Supplementary information

Myelopoiesis is regulated by osteocytes through Gsα-dependent signaling

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Supplemental Material and Methods

Primers

The genotypes of all experimental mice were determined by PCR analysis of genomic DNA extracted from tail biopsies using following primers: Cre-transgene:
forward 5'-CGCGGTCTGGCAGTAAAAACTATC-3' and reverse: 5'-CCCACCGTCAGTACGTGAGATATC-3'. Gsα allele: forward: 5'-GAGAGCGAGACGAAGACAGC-3' and reverse: 5'-TCGGGCCTCTGGCGGAGCTT-3'. PPR allele: forward:5'-ATGAGGTCTGAGGTACATGGCTCTGA-3'and reverse 5'-CCTGCTGAC CTCTCTGAAAGAATG T -3'

Tissue specific DNA recombination of DMP1-Cre; $Gs\alpha^{flox/flox}$ alleles was evaluated by multiplex PCR (Extract-N-Amp Tissue PCR Kit, Sigma) on DNA isolated from skeletal tissues (femur and calvaria) and hematopoietic tissues (bone marrow, spleen, and liver) of OCY-GsαKO mice. Three primers spanning the floxed exon E1 of the Gsα gene were used: G1, 5'-GAGAGCGAGAGGAAGACAGC-3'; G2, 5'-TCGGGCCTCTGGCGGAGCTT-3'; and G3, AGCCCTACTCTGTCGCAGTC-3' (G3).

Gene expression analysis

For osteocyte specific gene expression, RNA was isolated from OEBE homogenized in Trizol (Invitrogen) using a tissue homogenizer. RNA was then extracted by combining the manufacturer's Trizol protocol with GenElute Mammalian Total RNA Kit (Sigma). One μg RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad), and amplified by real-time PCR using SYBR GREEN PCR Master Mix (Applied Biosystems). Primers sequences are as follow:
SOST: 5'-GGTGGCCTCGTGCAAGTGCAA-3', 5'-TAGGCGTTCTCCAGCTCCG-3';
TPO: 5'-GTGGCAAGACTAACTCTGTCC-3', 5'-GGAGTCACGCAGCAGTTTATTTA-3'
TNFα: 5'-AGACCCTCACACTCAGATCATCTTC-3', 5'-CCACTTGGTGGTTTGCTACGA-3';
SDF-1: 5'-GAGCCAACGTCAAGCATCTG-3', 5'-CAGCCGTGCAACAATCTGAA-3'
GM-CSF: 5'-TCGTCTCTAACGAGTTCTCCTTCAA-3', 5'-CCGTAGACCCTGCTCGAATATC-3';
G-CSF: 5'-CCCCACCTTGGACTTGCTT-3', 5'-GCCACCCCTAGGTTTTCCAT-3';
IL-12: 5'-CAATCACGCTACCTCCTCTTTT-3',5'-CAGCAGTGCAGGAATAATGTTTC-3';
IL-6: 5'-CTGCAAGAGACTTCCATCCAG-3', 5'-AGTGGTATAGACAGGTCTGTTGG-3';
IL-4: 5'-GAGACTCTTTCGGGCTTTTCG-3', 5'-GTGGACTTGGACTCATTCATGGT-3';
β2m: 5'-TTCACCCCCACTGAGACTGAT-3', 5'-GTCTTGG

Bone radiography, histomorphometry analyses, and electron microscopy

Bone mineral density (BMD) and content (BMC) were measured by Lunar PIXImus II densitometer (GE Medical System Luna). To visualize the osteocyte lacunae-canaliculi network, non-decalcified femurs were embedded in methylmethacryate and the surface was polished with different diamond suspensions until smooth. The surface was acid-etched with 37% phosphoric acid followed by 5% sodium hypochlorite to remove bone mineral. The air-dried samples were coated with gold and palladium, and examined by a scanning electron microscope (SEM).

