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Supplemental Information

Endogenous Bone Marrow MSCs Are Dynamic, Fate-Restricted Participants in Bone Maintenance and Regeneration

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Inventory of Supplemental Information

Figure S1.

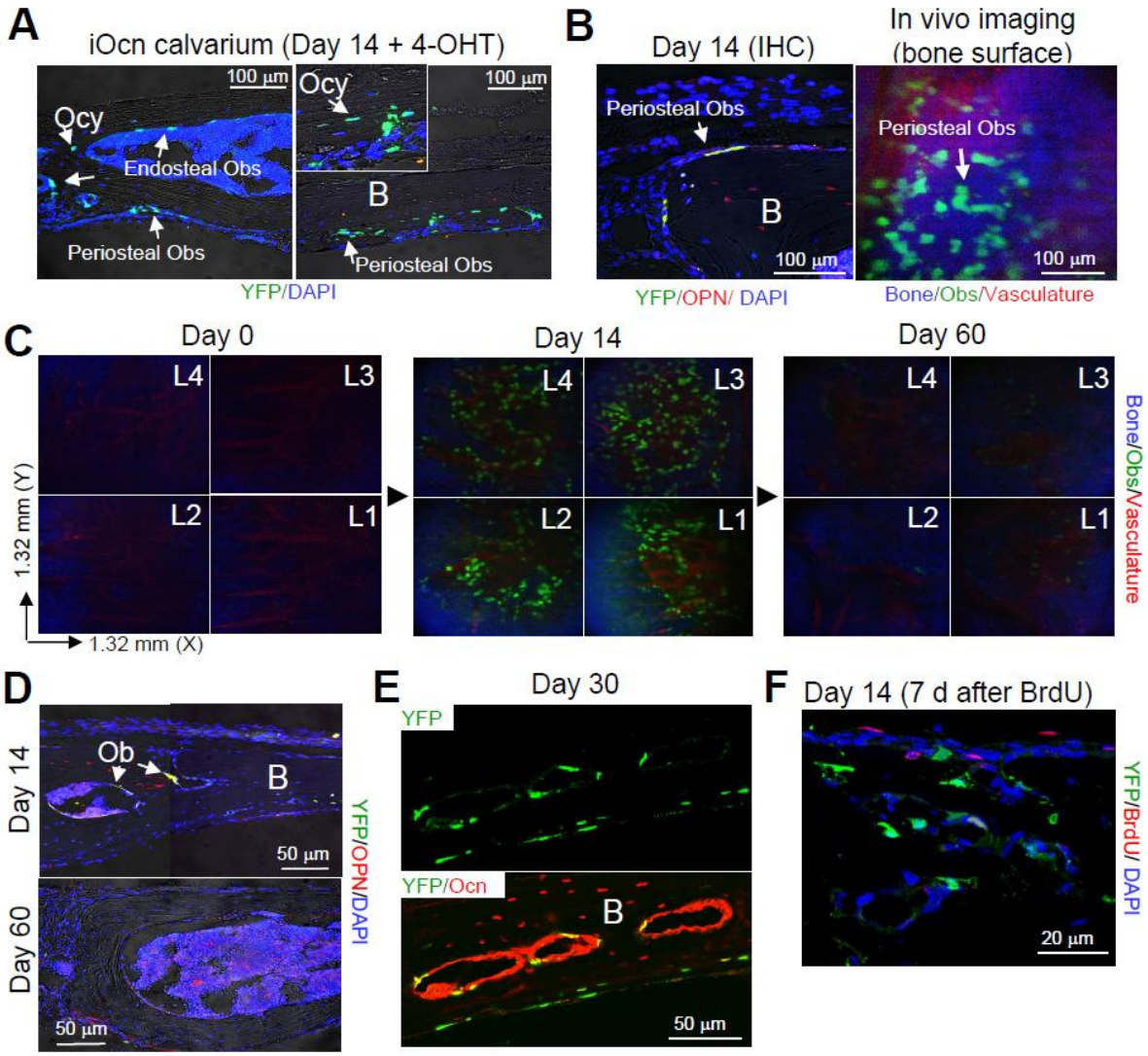
A
iOcn calvarium (Day 14 + 4-OHT)
100 μm
Ocy
Endosteal Obs
Periosteal Obs
Ocy
100 μm
B
Periosteal Obs
YFP/DAPI
B
Day 14 (IHC)
Periosteal Obs
B
100 μm
YFP/OPN/ DAPI
In vivo imaging
(bone surface)
Periosteal Obs
100 μm
Bone/Obs/Vasculature
C
Day 0
Day 14
Day 60
L4
L3
L2
L1
1.32 mm (Y)
1.32 mm (X)
Bone/Obs/Vasculature
D
Day 14
Ob
B
50 μm
YFP/OPN/DAPI
Day 60
50 μm
E
Day 30
YFP
YFP/Ocn
B
50 μm
F
Day 14 (7 d after BrdU)
20 μm
YFP/BrdU/ DAPI

