRIzol (Thermo Fisher Scientific) followed by Direct-zol RNA extraction (Zymo Research) according to manufacturers' instructions. RNA was treated with DNase I (Invitrogen) and cDNA was synthesized by Superscript II reverse transcriptase (Invitrogen). qPCR was performed as described previously (3). Relative quantification of gene expression was

determined by the ΔΔCt method. Data was normalized to human *HPRT* gene expression. Primer sequences are listed in Supplemental Table 2.

Characterization of hiPSC-OCs

Osteoclast formation and bone resorption were examined by TRAP staining and bone resorption assays as described previously (3, 4). hiPSC OC precursors were plated on 12 mm glass slides in 24-well plates ($0.3x10^6$ cells per well) for TRAP staining and on bone chips ($0.1x10^6$ cells per bone chip) for bone resorption assays. Briefly, cells were washed twice with PBS, fixed with 2.5% glutaraldehyde and stained with TRACP (Lymphocyte Acid Phosphatase Kit; Sigma-Aldrich). TRAP images were taken by a Z1 Observer microscope (Zeiss). To analyze the resorption pits on bone chips, images from 6-8 bone chips were taken for each group and percent resorption was calculated as the ratio of resorbed area to total area using Image J software. To measure the size of osteoclasts, we chose the "region of interest" function in image J software to determine the osteoclast surface of more than 400 multinucleated ($n \geq 3$) TRAP positive cells per group. We selected the larger cells from three independent technical triplicates.

Generation of isogenic hiPSCs by CRISPR/Cas9 technology

We corrected the mutant allele (Ser375 deletion in *ANKH*) in hiPSCs from a CMD patient or created the mutant allele in hiPSCs from a healthy control individual by introducing a Phe377del mutation in *ANKH* using the CRISPR/Cas9 system. The CRISPR/Cas9 plasmid (Addgene) co-expressing a fused guide RNA/tracr RNA and Cas9 protein along with a targeting vector was electroporated into singularized hiPSCs. The tracr RNA recognized the guide RNA site and the Cas9 protein resulted in a targeted double strand break in the genome. The targeting vector was used to repair this double stranded break through the homology directed repair pathway. The targeting vector contained the desired genotype built into the homology arms along with a loxp-flanked selection cassette. After electroporation the cells were cultured and underwent antibiotic selection with G418. Once colonies appeared, they were picked and plated as isolated clones. These clones were genotyped by long-range PCR and sequencing for proper targeting and incorporation site of the desired allele. Silent mutations were introduced while correcting Ser375del mutation to minimize recutting a repaired site by subsequent nuclease binding. Positive clones were transfected with a plasmid transiently expressing CRE to remove the selection cassette.

Immunoblotting

Cells were lysed by RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1% NP40, 0.5% deoxycholate and 0.1% SDS) containing 1xHALT protease inhibitor cocktail (Thermo Fisher Scientific). The concentration of protein within the soluble protein lysate was determined by BCA protein assay (Thermo Fisher Scientific). The samples were run on SDS-PAGE gels and transferred onto PVDF membranes (BioRad) using a semi-dry transfer apparatus (Transblot; BioRad). The membranes were blocked in 5% skim milk Tris-buffered saline/Tween-20 (TBST) overnight and then probed with 1:1000 ANKH antibody (catalogue number AP9741b, Abgent) or 1:1000 ATP6v0d2 antibody (catalogue number sc-517031, Santa Cruz Biotechnology) in 1% skim milk TBST and subsequent goat anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibody (Thermo Fisher Scientific). GAPDH antibody (catalogue number sc-25778, Santa Cruz Biotechnology) served as internal control. Protein bands were detected by enhanced chemiluminescent detection reagent (Azure Biosystems) and visualized by an Azure c600 imaging system (Azure Biosystems). Densitometric analyses of immunoblots were performed using Image J software.

Supplemental References

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4. Chen IP, Wang CJ, Strecker S, Koczon-Jaremko B, Boskey A, and Reichenberger EJ. Introduction of a Phe377del mutation in ANK creates a mouse model for craniometaphyseal dysplasia. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2009;24(7):1206-15.