# Csf1r⁻ macrophages govern the second wave of neutrophils for bone repair

Zhifeng He[1]†, Linan Shi[2]†, Yuko Nakamichi[1], Kohei Murakami[3], Toshihide Mizoguchi[4], Yuki Matsushita[5], Hirotaka Matsumoto[6], Shinichirou Ito[7], Shunsuke Uehara[8], Rina Iwamoto[1], Takumi Takahashi[1], Zizhao Tian[9], Toru Hiraga[10], Ruoxuan Li[1], Masataka Kasahara[7], Kazuo Okamoto[11], Hiroshi Takayanagi[12], Martin M Matzuk[13], Nobuyuki Udagawa[8], Yasuhiro Kobayashi[1]*

[1]Department of Hard Tissue Research, Institute for Oral Science, Matsumoto Dental University
[2]Department of Stomatology, Affiliated Hangzhou First People' s Hospital, School of Medicine, Westlake University,
[3]Department of Veterinary Immunology, Okayama University of Science,
[4]Institute for Oral Science, Tokyo Dental College,
[5]Department of Skeletal Development and Regenerative Biology, Nagasaki University Graduate School of Biomedical Sciences,
[6]School of Information and Data Science, Nagasaki University,
[7]Department of Pharmacology, Tokyo Dental College,
[8]Department of Biochemistry, Matsumoto Dental University,
[9]Department of Biomedical Sciences, City University of Hong Kong,
[10]Department of Histology and Cell Biology, Matsumoto Dental University,
[11]Cancer Research Institute, Division of Immune Environment Dynamics, Kanazawa University,
[12]Department of Immunology, Graduate School of Medicine, The University of Tokyo,
[13]Department of Pathology& Immunology and Center for Drug Discovery, Baylor College of Medicine,

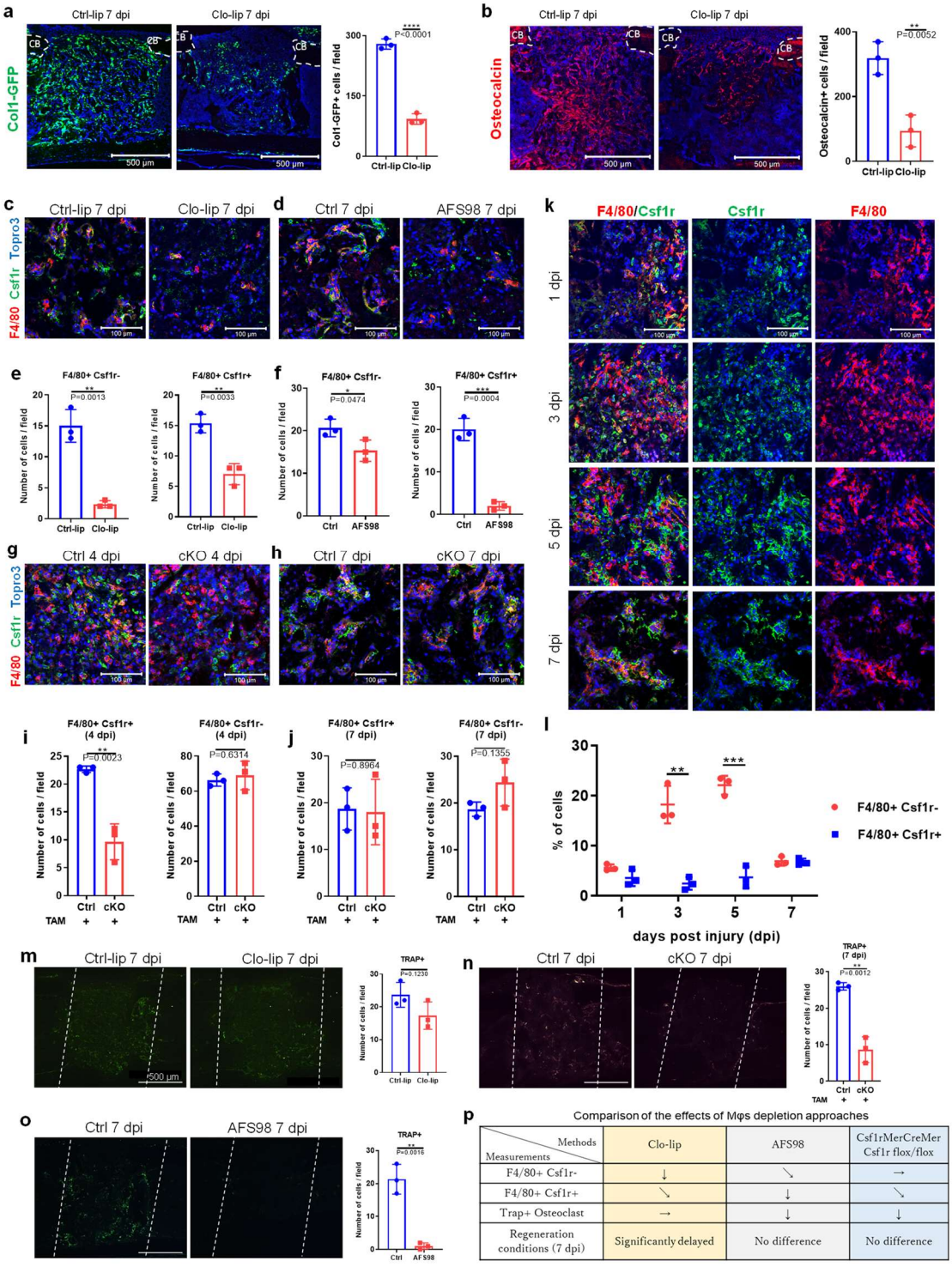
†These authors contributed equally to this work.

