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Supplemental information

Regional specialization and fate specification of bone stromal cells in skeletal development

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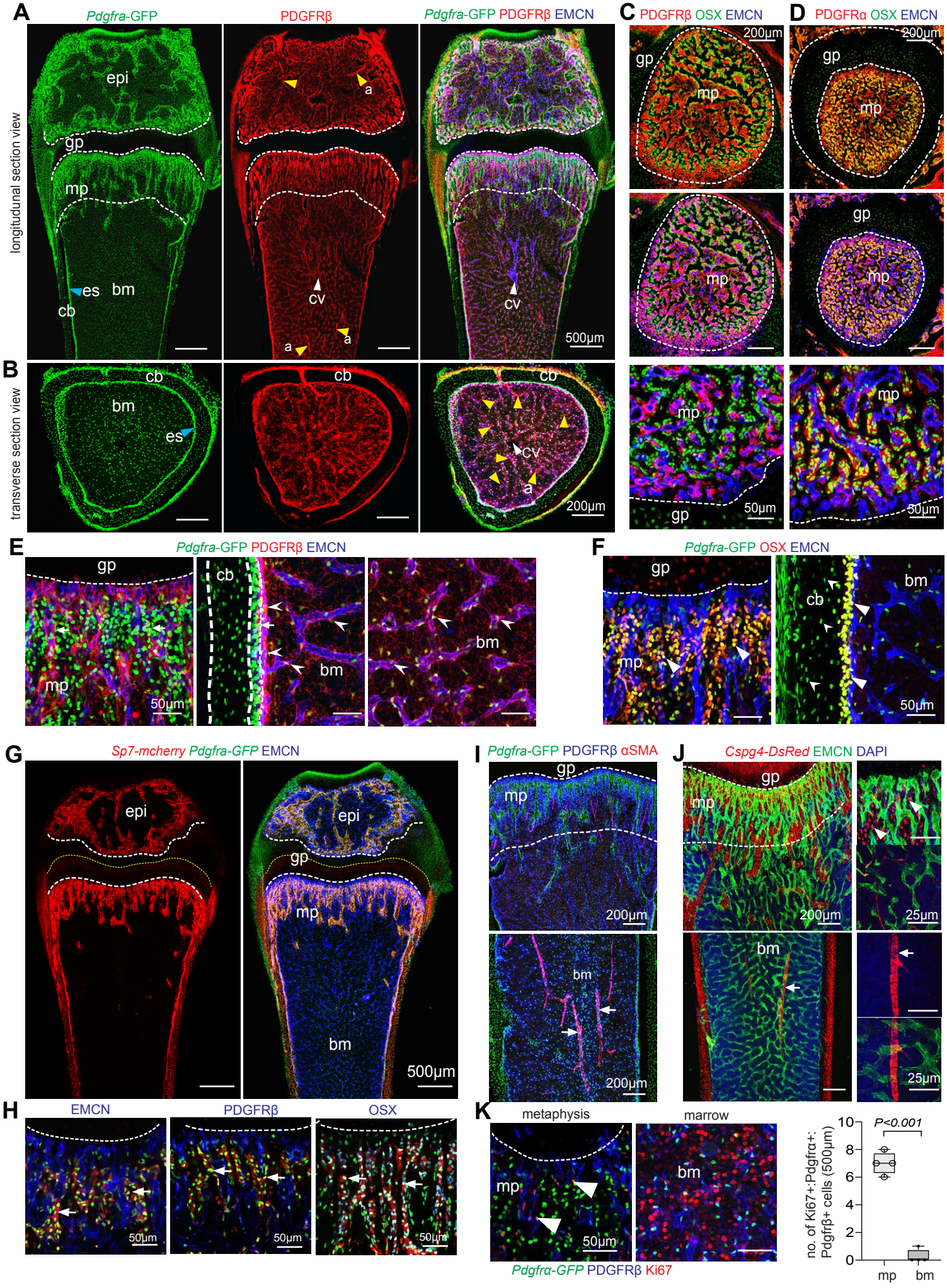

A
Pdgfra-GFP
PDGFRβ
Pdgfra-GFP PDGFRβ EMCN
longitudunal section view
epi
gp
mp
es
bm
cb
cv
a
500μm
B
transverse section view
200μm
C PDGFRβ OSX EMCN
D PDGFRα OSX EMCN
50μm
E
Pdgfra-GFP PDGFRβ EMCN
F
Pdgfra-GFP OSX EMCN
G
Sp7-mcherry Pdgfra-GFP EMCN
H
EMCN
PDGFRβ
OSX
I
Pdgfra-GFP PDGFRβ αSMA
J
Cspg4-DsRed EMCN DAPI
25μm
K
metaphysis
marrow
Pdgfra-GFP PDGFRβ Ki67
P<0.001
no. of Ki67+;Pdgfra+; Pdgfrβ+ cells (500μm)
10
8
6
4
2
0
mp
bm

Supplementary Figure 1 (Related to Figure 1). Identifications and characterization of cell populations in long bone.

A and **B.** Tile scan confocal image show longitudinal (**A**) and transverse (**B**) sections of 3-week-old *Pdgfra-GFP* reporter bone co-stained with PDGFRβ (red) and EMCN (blue). Endosteum (es, blue arrowheads) near cortical bone (cb), arteries (a, yellow arrowheads), and central vein (cv, white arrowheads) are indicated.

C and **D.** Representative image of transverse sections through 3-week-old wild-type femoral metaphysis. Bottom panels show high magnification. PDGFRβ+ cells (red) are perivascular (**C**. Osterix+ (OSX, green) cells express PDGFRα (**D**). EMCN (blue).