Immunohistochemistry

Femurs or tibia were fixed in 10% neutral formalin at 4^{o}C overnight, decalcified in 20% EDTA pH 8 for 7–15 days, embedded in paraffin, and sectioned at 5mm. Immunohistochemistry was performed using biotinylated anti-sclerostin (R&D Systems, 1:50) antibody and ABC Elite kits (Vector Labs).

Supplementary Figure Legends

Figure S1. Skeletal and peripheral blood phenotypes of wild-type and DMP1-Cre mice matched by genetic background and 3-week old OCY-GsαKO mice. (A) Whole body bone mineral density (BMD) (g/cm^2), (B) bone mineral content (BMC), and (C) number of leukocytes, (D) % neutrophils, (E) number of platelets, and (F) number of lymphocytes in 21-week old DMP1-cre mice matched by genetic background (n=4). Peripheral blood phenotype of 3-week old control and OCY-GsαKO mice showing (G) number of leukocytes, (H) % neutrophils, (I) number of platelets, and (J) number of lymphocytes (n=5). Error bars, mean ± SEM. *, $p < 0.05$ by t-test.

Figure S2. Immunophenotypic analysis of hematopoietic cells in BM and spleen showing frequency of B-cells (B220+ IgM+), granulocytes (Gr1+ CD11b+), erythroid cells (Ter119+ CD45-), monocytes (F4/80- CD11b+), macrophages (F4/80+ CD11b+), T-cell (CD4+), and cytotoxic T-cells (CD8a+) cells in BM (A,B) and spleens (C,D) of 7-week (A,C) and 21-week (B,D) control and OCY-GsαKO female mice ($n \geq 6$). Also showing frequency of LKS (Lin- c-Kit+ Sca-1+), SLAM (Lin- c-Kit+ Sca-1+ CD150+ CD48-), multipotent progenitors (MPP: Lin- c-kit+ Sca-1+ Thy1.1- Flk-2+), common myeloid progenitors (CMP: IL-7Rα- Lin- c-kit+ Sca-1- FcγR^{lo} CD34+), granulocyte macrophage progenitors (GMP: IL-7Rα- Lin- c-kit+ Sca-1- FcγR+ CD34+), and megakaryocyte erythroid progenitors (MEP: IL-7Rα- Lin- c-kit+ Sca-1- FcγR^{lo} CD34-) in (E) bone marrow and (F) spleen from control and OCY-GsαKO female mice (n = 6). Error bars, mean ± SEM. *, $P < 0.05$ by t-test.

Figure S3. Osteocyte-enriched bone explant (OEBE) generation and gene expressions of cytokines in OEBE from control and OCY-GsαKO mice. (A) OEBEs were generated from femurs and tibia diaphyses after flushing out BM and removing endosteal and periosteal osteoblasts by serial collagenase and EDTA digestions. Hemotoxylin and eosin staining shows presence of only the matrix-embedded osteocytes, lower panel (4x), top panel (20X). (B) After 7 days of *in vitro* culture, the OEBE had 80% of lacunae filled with osteocytes (n = 3). (C)

Relative mRNA expressions of IL-4, IL-6, GM-CSF, SDF-1, TNFα, thrombopoietin (TPO), and IL-12 in OEBE from control and OCY-GsαKO female mice (n = 3). (D) IL-12 in OEBE conditioned media from control and OCY-G_sαKO mice (n = 4). Error bars, mean ± SEM. *, $p < 0.05$ by t-test.

Figure S4. Scanning electron microscopy evaluation of cortical bone from 7-week old control and OCY-GsαKO female mice ($n = 4$) showing (j) representative images at 2000X magnification, (k) osteocytes / field, (l) number of canaliculi / ostocytes. Error bars, mean ± SEM. *, $p < 0.05$ by t-test.