27 *Corresponding author

28 E-mail address: yasuhiro.kobayashi@mdu.ac.jp

29 1780 Hiro-oka Gobara, Shiojiri, Nagano 399-0781, Japan

30 Tel: +81-263-51-2238, Fax: +81-263-51-2223




p

Comparison of the effects of Mφs depletion approaches

| Methods / Measurements | Clo-lip | AFS98 | Csf1rMerCreMer Csf1r flox/flox |
|---|---|---|---|
| F4/80+ Csf1r- | ↓ | ↘ | → |
| F4/80+ Csf1r+ | ↘ | ↓ | ↘ |
| Trap+ Osteoclast | → | ↓ | ↓ |
| Regeneration conditions (7 dpi) | Significantly delayed | No difference | No difference |

**Figure. S1 Mφ subpopulations distinguished by F4/80 and Csf1r expression, and osteoclasts targeted by various depletion strategies.**

**(a)** Immunofluorescent analysis of *Col1*-GFP(+) cells at the bone injury site at 7 dpi, n = 3.

**(b)** Immunofluorescent analysis of osteocalcin(+) cells at the bone injury site at 7 dpi, n = 3.

**(c- f)** Immunofluorescent analysis of F4/80(+)Csf1r(+) and F4/80(+)Csf1r(–) cells at the bone injury sites at 7 dpi from Ctrl-lip or Clo-lip-treated (**c, e**), and AFS98 or saline-treated mice (**d, f**), n = 3.

**(g- j)** Immunofluorescent analysis of F4/80(+)Csf1r(+) and F4/80(+)Csf1r(–) cells at the bone injury site at 4 and 7 dpi in *Csf1r*MerCreMer; *Csf1r* fl/fl and control mice (genotypes of control mice: $Cre^{-}$, $Cre^{+/+}$, $Cre^{fl/+}$), n = 3.

**(k)** Immunofluorescent analysis of F4/80(+)Csf1r(+) and F4/80(+)Csf1r(–) cells at the bone injury sites at 1, 3, 5 and 7 dpi.

**(l)** Percentage of F4/80(+)Csf1r(+) and F4/80(+)Csf1r(–) cells at the bone injury sites at 1, 3, 5 and 7 dpi, n = 3.

**(m- o)** TRAP staining using the ELF 97 phosphatase substrate of the bone injury sites at 7 dpi by macrophage depletion methods using Clo-lip **(m)**, macrophage-lineage cell specific deletion of the *Csf1r* gene **(n)**, and AFS98 **(o)**, n = 3. The region between the two dash lines indicates the injury site.