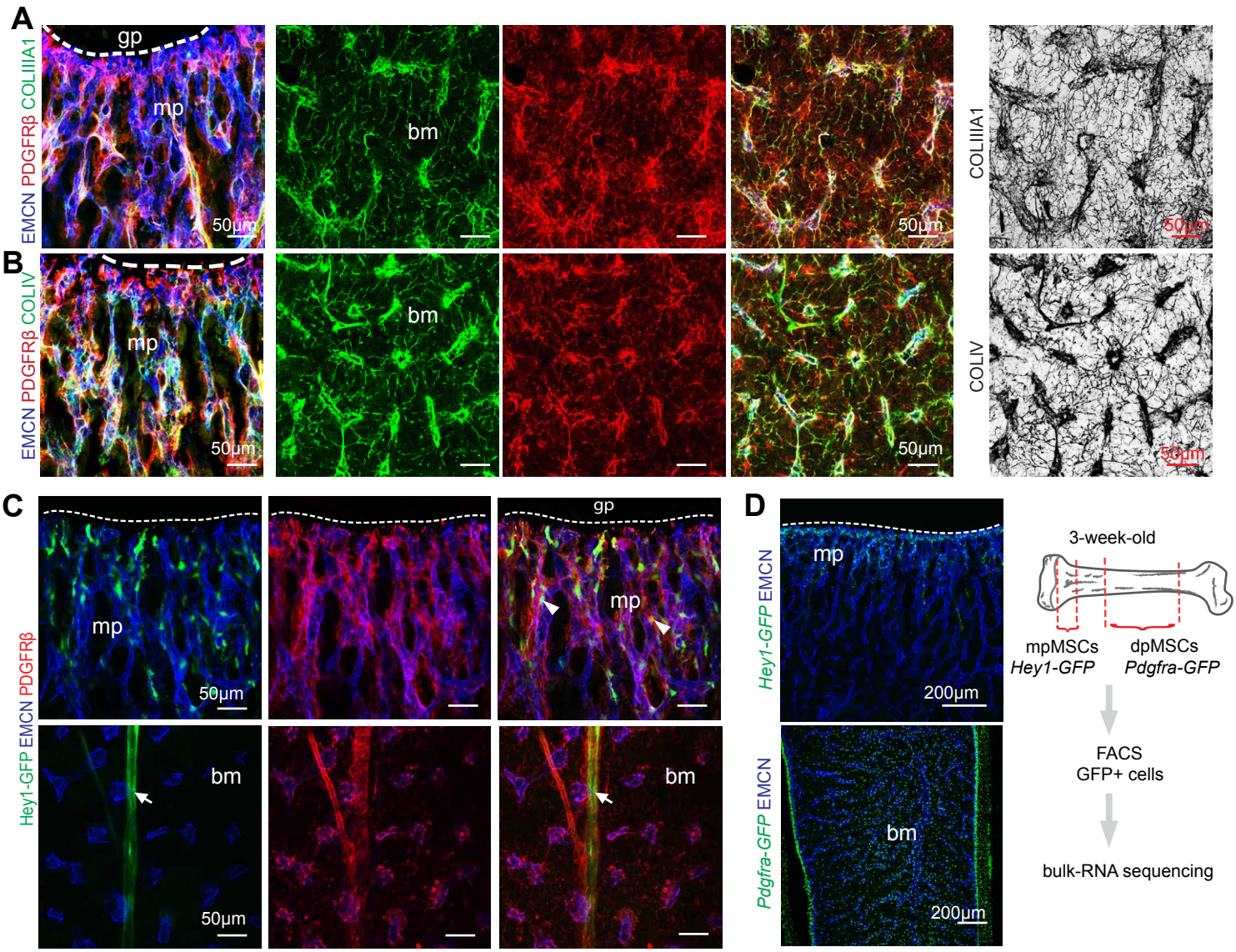
E and **F.** High magnification images of *Pdgfra-GFP*+ (green) cells in metaphysis (left, arrows), endosteum (center) and bone marrow (right, arrowheads) co-stained for PDGFRβ+ (red) and EMCN (blue) (**E**). OSX+ is co-expressed (arrowheads) with *Pdgfra-GFP* in metaphysis (left) and endosteum (eo) but not in bone marrow (bm), compact bone (cb) or periosteum (**F**).

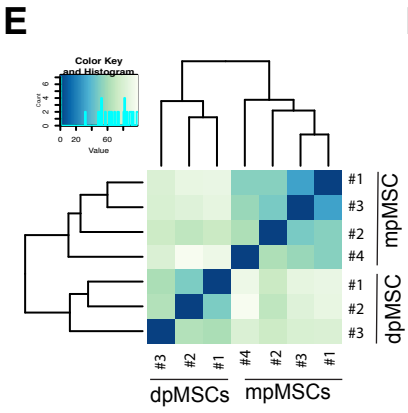
G and **H.** Tile scan confocal image showing *Sp7-mcherry* (red) signal in bone cells and co-expression with *Pdgfra-GFP*. In addition, *Pdgfra-GFP* is expressed in articular cartilage and resting zone chondrocytes, whereas *Sp7-mcherry* marks hypertrophic chondrocytes (**G**). High magnification images show *Pdgfra-GFP/Sp7-mcherry* double positive cells (arrows) and *Pdgfra-GFP*+ (arrowheads) (left). *Pdgfra-GFP+/Sp7+* cells can be separated in PDGFRβ+ (arrows) and PDGFRβ- (arrowheads) populations (center). *Pdgfra-GFP*+ *Sp7-mcherry*+/OSX+ cells (right, arrows) in metaphysis (**H**).

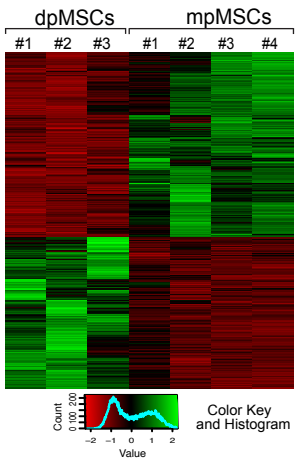
I. Representative confocal image of 3-week-old *Pdgfra-GFP* (green) femoral metaphysis and BM co-stained for αSMA (red) and EMCN (blue). Arrows indicate arteries.

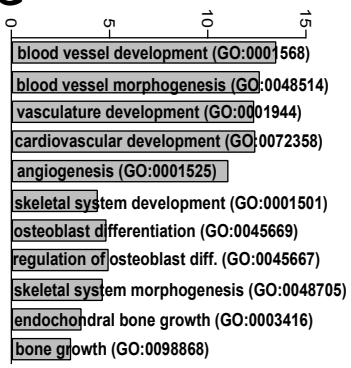
J. 3-week-old *Cspg4-DsRed* (red) metaphysis and BM co-stained with EMCN (green) and DAPI (blue). Cells in metaphysis (arrowheads), growth plate and compact bone express *Cspg4-DsRed*. Periarterial SMCs express *Cspg4-DsRed* (arrows).

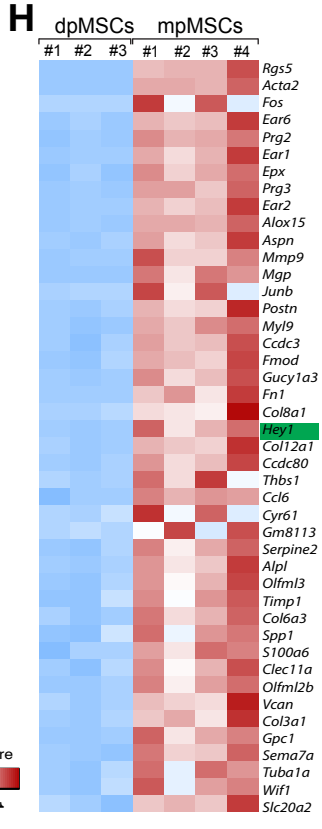
K. *Pdgfra-GFP*+ PDGFRβ+ BMSCs in metaphysis are frequently positive for the proliferation marker Ki67 (arrowheads), whereas BM contains mostly Ki67+ hematopoietic cells (n=4; data are presented as mean sem, p-values, two-tailed unpaired t-test).