Figure S1. *In vivo* tracking of pulse-labeled mature osteoblasts. (A) Fourteen days after inducible YFP expression in iOcn mouse (*Ocn-Cre-ER*$^{+}$*Rosa-EYFP*$^{+}$, 4-6 wk ages) by tamoxifen (4-OHT) injection, endosteal and periosteal Ocn/YFP^{+} osteoblasts in calvaria were analyzed by anti-GFP/YFP immunohistochemistry (to obtain enhanced YFP signal). Cells and bone structure are visualized by DAPI (blue) and transmission respectively. Arrows indicate Ocn/YFP^{+} osteoblasts (Ob), osteocytes (Ocy). **(B)** Flat and elongated periosteal Ocn/YFP^{+} osteoblasts (or bone lining cells) were visualized by anti-GFP/YFP (Green) and anti-osteopontin (red) immunohistochemistry (left) and by *in vivo* microscopy (right). **(C)** Four sequential images (L1 - L4; 660 μm^2 each) of the first cavity in upper left side of sagittal and coronal suture intersection were repetitively acquired from the same iOcn mouse at the indicated time points. Images of L1 position were used for counting osteoblast number in Figure 1. **(D and E)** Analysis of YFP^{+} osteoblasts in iOcn mouse calvaria at 14 days (D, Day 14), 30 days (E, Day 30), or 60 days (D, Day 60) after tamoxifen injection by anti-GFP/YFP (green) and anti-osteopontin (D, red) or anti-osteocalcin (E, red) immunohistochemistry. **(F)** Fourteen days after tamoxifen injection (7 days after BrdU injection (100 mg/kg/day x 3), YFP+ cells in iOcn mouse femurs were analyzed by anti-GFP/YFP (green) and anti-BrdU (red) immunohistochemistry. B, bone.

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Figure S2.