**(p)** Summary of Figure 1 and Fig. S1: The effects of three Mφ depletion strategies (Clo-lip, AFS98, and *Csf1r* conditional knockout) on F4/80(+)Csf1r(–), F4/80(+)Csf1r(+) cell faction, and osteoclasts, as well as bone regeneration outcomes. Scale bars with its value are shown in each image. Data are shown in mean± S.D. Statistical significance was determined by unpaired Student's t-tests. P- values are shown in each graph (**: $p < 0.01$; ***: $p < 0.001$).

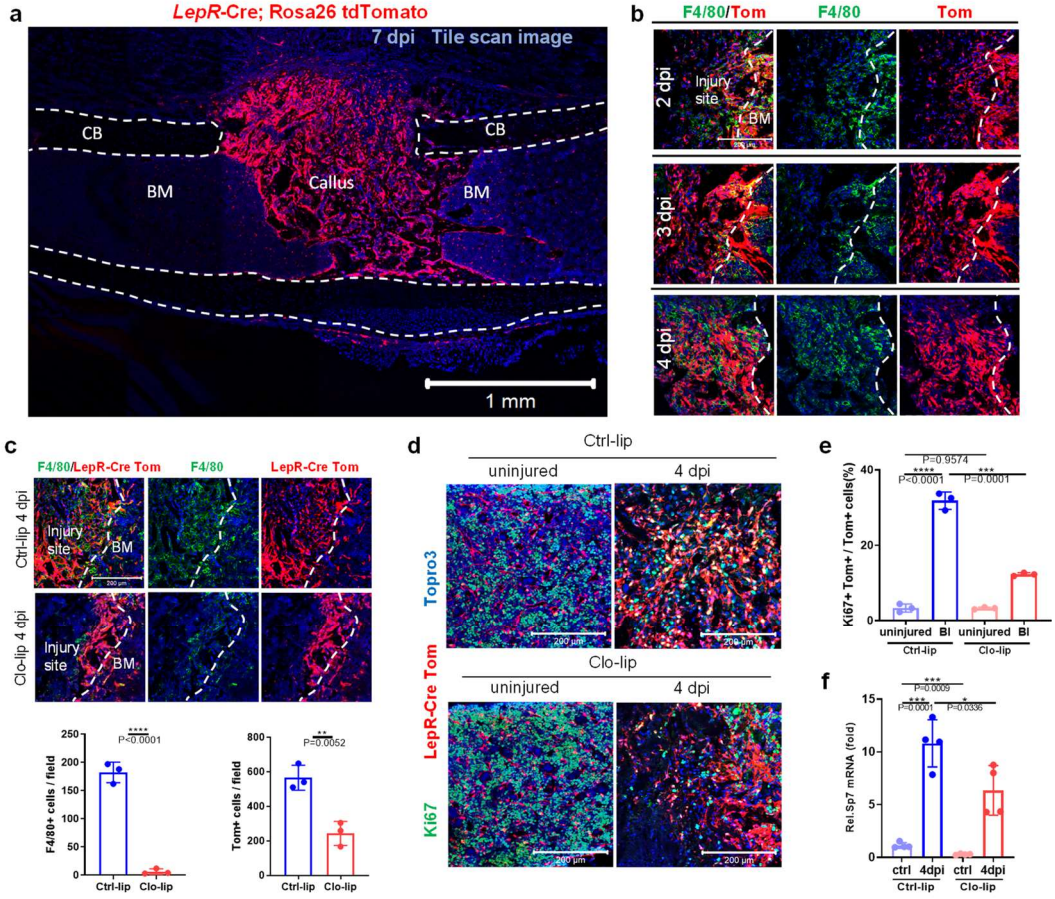

a
LepR-Cre; Rosa26 tdTomato
7 dpi Tile scan image
CB
CB
BM
Callus
BM
1 mm
b
F4/80/Tom
F4/80
Tom
2 dpi
Injury site
BM
3 dpi
4 dpi
c
F4/80/LepR-Cre Tom
F4/80
LepR-Cre Tom
Ctrl-lip 4 dpi
Clo-lip 4 dpi
Injury site
BM
F4/80+ cells / field
P<0.0001
Ctrl-lip
Clo-lip
Tom+ cells / field
P=0.0052
d
Ctrl-lip
uninjured
4 dpi
Topro3
LepR-Cre Tom
Ki67
Clo-lip
e
Ki67+ Tom+ / Tom+ cells(%)
P=0.9574
P<0.0001
P=0.0001
uninjured
BI
Ctrl-lip
Clo-lip
f
Rel.Sp7 mRNA (fold)
P=0.0009
P=0.0001
P=0.0336
ctrl
4dpi

**Figure. S2 Osteodirective Mφs support the proliferation and differentiation of stromal cells.**

**(a)** Immunofluorescent analysis of Tomato(+) cells in *LepR*-Cre; tdTomato mice at the bone injury sites at 7 dpi. Dash lines indicate the cortical bone surface.

**(b)** Immunofluorescent analysis of F4/80(+) cells in *LepR*-Cre; tdTomato mice in the bone injury sites at 2, 3 and 4 dpi. Dash lines indicate the boundary between injury site and uninjured bone marrow.

**(c)** Immunofluorescent analysis of F4/80(+) cells in *LepR*-Cre; tdTomato mice in the bone injury sites at 4 dpi following treatment with Ctrl-lip and Clo-lip. The number of F4/80(+) cells (left) and Tomato(+) cells (right) at the bone injury sites at 4 dpi treated with Ctrl-lip and Clo-lip,.n = 3. Dash lines indicate the boundary between injury site and uninjured bone marrow.

**(d)** Immunofluorescent analysis of Ki67(+) cells in *LepR*-Cre tdTomato mice at the bone injury site at 4 dpi and in the uninjured bone marrow area of the diaphysis following Ctrl-lip and Clo-lip treatment.

**(e)** Percentage of Ki67(+) *LepR*-Cre-labeled Tomato(+) cells among all Tomato(+) cells at the bone injury sites and in the uninjured area at 4 dpi following Ctrl-lip and Clo-lip treatment, n = 3.

**(f)** Quantitative RT-PCR analyses of *Sp7* mRNA expression in bone tissues from bone injury sites (4 dpi) and from control uninjured region of contralateral tibiae following Ctrl-lip and Clo-lip treatment, n = 4.

Scale bars: 1 mm in (a), 200 μm in (b), (c) and (f). Data are shown in mean± S.D. Statistical significance was determined by unpaired Student's t-tests. P- values are shown in each graph.