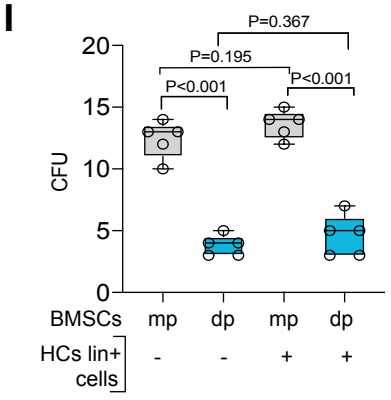

A
EMCN PDGFRβ COLIIIA1
gp
mp
bm
50μm
COLIIIA1
B
EMCN PDGFRβ COLIV
COLIV
C
EMCN PDGFRβ
Hey1-GFP EMCN PDGFRβ
D
Hey1-GFP EMCN
Pdgfra-GFP EMCN
200μm
3-week-old
mpMSCs
Hey1-GFP
dpMSCs
Pdgfra-GFP
FACS
GFP+ cells
bulk-RNA sequencing

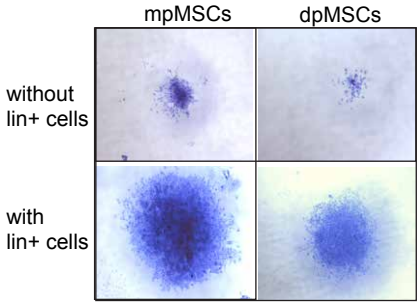

E
Color Key and Histogram
mpMSC
dpMSC
dpMSCs
mpMSCs


F
dpMSCs
mpMSCs
Color Key and Histogram


G
blood vessel development (GO:0001568)
blood vessel morphogenesis (GO:0048514)
vasculature development (GO:0001944)
cardiovascular development (GO:0072358)
angiogenesis (GO:0001525)
skeletal system development (GO:0001501)
osteoblast differentiation (GO:0045669)
regulation of osteoblast diff. (GO:0045667)
skeletal system morphogenesis (GO:0048705)
endochondral bone growth (GO:0003416)
bone growth (GO:0098868)


H
dpMSCs
mpMSCs
Rgs5
Acta2
Fos
Ear6
Prg2
Ear1
Epx
Prg3
Ear2
Alox15
Aspn
Mmp9
Mgp
Junb
Postn
Myl9
Ccdc3
Fmod
Gucy1a3
Fn1
Col8a1
Hey1
Col12a1
Ccdc80
Thbs1
Ccl6
Cyr61
Gm8113
Serpine2
Alpl
Olfml3
Timp1
Col6a3
Spp1
S100a6
Clec11a
Olfml2b
Vcan
Col3a1
Gpc1
Sema7a
Tuba1a
Wif1
Slc20a2
Row Z-Score


I
P=0.367
P=0.195
P<0.001
P<0.001
CFU
BMSCs
mp
dp
mp
dp
HCs lin+ cells
-
-
+
+


mpMSCs
dpMSCs
without lin+ cells
with lin+ cells

Supplementary Figure 2 (Related to Figure 1 and 2). Analysis of mpMSCs and dpMSCs in long bone.

A and **B.** Confocal image showing 3-week-old femoral metaphysis (mp) and bone marrow (bm). Collagen type III alpha 1 chain (COLIIIA1, green) (**A**) and Collagen type IV (COLIV, green) decorate reticular fibers emerging from perivascular PDGFRβ+ dpMSCs (red) (**B**).

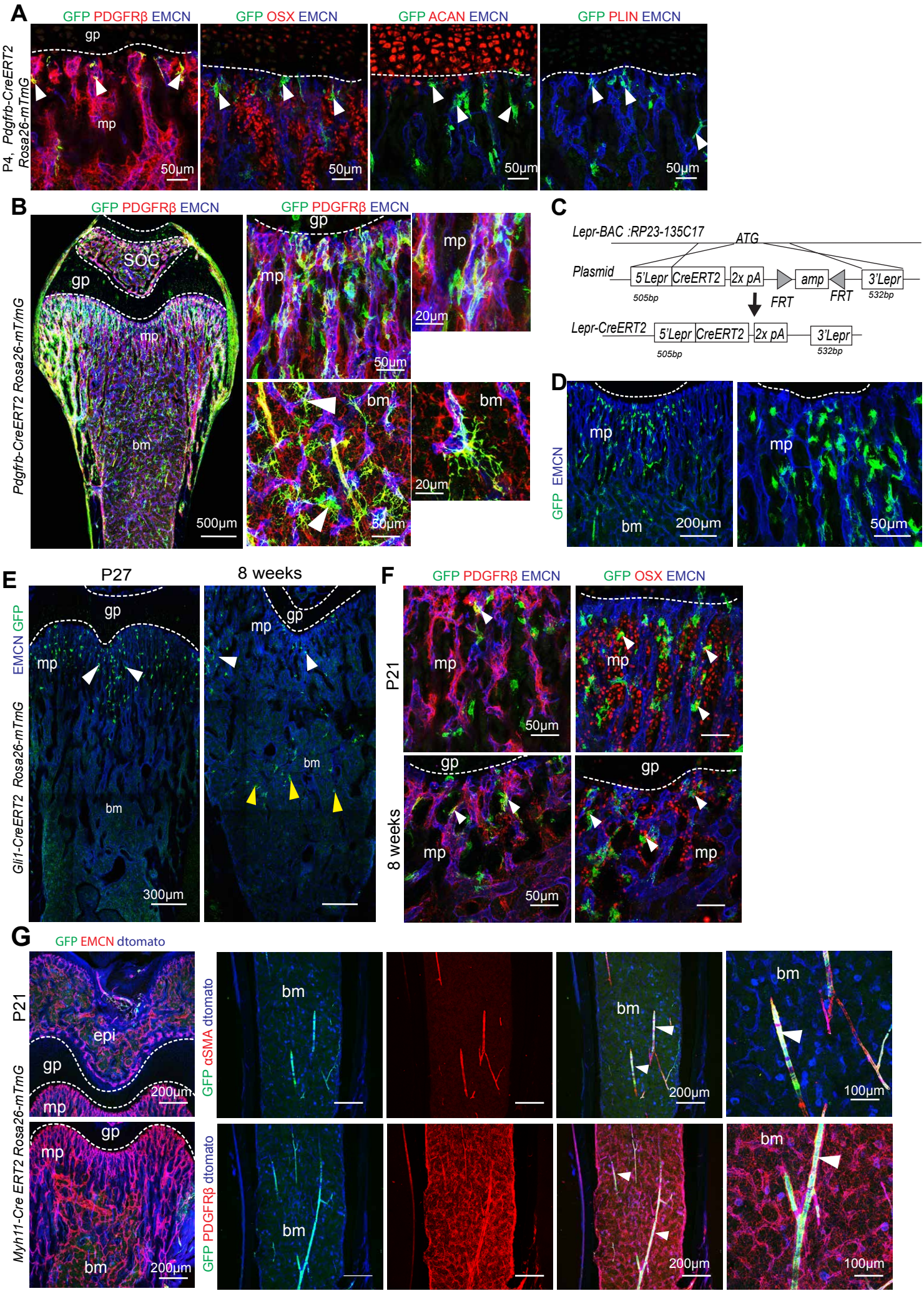
Vessels, EMCN (blue). Images on the right show COLIIIA1 or COLIV signal, respectively.
C. *Hey1-GFP* reporter in 3-week-old femur labels PDGFRβ+ (red) mpMSCs (arrowheads) in metaphysis and arterial ECs in BM (arrows).