Figure S5. Megakaryocyte analysis. (A) Immunophenotypic analysis of hematopoietic cells in BM showing absolute counts of megakaryocytes (CD41+ CD42d+). (B) Megakaryocyte colonies in growth-factors-supplemented MegaCult-C media from $1X10^5$ bone marrow cells from 7-week old control or OCY-GsαKO mice (n=3). (C) Number of megakaryocytic colonies in growth-factor-deficient MegaCult-C media formed after co-culture of BM cells from control mice with conditioned media from OEBE from control or OCY-G_sαKO mice and in the presence or absence of a G-CSF neutralizing antibody (n = 3). Error bars, mean ± SEM. *, $P < 0.05$ by t-test.

Figure S6. G-CSF gene expression in response to forskolin treatment of OEBE. OEBE from 7-week old control and OCY-GsαKO mice were serum starved overnight and were treated with 10μM forskolin for 4hr and G-CSF mRNA expression was examined by qPCR (n = 3). Error bars, mean ± SEM. *, $P < 0.05$ by t-test.

Fig. S1

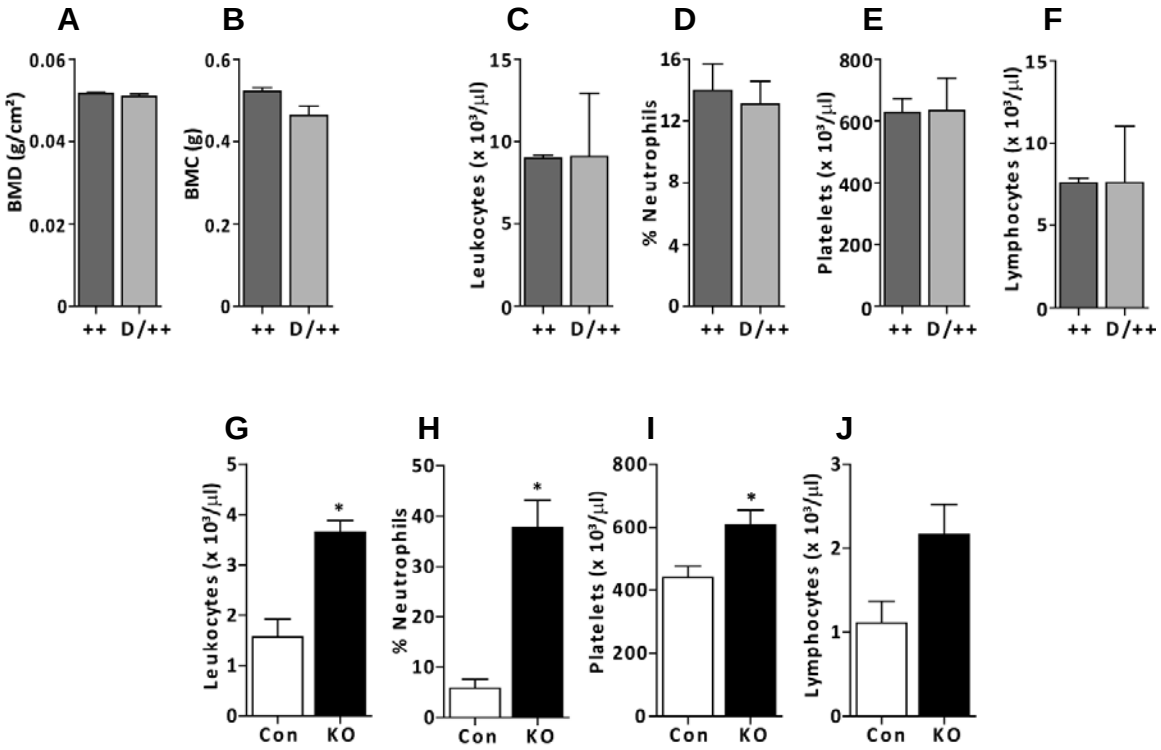

A
B
C
D
E
F
BMD (g/cm²)
BMC (g)
Leukocytes (x 10³/μl)
% Neutrophils
Platelets (x 10³/μl)
Lymphocytes (x 10³/μl)
++
D/++
G
H
I
J
Con
KO
*