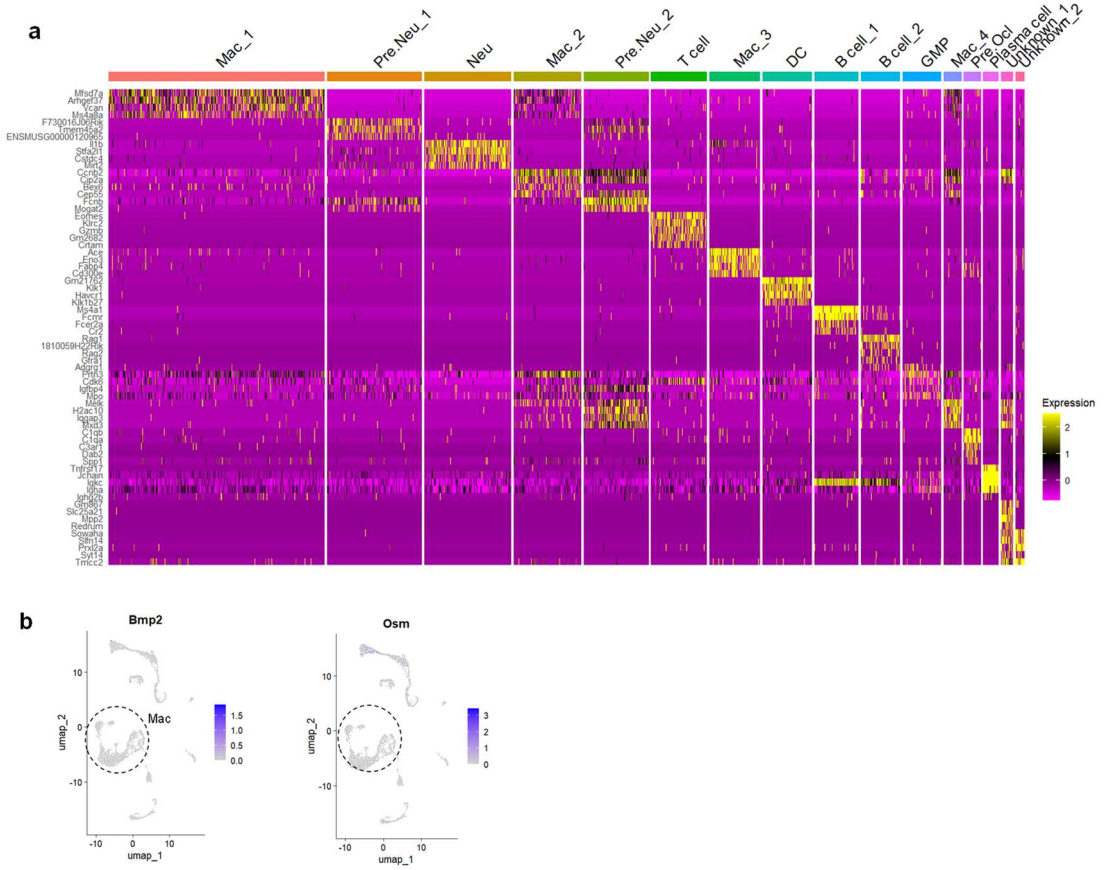