D. Schematic diagram of spatial dissection of lone bone and confocal images showing *Hey1-GFP*+ cells from metaphysis and *Pdgfra-GFP*+ cells from BM used for bulk RNA sequencing.

E and **F.** Hierarchical clustering of bulk-RNA sequencing data of mpMSCs and dpMSCs (**E**). Heatmap showing differentially expressed genes (**F**).

G. Gene-set enrichment analysis of gene ontology (GO) shows enrichment of differentially expressed genes involved in vascular and skeletal development in mpMSCs relative to dpMSCs.
H. Heatmap of mpMSC and dpMSC bulk RNA-sequencing data highlighting selected differentially expressed genes including *Hey1* (n=3, 4; Fpkm: Fragments per kilobase million).

I. Fibroblastic colony forming units (CFU) assay. The number of colonies formed was not changed by the depletion of hematopoietic cells, but the size of the colonies was bigger in co-culture with hematopoietic cells (bottom) (n=5; data are presented as mean sem, p-values, two-tailed unpaired t-test).


A
GFP PDGFRβ EMCN
GFP OSX EMCN
GFP ACAN EMCN
GFP PLIN EMCN
P4, Pdgfrb-CreERT2 Rosa26-mTmG
gp
mp
50μm
B
GFP PDGFRβ EMCN
Pdgfrb-CreERT2 Rosa26-mT/mG
SOC
gp
mp
bm
500μm
20μm
50μm
C
Lepr-BAC :RP23-135C17
ATG
Plasmid
5'Lepr
CreERT2
2x pA
amp
3'Lepr
505bp
FRT
532bp
Lepr-CreERT2
D
GFP EMCN
200μm
E
P27
8 weeks
EMCN GFP
Gli1-CreERT2 Rosa26-mTmG
300μm
F
GFP PDGFRβ EMCN
GFP OSX EMCN
P21
8 weeks
G
GFP EMCN dtomato
P21
epi
Myh11-Cre ERT2 Rosa26-mTmG
GFP αSMA dtomato
GFP PDGFRβ dtomato
100μm

Supplementary Figure 3 (Related to Figure 3). Genetic fate tracking of BMSCs *in vivo*.

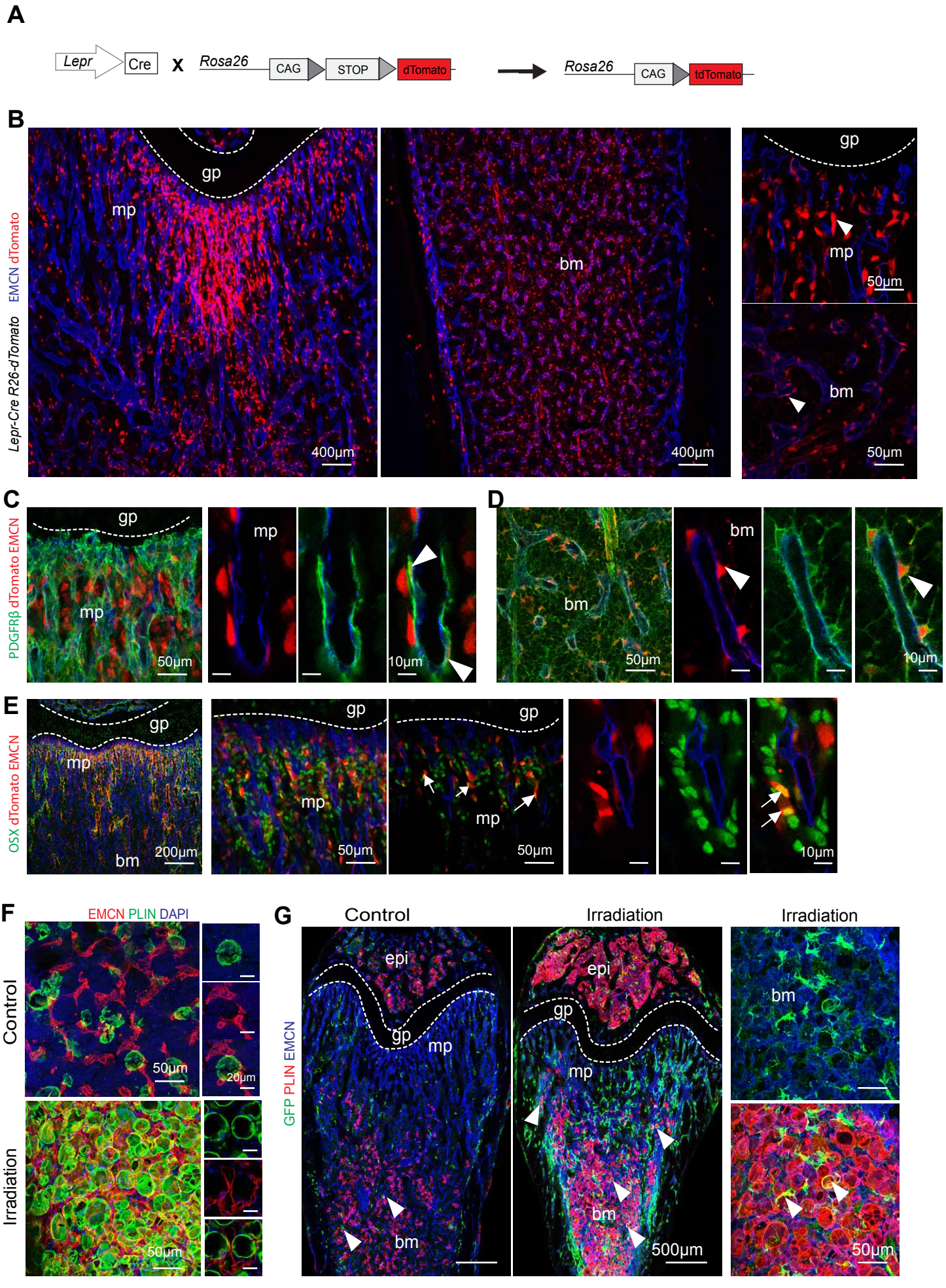
A. Representative confocal image of P4 *Pdgfrb-CreERT2 Rosa26-mTmG* bone after tamoxifen administration at P1-3. Note that GFP+ cells (arrowheads) are stained for PDGFRβ+ (red) but not Osterix (OSX), Aggrecan (ACAN) or Perilipin (PLIN). ECs, EMCN (blue).

B. Tile scan confocal image of P21 *Pdgfrb-Cre R26-mTmG* femur stained for PDGFRβ (red) and EMCN (blue). GFP+ (green) cells are widely distributed in bone. High magnification images (right) show that GFP+ cells in metaphysis and marrow are morphologically different. In BM, GFP labels reticular cells (arrowheads) and SMCs covering arteries.

C and **D**. Experimental strategy for generation of tamoxifen-inducible *Lepr-CreERT2* transgenic mice (**C**). GFP cells in the metaphysis of P21 *Lepr-CreERT2 R26-mTmG* femur (**D**).