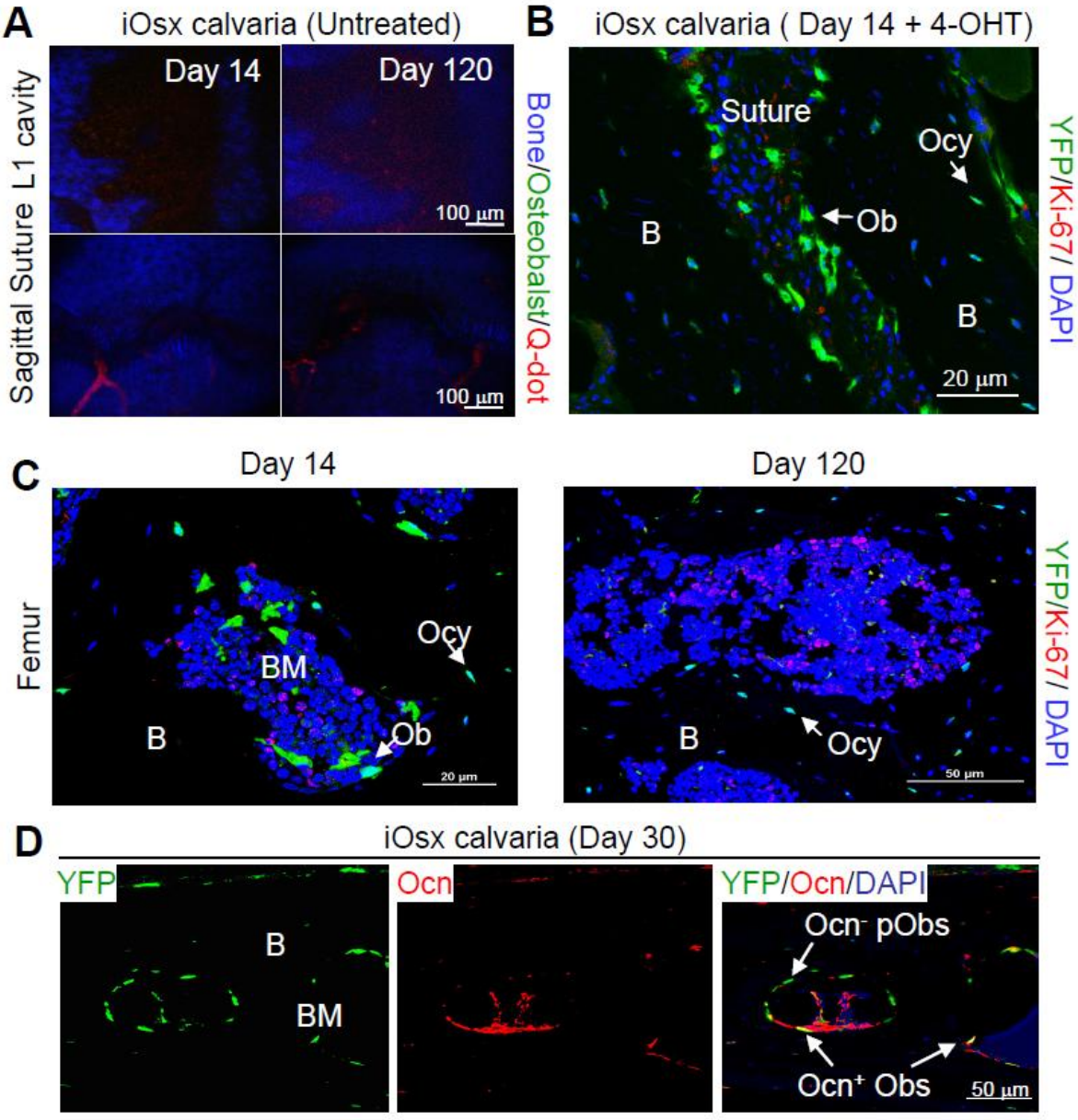
A
iOsx calvaria (Untreated)
Day 14
Day 120
Sagittal Suture L1 cavity
100 μm
100 μm
Bone/Osteobalst/Q-dot
B
iOsx calvaria (Day 14 + 4-OHT)
Suture
Ocy
Ob
B
B
20 μm
YFP/Ki-67/ DAPI
C
Day 14
Day 120
Femur
Ocy
BM
B
Ob
20 μm
B
Ocy
50 μm
YFP/Ki-67/ DAPI
D
iOsx calvaria (Day 30)
YFP
B
BM
Ocn
YFP/Ocn/DAPI
Ocn⁻ pObs
Ocn⁺ Obs
50 μm