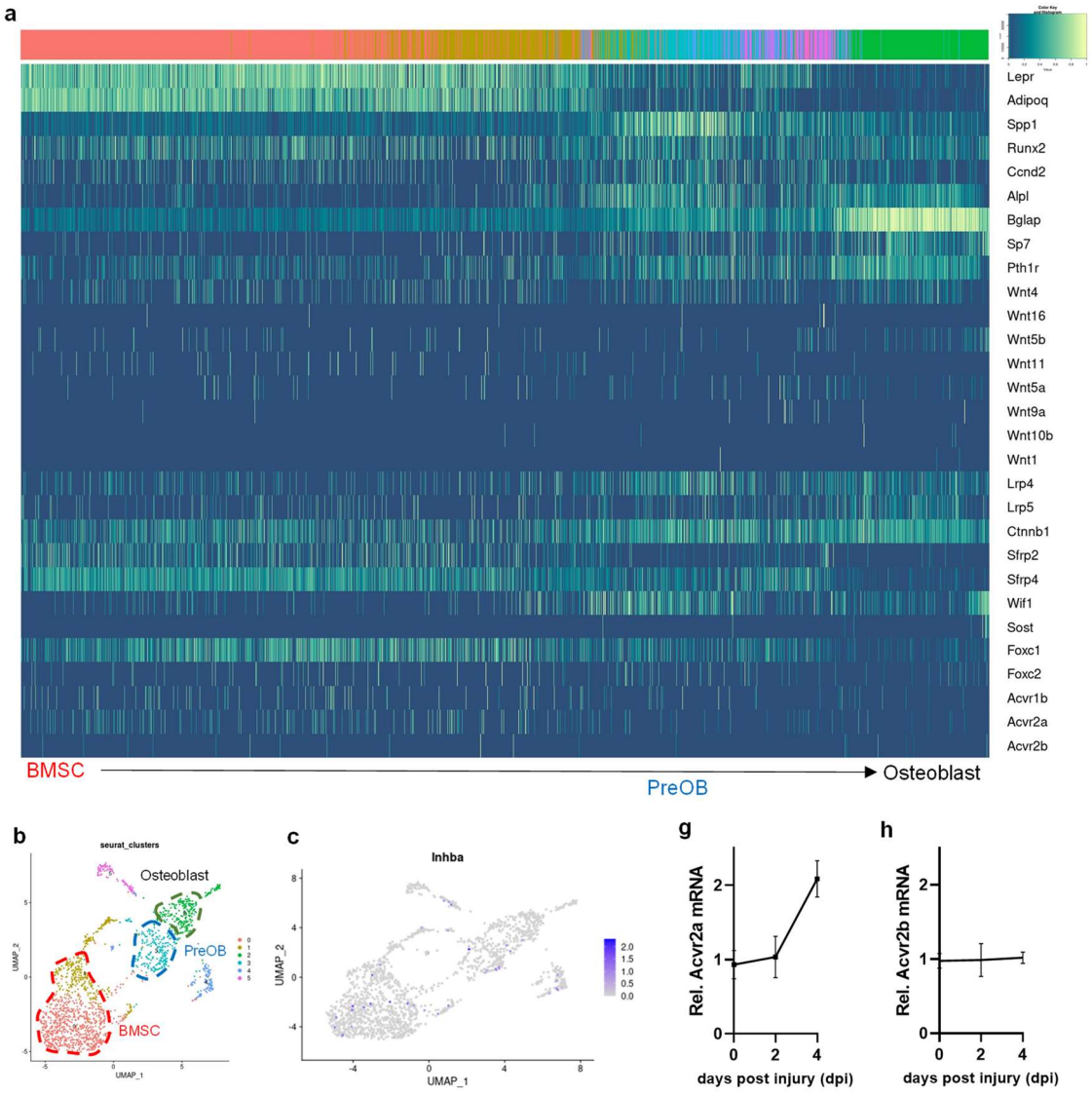