E and **F**. Tile scan confocal images of P27 (left) and 8 week-old (right) *Gli1-CreERT2 R26-mTmG* femurs after tamoxifen induction at P22-24. Note expansion of GFP+ cells from metaphysis (white arrowheads) into bone marrow (yellow arrowheads) (**E**). *Gli1-CreERT2* labels PDGFRβ+ mpMSCs and OSX+ cells (arrowheads) in metaphysis (mp) (**F**).

G. Representative confocal image of P21 *Myh11-CreERT2 R26-mTmG* femur after tamoxifen treatment at P1-3. GFP+ αSMA+ cells cover arteries (arrowheads) and express PDGFRβ.


A
Lepr
Cre
X
Rosa26
CAG
STOP
dTomato
Rosa26
CAG
tdTomato
B
Lepr-Cre R26-dTomato
EMCN dTomato
gp
mp
bm
400μm
50μm
C
PDGFRβ dTomato EMCN
10μm
D
E
OSX dTomato EMCN
200μm
F
EMCN PLIN DAPI
Control
Irradiation
20μm
G
Control
Irradiation
GFP PLIN EMCN
epi
500μm

Supplementary Figure 4 (Related to Figure 3 and 4). Labeling of BMSCs by constitutive *Lepr-Cre* and effect of irradiation.

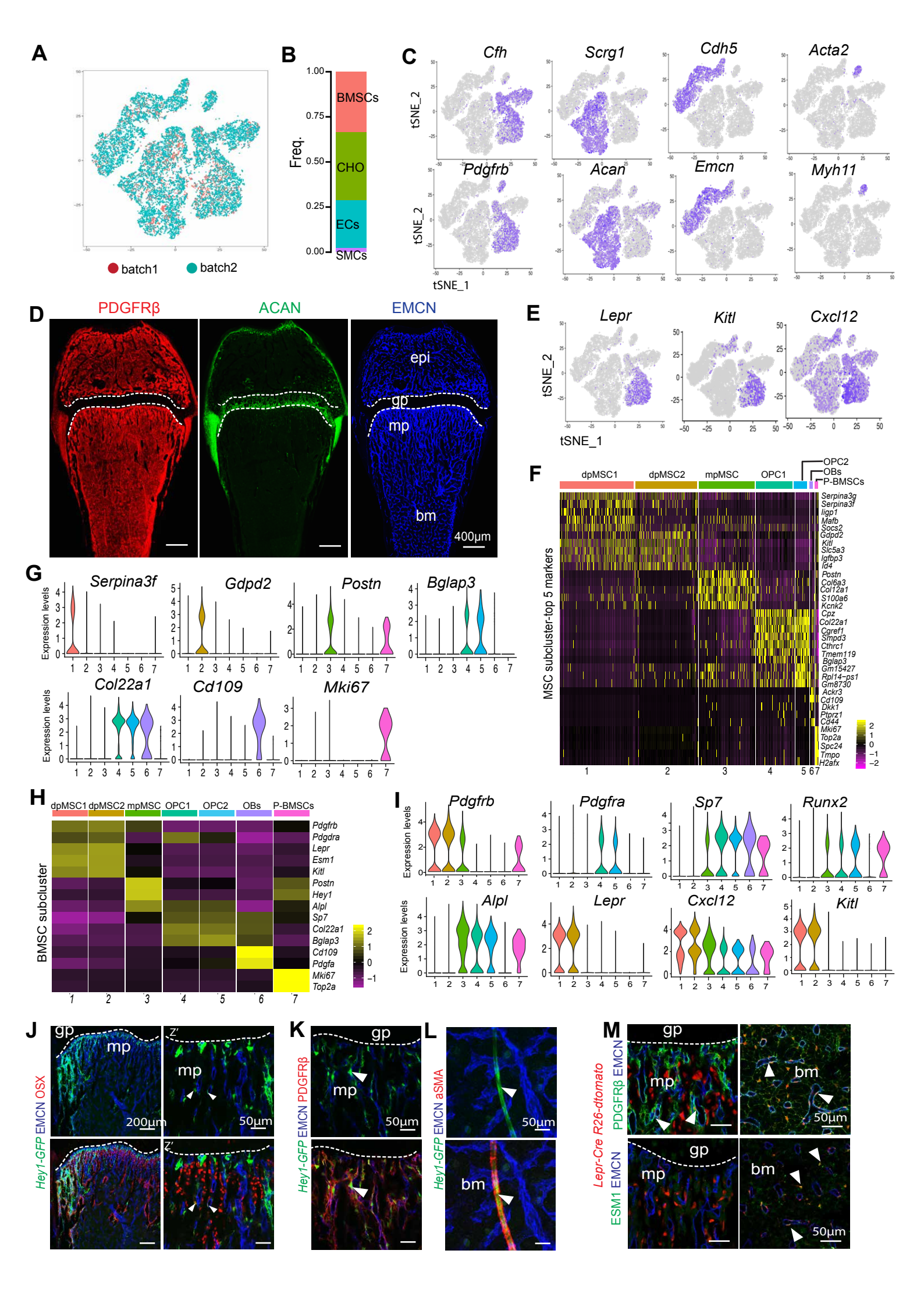
A. Mating scheme for the analysis of constitutive *Lepr-Cre* mice with the *Rosa26-dTomato* reporter.

B. Representative confocal image of 3-week-old *Lepr-Cre R26-dTomato* bone showing genetically labeled (red) cells in metaphysis (mp) near the growth plate (gp) and in BM. High magnification images (right) show that *Lepr-Cre*-labeled cells (arrowheads) are perivascular in metaphysis and bone marrow (right).

C - **E**. Confocal images showing PDGFRβ+ (green) cells with low *Lepr-Cre R26-dTomato*+ levels (red) in mp (**C**) and bm (**D**) (arrowheads). mpMSCs with strong dTomato signal in metaphysis (mp) co-express (arrows) OSX+ (green) (**E**).

F. Emergence of PLIN+ adipocytes after irradiation. ECs, EMCN (red); Nuclei, DAPI (blue).

G. Tile scan confocal image of *Pdgfrb-CreERT2 R26-mTmG* femur at 8 weeks. At one week after irradiation (9 Gy), GFP+ PLIN+ (arrowheads) adipocytes are prominent in epiphysis (epi) and BM relative to control. High magnification of BM is shown on the right.


A
batch1
batch2
B
Freq.
BMSCs
CHO
ECs
SMCs
C
Cfh
Scrg1
Cdh5
Acta2
Pdgfrb
Acan
Emcn
Myh11
tSNE_1
tSNE_2
D
PDGFRβ
ACAN
EMCN
epi
gp
mp
bm
400μm
E
Lepr
Kitl
Cxcl12
F
dpMSC1
dpMSC2
mpMSC
OPC1
OPC2
OBs
P-BMSCs
MSC subcluster-top 5 markers
G
Serpina3f
Gdpd2
Postn
Bglap3
Col22a1
Cd109
Mki67
Expression levels
H
BMSC subcluster
Pdgfrb
Pdgdra
Lepr
Esm1
Kitl
Postn
Hey1
Alpl
Sp7
Col22a1
Bglap3
Cd109
Pdgfa
Mki67
Top2a
I
Pdgfrb
Pdgfra
Sp7
Runx2
Alpl
Lepr
Cxcl12
Kitl
J
gp
mp
z'
200μm
50μm
Hey1-GFP EMCN OSX
K
Hey1-GFP EMCN PDGFRβ
L
Hey1-GFP EMCN aSMA
bm
M
Lepr-Cre R26-dtomato
PDGFRβ EMCN
ESM1 EMCN

Supplementary Figure 5 (Related to Figure 4). Transcriptional landscape of BMSCs at single-cell resolution.