Fig. S2

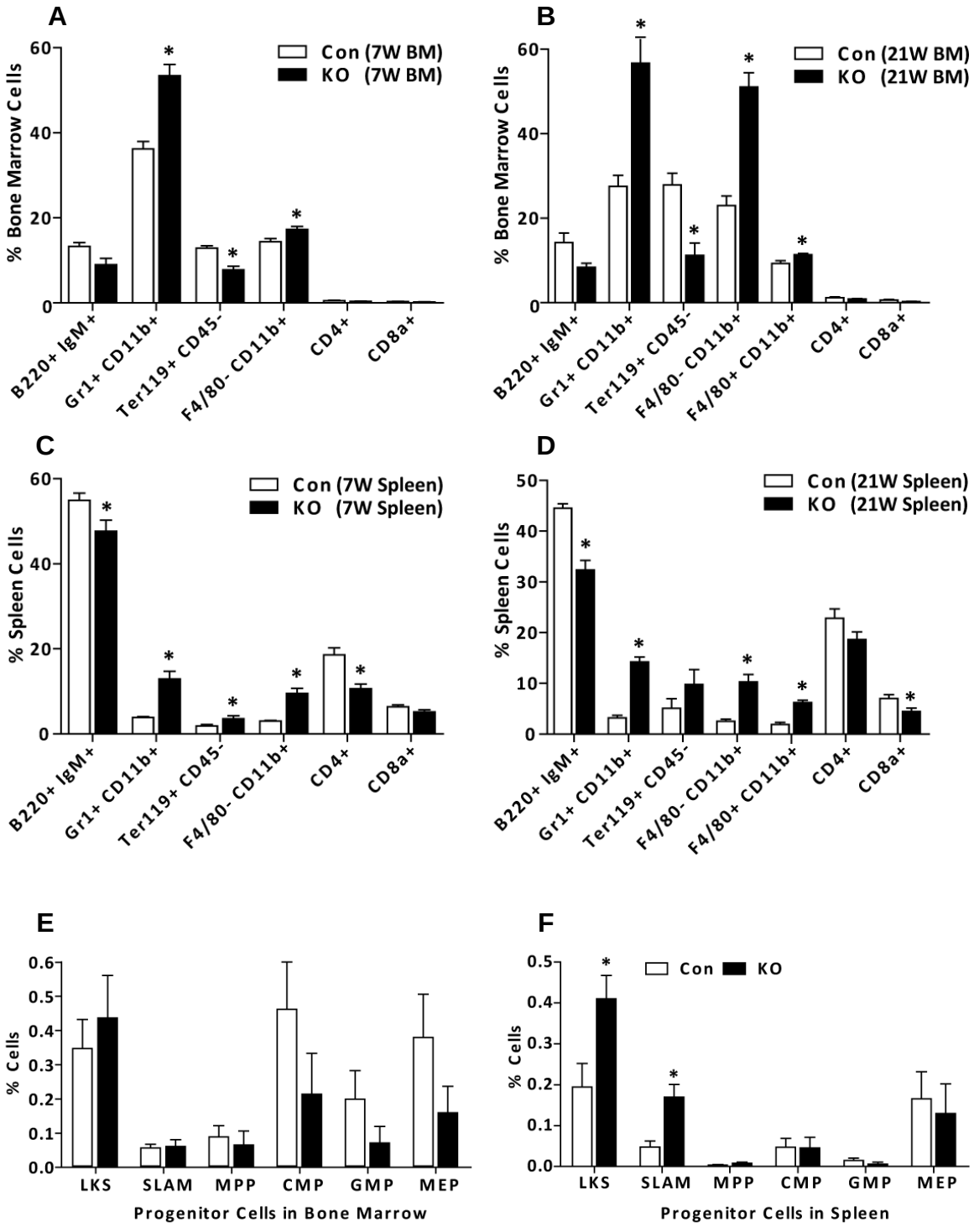

A
% Bone Marrow Cells
Con (7W BM)
KO (7W BM)
B220+ IgM+
Gr1+ CD11b+
Ter119+ CD45-
F4/80- CD11b+
CD4+
CD8a+
B
% Bone Marrow Cells
Con (21W BM)
KO (21W BM)
F4/80+ CD11b+
C
% Spleen Cells
Con (7W Spleen)
KO (7W Spleen)
D
% Spleen Cells
Con (21W Spleen)
KO (21W Spleen)
E
% Cells
LKS
SLAM
MPP
CMP
GMP
MEP
Progenitor Cells in Bone Marrow
F
Con
KO
Progenitor Cells in Spleen