a
Mac_1
Pre.Neu_1
Neu
Mac_2
Pre.Neu_2
T cell
Mac_3
DC
B cell_1
B cell_2
GMP
Mac_4
Pre.Ocl
Plasma cell
Unknown_1
Unknown_2
Expression
2
1
0
b
Bmp2
Osm
Mac
umap_1
umap_2
1.5
1.0
0.5
0.0
3
2
1
0

**Figure. S3 Presentative gene expression pattern in bone marrow F4/80(+) and Csf1r(+) cells.**

**(a)** Heatmap pf top5 marker genes that were differentially expressed in each cluster.

**(b)** Expression patterns of osteogenic factor encoding genes *Bmp2* and *Osm* (encoding Oncostatin M) in the UMAP visualization. The dashed line enclosed macrophage-lineage clusters.


a
Lepr
Adipoq
Spp1
Runx2
Ccnd2
Alpl
Bglap
Sp7
Pth1r
Wnt4
Wnt16
Wnt5b
Wnt11
Wnt5a
Wnt9a
Wnt10b
Wnt1
Lrp4
Lrp5
Ctnnb1
Sfrp2
Sfrp4
Wif1
Sost
Foxc1
Foxc2
Acvr1b
Acvr2a
Acvr2b
BMSC
PreOB
Osteoblast
b
seurat_clusters
Osteoblast
PreOB
BMSC
UMAP_1
UMAP_2
c
Inhba
UMAP_1
UMAP_2
g
Rel. Acvr2a mRNA
days post injury (dpi)
h
Rel. Acvr2b mRNA
days post injury (dpi)

118

119

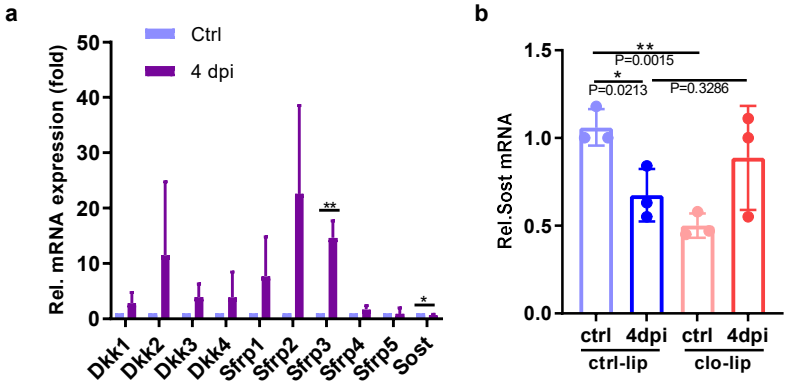
**Figure. S4 The source of activin A production.**