A and **B.** t-SNE plot combining two independent scRNA-seq experiments (**A**). Relative frequency of BMSCs, chondrocytes (CHO), ECs and smooth muscle cells (SMCs) **(B).**

C. Feature plots showing marker genes for individual clusters: BMSCs (*Cfh, Pdgfrb*), chondrocytes (*Scrg1, Acan*), ECs (*Cdh5, Emcn*) and SMCs (*Acta2, Myh11*).

D. Tile scan confocal image of 3-week-old femur immunostained for the indicated markers.

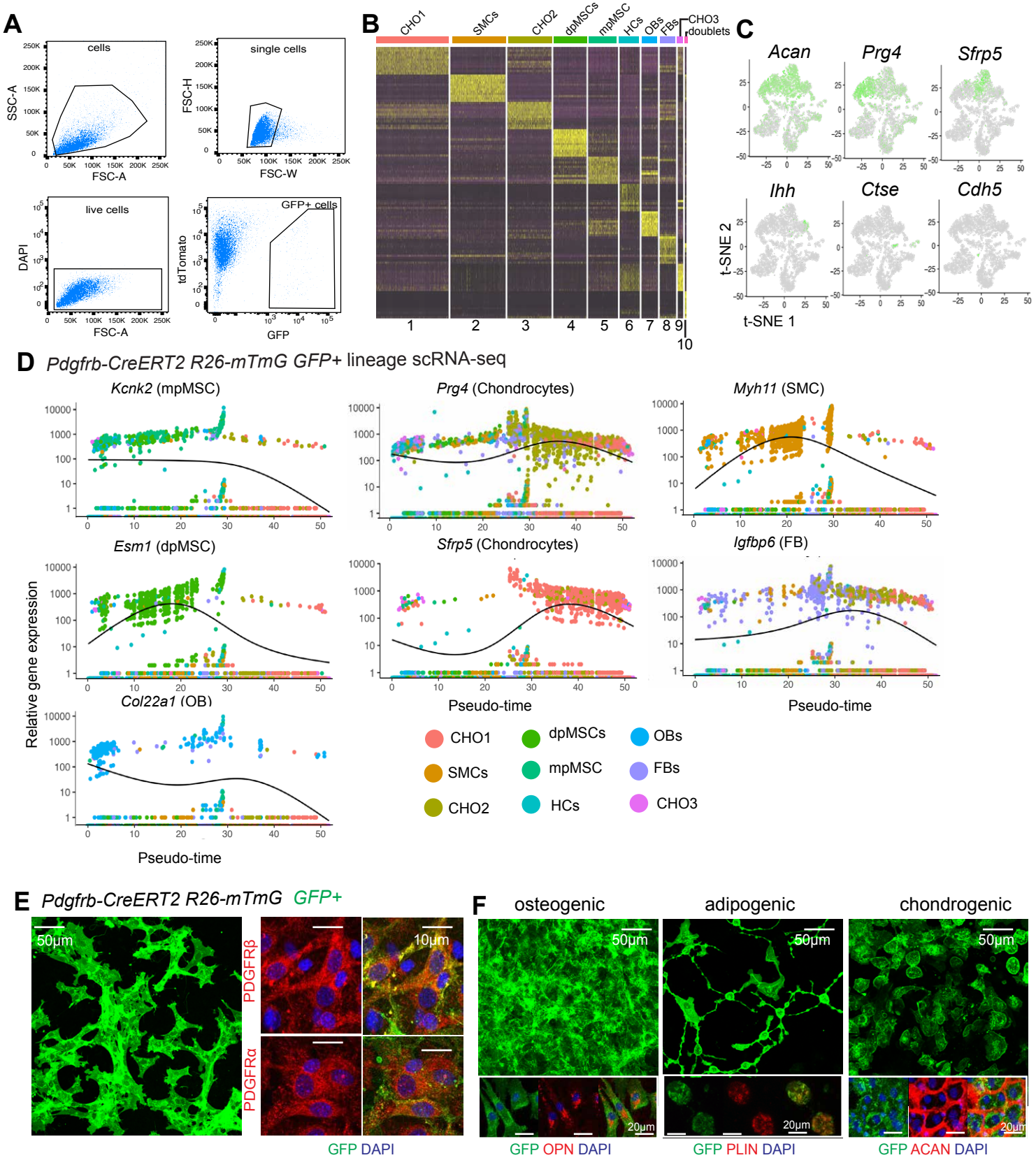
E. Feature plots showing expression of the niche factors *Kitl* and *Cxcl12* in *Lepr*+ BMSCs (dpMSCs).

F. Heatmap showing the top 5 differentially expressed genes for each subcluster.

G-I. Violin plots showing expression of marker genes for each subcluster (**G**). Heatmap showing cluster-specific expression of different markers (**H**). Violin plots showing expression of BMSC and osteoprogenitor marker genes in subclusters (**I**).

J-L. *Hey1-GFP* (green) reporter expression in the metaphysis of 3-week-old femur. High magnification single plane image shows GFP signal in cells with weak OSX (red) expression (arrowheads) (**J**). *Hey1-GFP* expression in PDGFRβ+ mpMSCs (arrowheads) (**K**) and in arteries in BM (**L**). ECs, EMCN (blue).

M. Representative confocal image of 3-week-old *Lepr-Cre R26-dtomato* (red) femoral metaphysis (left) and BM (right) co-stained with PDGFRβ (green), EMCN (blue) and ESM1 (green), as indicated. Arrowheads mark double positive cells.



Supplementary Figure 6 (Related to Figure 5). scRNA sequencing and differentiation of PDGFR+ BMSCs.