Fig. S3

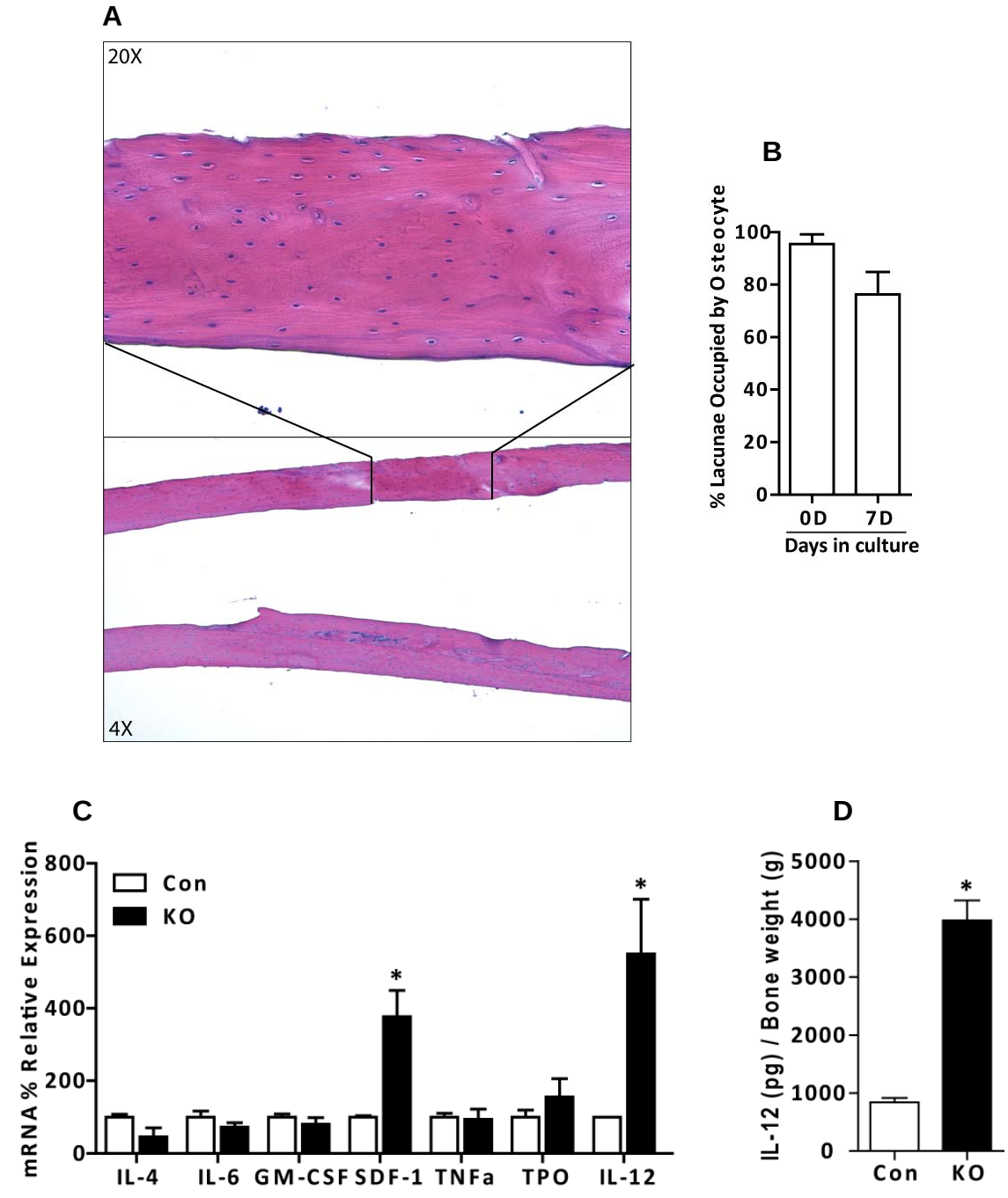

A
20X
4X
B
% Lacunae Occupied by Osteocyte
100
80
60
40
20
0
0D
7D
Days in culture
C
mRNA % Relative Expression
800
600
400
200
0
Con
KO
*
*
IL-4
IL-6
GM-CSF
SDF-1
TNFa
TPO
IL-12
D
IL-12 (pg) / Bone weight (g)
5000
4000
3000
2000
1000
0
*
Con
KO