**(a)** Heatmap of representative pseudotime-dependent genes from single-cell RNA-seq analyses of *Cxcl12*-CreERT2; tdTomato (+) cells isolated from femurs at 14 days after bone marrow ablation (GSE136973).

**(b)** UMAP visualization of 6 clusters using Seurat, highlighting three major classes of osteoblastic cells.

**(c)** Expression patterns of *Inhba* in the UMAP visualization.

**(d, e)** Quantitative RT-PCR analyses of *Acvr2a* and *Acvr2b* mRNA expression in bone tissues from bone injury sites at 0, 2, and 4 dpi, (n = 3 - 4 mice per each group).


a
Ctrl
4 dpi
Rel. mRNA expression (fold)
50
40
30
20
10
0
**
*
Dkk1
Dkk2
Dkk3
Dkk4
Sfrp1
Sfrp2
Sfrp3
Sfrp4
Sfrp5
Sost
b
**
P=0.0015
*
P=0.0213
P=0.3286
Rel.Sost mRNA
1.5
1.0
0.5
0.0
ctrl
4dpi
ctrl
4dpi
ctrl-lip
clo-lip

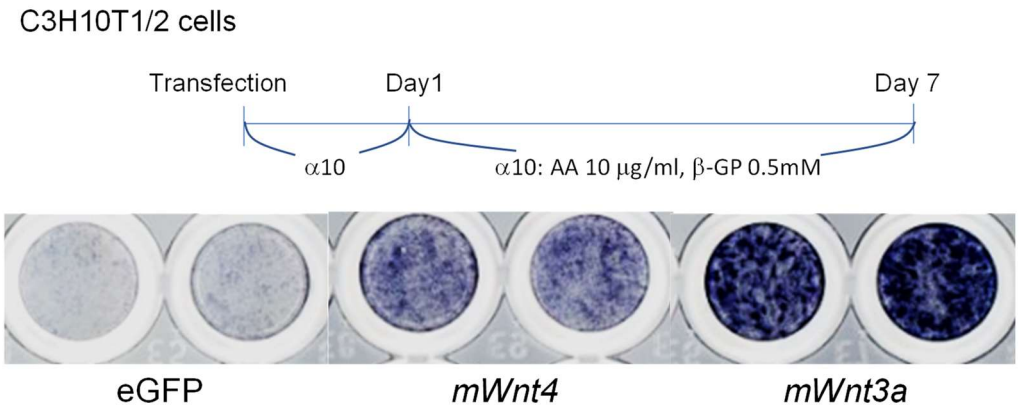
147

148

**Figure. S5 Wnt inhibtors gene expression in bone repair.**

**(a)** Quantitative RT-PCR analyses of Wnt inhibitors including *Dkk1* to *4*, *Sfrp1* to *5* and *Sost* mRNA expression in bone tissues from bone injury sites (4 dpi) and uninjured region of contralateral tibiae (ctrl at 4dpi). n = 3 mice per each group.

**(b)** Quantitative RT-PCR analyses of *Sost* mRNA expression in bone tissues from bone injury sites (4 dpi) and uninjured region of contralateral tibiae (ctrl at 4dpi) treated with Ctrl-lip and Clo-lip (n = 4 mice per each group).


C3H10T1/2 cells
Transfection
Day1
Day 7
α10
α10: AA 10 μg/ml, β-GP 0.5mM
eGFP
mWnt4
mWnt3a

177

**Figure. S6 Wnt4 overexpression induced osteoblastogenesis in C3H10T1/2 cells.**

Detection of ALP activity of C3H10T1/2 cells after transfection by eGFP, mWnt4 and mWnt3a for 24 hours, and cultured in 96-well-plate for 7 days.