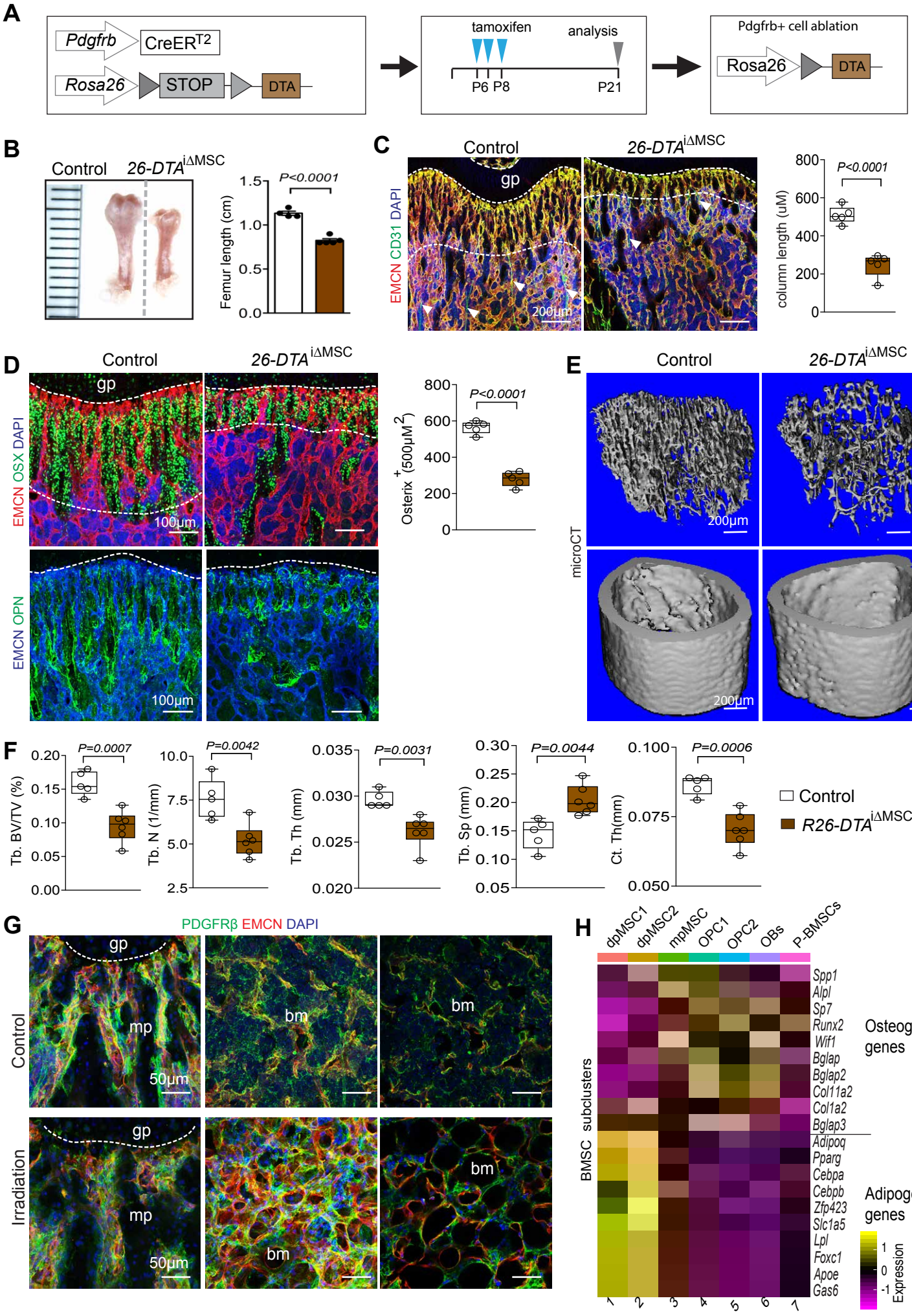
A. Representative FACS dot plots showing sorting of Pdgfrb-CreERT2-labeled GFP+ cells for scRNA-seq.

B. Heatmap of top 12 most significantly expressed genes in all clusters sorted from Pdgfrb-CreERT2 labeled bone.

C. Feature plots showing the chondrocyte (CHO) marker Acan and the subcluster markers Prg4, Sfrp5 and Ihh. Ctse+ cells and Cdh5+ ECs are also shown.

D. Relative gene expression in GFP+ cells displayed in pseudo-time.

E and **F**. Confocal image of Pdgfrb-CreERT2-labeled GFP+ mpMSCs (green) immunostained for PDGFRα and PDGFR (**E**) and after differentiation to OPN+ osteogenic cells, PLIN+ adipocytes and ACAN+ chondrocytes (**F**). Nuclei, DAPI (blue).


A
Pdgfrb
CreER^{T2}
Rosa26
STOP
DTA
tamoxifen
analysis
P6 P8
P21
Pdgfrb+ cell ablation
Rosa26
DTA
B
Control
26-DTA^{iΔMSC}
Femur length (cm)
P<0.0001
C
Control
26-DTA^{iΔMSC}
gp
EMCN CD31 DAPI
200μm
column length (uM)
P<0.0001
D
Control
26-DTA^{iΔMSC}
gp
EMCN OSX DAPI
100μm
EMCN OPN
100μm
Osterix^{+} (500μM^2)
P<0.0001
E
Control
26-DTA^{iΔMSC}
microCT
200μm
200μm
F
Tb. BV/TV (%)
P=0.0007
Tb. N (1/mm)
P=0.0042
Tb. Th (mm)
P=0.0031
Tb. Sp (mm)
P=0.0044
Ct. Th(mm)
P=0.0006
Control
R26-DTA^{iΔMSC}
G
PDGFRβ EMCN DAPI
Control
Irradiation
gp
mp
bm
50μm
H
dpMSC1
dpMSC2
mpMSC
OPC1
OPC2
OBs
P-BMSCs
BMSC subclusters
Spp1
Alpl
Sp7
Runx2
Wif1
Bglap
Bglap2
Col11a2
Col1a2
Bglap3
Osteogenic genes
Adipoq
Pparg
Cebpa
Cebpb
Zfp423
Slc1a5
Lpl
Foxc1
Apoe
Gas6
Adipogenic genes
Expression
1
2
3
4
5
6
7

Supplementary Figure 7 (Related to Figure 6). Function of PDGFRβ+ BMSCs in bone formation and differentiation in response to stress.

A. Scheme depicting tamoxifen-induced PDGFRβ+ cell ablation.

B. Image of 3-week-old control and *R26-DTA*$^{i\Delta MSC}$ femur (left) and quantification of femur length (right) (n=5; data are presented as mean sem, p-values, two-tailed unpaired t-test).