Figure S2. *In vivo* tracking of pulse-labeled Osx+ preosteoblasts. (A) No basal labeling of iOsx mice. Without tamoxifen (4-OHT) injection, the first cavity and suture in upper left side of sagittal and coronal suture intersection of iOsx mouse (*Osx-Cre-ER*$^{+}$*Rosa-EYFP*$^{+}$, 4-6 wk ages) were sequentially imaged at the indicated time points. **(B)** Fourteen days after inducible YFP expression in iOsx mouse by tamoxifen (4-OHT) injection, Osx/YFP^{+} preosteoblasts in calvarial suture were analyzed by anti-GFP/YFP (green) and anti-Ki67 (red) immunohistochemistry. Cells and bone structure are visualized by DAPI (blue). **(C)** Analysis of Osx/YFP^{+} cells in iOsx mouse femurs at 14 days or 120 days after tamoxifen injection by anti-GFP/YFP (green) and anti-Ki-67 (red) immunohistochemistry. Arrows indicate YFP^{+} osteoblasts (Ob) and YFP^{+} osteocytes (Ocy). **(D)** Thirty days after tamoxifen injection, YFP^{+} cells in iOsx mouse femurs were analyzed by anti-GFP/YFP (green) and anti-osteocalcin (red) immunohistochemistry. Ocn^{+} Obs, YFP^{+}Ocn^{+} cells; Ocn^{-} pObs, YFP^{+}Ocn^{-} cells; B, bone; BM, bone marrow.

Figure S3.

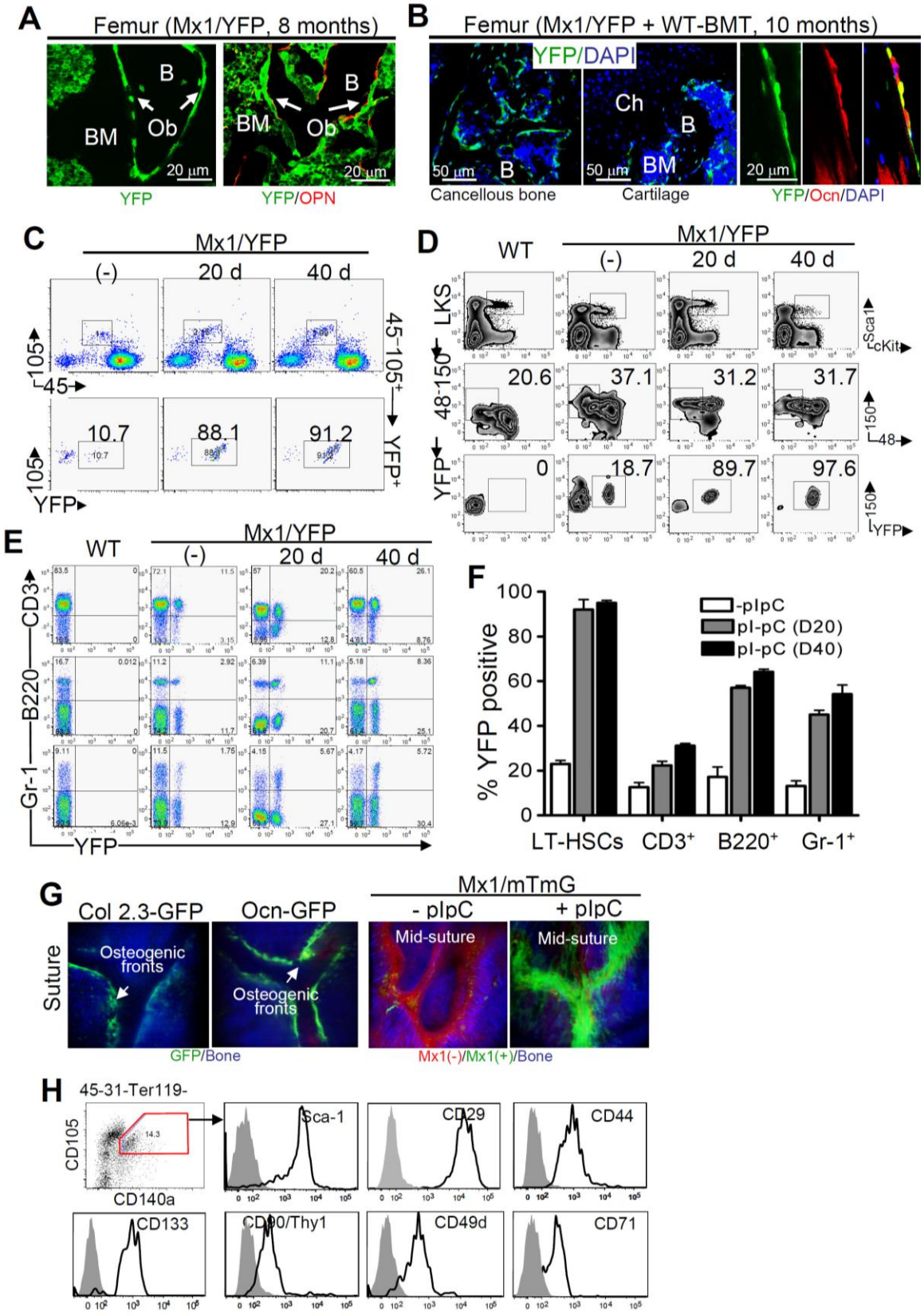
A
Femur (Mx1/YFP, 8 months)
B
Ob
BM
20 μm
YFP
YFP/OPN
B
Femur (Mx1/YFP + WT-BMT, 10 months)
YFP/DAPI
Ch
B
BM
50 μm
20 μm
Cancellous bone
Cartilage
YFP/Ocn/DAPI
C
Mx1/YFP
(-)
20 d
40 d
45-105+
YFP+
10.7
88.1
91.2
D
WT
Mx1/YFP
LKS
Sca1
cKit
48-150+
20.6
37.1
31.2
31.7
YFP+
0
18.7
89.7
97.6
E
WT
Mx1/YFP
CD3
B220
Gr-1
YFP
F
% YFP positive
-pIpC
pI-pC (D20)
pI-pC (D40)
LT-HSCs
CD3+
B220+
Gr-1+
G
Mx1/mTmG
Col 2.3-GFP
Ocn-GFP
- pIpC
+ pIpC
Suture
Osteogenic fronts
Mid-suture
GFP/Bone
Mx1(-)/Mx1(+)/Bone
H
45-31-Ter119-
CD105
CD140a
14.3
Sca-1
CD29
CD44
CD133
CD90/Thy1
CD49d
CD71