Fig. S4

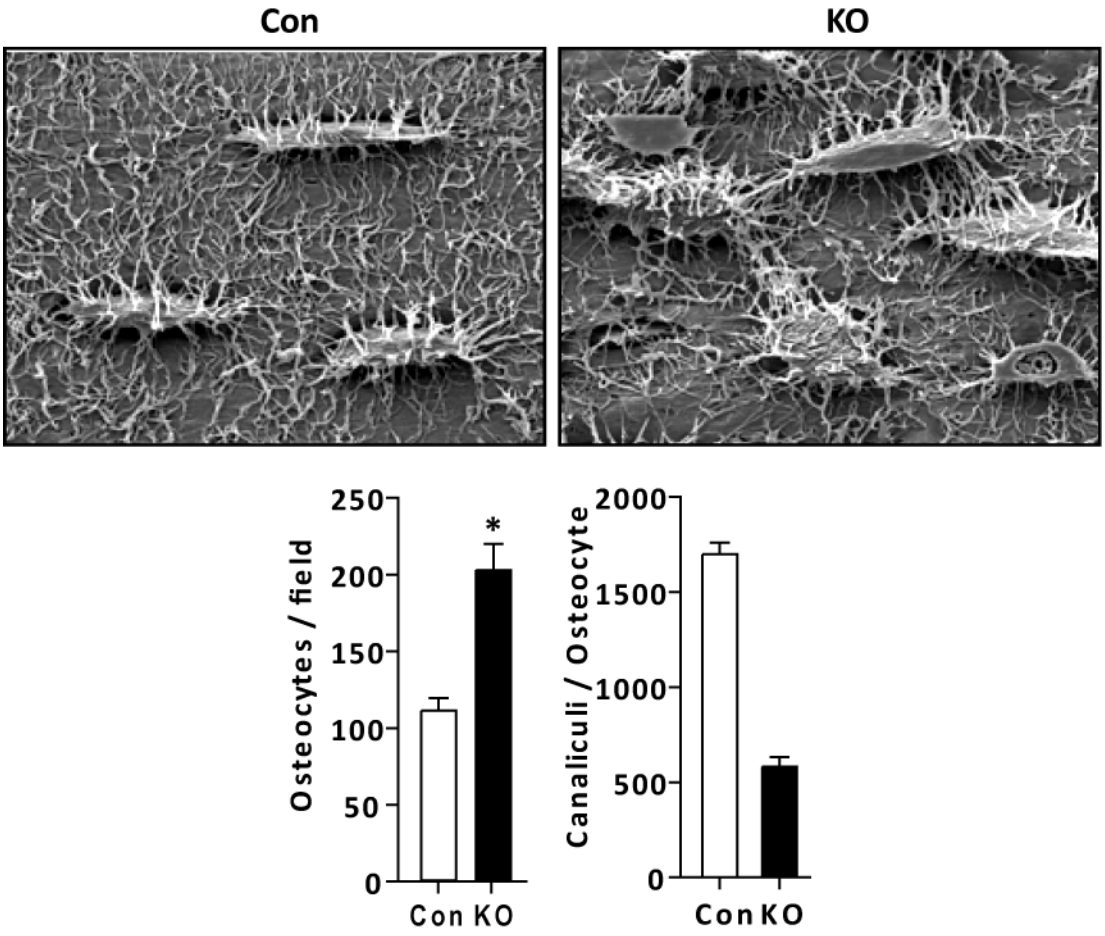

Con
KO
Osteocytes / field
250
200
150
100
50
0
*
Con KO
Canaliculi / Osteocyte
2000
1500
1000
500
0
Con KO

Fig. S5

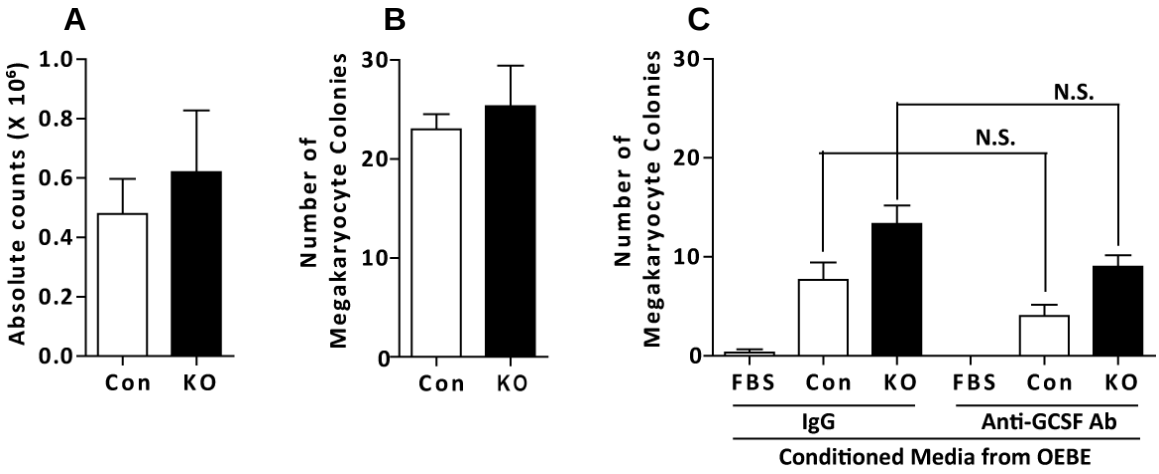
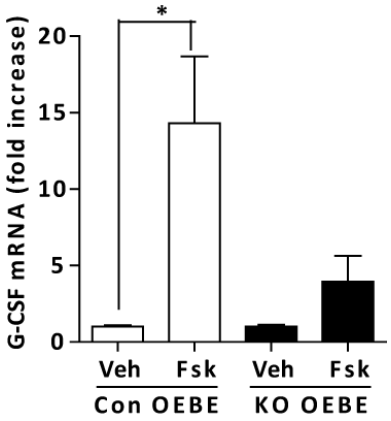

A
Absolute counts (X 10⁶)
1.0
0.8
0.6
0.4
0.2
0.0
Con
KO
B
Number of Megakaryocyte Colonies
30
20
10
0
Con
KO
C
Number of Megakaryocyte Colonies
30
20
10
0
N.S.
N.S.
FBS
Con
KO
FBS
Con
KO
IgG
Anti-GCSF Ab
Conditioned Media from OEBE

Fig. S6


*
G-CSF mRNA (fold increase)
20
15
10
5
0
Veh
Fsk
Veh
Fsk
Con OEBE
KO OEBE