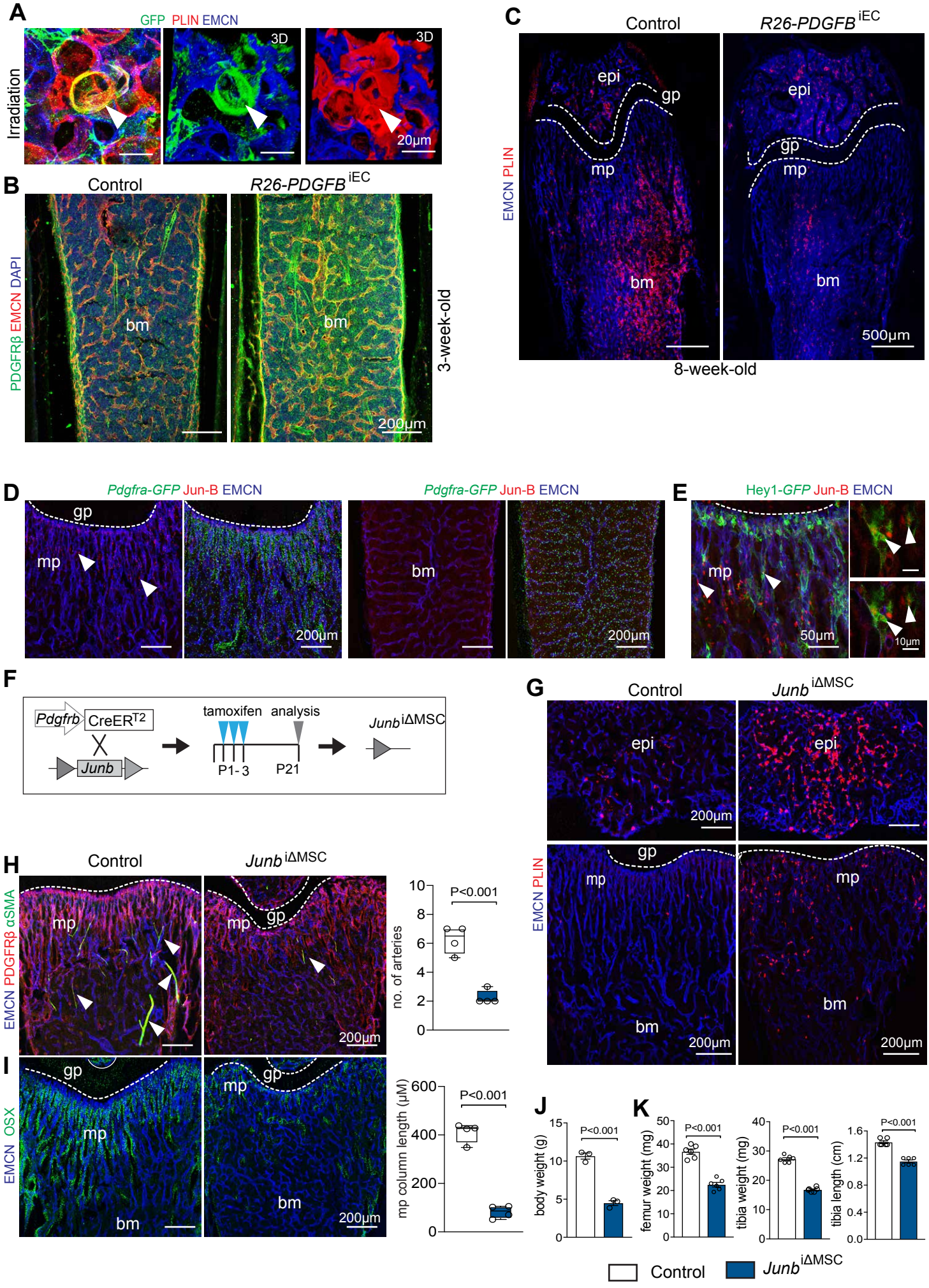
C. Representative confocal image of 3-week-old control and *R26-DTA*$^{i\Delta MSC}$ mutant femoral EMCN+ (red) and CD31+ (green) blood vessels in proximity of growth plate (gp). Nuclei, DAPI (blue). Metaphyseal vessel column length (indicated by dashed lines) is significantly reduced in *R26-DTA*$^{i\Delta MSC}$ mutants (n=5; data are presented as mean sem, p-values, two-tailed unpaired t-test).

D. The number of Osterix+ (OSX, green) cells is significantly reduced in *R26-DTA*$^{i\Delta MSC}$ mutant bone and reduced Osteopontin (OPN) staining in mutant (below). (n=5; data are presented as mean sem, p-values, two-tailed unpaired t-test).

E and **F.** Representative μCT images of trabecular bone in 3-week-old control and *R26-DTA*$^{i\Delta MSC}$ mutant femur (**E**). Quantification of μCT data shows that trabecular bone (Tb) volume (BV/TV, bone volume/total volume), number, thickness, and compact bone thickness (Ct. Th.) are significantly decreased, whereas trabecular bone separation (Tb. Sp.) is increased in *R26-DTA*$^{i\Delta MSC}$ mutants (n=5; data are presented as mean sem, p-values, two-tailed unpaired t-test).

G. Representative confocal images showing PDGFRβ expression and morphology of PDGFRβ+ BMSCs after irradiation compared to control.

H. Heatmap of BMSC subclusters and expression of osteogenic and adipogenic gene signatures.


A
GFP PLIN EMCN
Irradiation
3D
3D
20μm
B
Control
R26-PDGFB iEC
PDGFRβ EMCN DAPI
bm
bm
200μm
3-week-old
C
Control
R26-PDGFB iEC
EMCN PLIN
epi
gp
mp
bm
epi
gp
mp
bm
500μm
8-week-old
D
Pdgfra-GFP Jun-B EMCN
gp
mp
200μm
Pdgfra-GFP Jun-B EMCN
bm
200μm
E
Hey1-GFP Jun-B EMCN
mp
50μm
10μm
F
Pdgfrb
CreER T2
Junb
tamoxifen
analysis
P1-3
P21
Junb iΔMSC
G
Control
Junb iΔMSC
epi
epi
200μm
EMCN PLIN
gp
mp
mp
bm
bm
200μm
200μm
H
Control
Junb iΔMSC
EMCN PDGFRβ αSMA
mp
mp
gp
200μm
no. of arteries
P<0.001
I
EMCN OSX
gp
mp
bm
gp
mp
bm
200μm
mp column length (μM)
P<0.001
J
body weight (g)
P<0.001
K
femur weight (mg)
P<0.001
tibia weight (mg)
P<0.001
tibia length (cm)
P<0.001
Control
Junb iΔMSC

Supplementary Figure 8 (Related to Figure 6 and 7). Endothelial PDGF-B signal and Jun-B in PDGFRβ+ suppresses adipogenic differentiation of BMSCs *in vivo*.

A. Higher magnification of irradiated *Pdgfrb-Cre R26-mTmG* bone showing GFP expression in PLIN+ adipocytes (arrowheads).

B. Representative confocal images showing increase in PDGFRβ+ (green) dpMSCs in 3-week-old *R26-PDGFB*^{iEC} gain-of-function femur relative to control. ECs, EMCN (red); nuclei, DAPI (blue).

C. Overview images showing decrease of PLIN+ adipocytes in 8-week-old *R26-PDGFB*^{iEC} gain-of-function femur relative to control. Growth plate is marked by dashed lines, key structures are labeled.

D and **E.** Representative confocal images show Jun-B (red) expression in metaphyseal *Pdgfra-GFP*+ (green) mpMSCs (arrowheads) but not in dpMSCs (**D**). *Hey1-GFP*+ mpMSCs express Jun-B (arrowheads) (**E**).

F. Experimental scheme for tamoxifen-inducible *Junb* inactivation with *Pdgfrb-CreERT2*.

G and **H.** Representative confocal images and quantitation showing reduced number of αSMA+ PDGFRβ+ arteries (arrowheads) and type H vessel columns in *Junb*$^{i\Delta MSC}$ femur (**G**) (n=4-6; data are presented as mean sem, p-values, two-tailed unpaired t-test). OSX+ cells and area containing these cells are reduced in mutant metaphysis (mp) (**H**).

I. Representative confocal image of femoral epiphysis (top), metaphysis and BM showing increase of PLIN+ adipocytes (red) in *Junb*$^{i\Delta MSC}$ mutants. ECs, EMCN (blue).

J and **K.** *Junb*$^{i\Delta MSC}$ mutant mice display reduced body weight (**J**) as well as reduced bone length and weight compared to control (**K**) (n=4-6; data are presented as mean sem, p-values, two-tailed unpaired t-test).