Figure S3. (A and B) *Mx1*-induced cells maintain long-term repopulation of osteoblasts *in vivo*. *Mx1*-induced osteoblasts (YFP^+) in femur sections of Mx1/YFP mice prepared 8 months after pIpC treatment (A, no irradiation and no WT-BMT) or 10 months after pIpC treatment (B, at 6-8 weeks after irradiation and wild type bone marrow transplantation, +WT-BMT) were analyzed by staining with anti-GFP/YFP (YFP) and or anti-osteopontin (A, OPN) anti-osteocalcin (B, Ocn, for mature osteoblasts) (right) (n = 3). B, bone; BM, bone marrow; Ob, YFP^+ osteoblasts; Ch, chondrocytes. **(C-F).** Labeling efficiency of osteogenic and hematopoietic cells by *Mx1*. **(C)** Flow cytometry analysis of *Mx1*-induced osteogenic cells. Collagenase-treated bone cells from *Mx1-Cre*$^+$ *Rosa-EYFP*$^+$ (Mx1/YFP) mice at indicated time points were stained with CD45 and CD105. YFP^+ cells within $CD45^-CD105^+$ fraction were analyzed. **(D)** Bone marrow cells from *Mx1* or wild type mice (WT) at the indicated time after pIpC treatment were stained with biotinylate lineage cocktail (B220, Mac1, Ter119, CD3, CD4, CD8; all equal amounts), c-Kit-APC, Sca-1-PE-Cy5.5, CD48-Pacific blue and CD150-PE antibodies followed by streptavidin-pacific orange. Percentage of Mx1/YFP^+ cells within long-term reconstituting hematopoietic stem cell populations (LT-HSCs, lineage$^-$$Kit^+Sca^+CD48^-CD150^+$) was analyzed. **(E)** Lymph node cells from the *Mx1* reporter or wild type mice (WT) were stained with CD3-PE-Cy5, B220-APC, and Gr1-PE antibodies. Percentage of Mx1/YFP^+ cells within indicated population was analyzed. **(F)** Mean percentage of Mx1/YFP^+ cells within indicated fractions in mice before (-) or 20 days (D20) and 40 days (D40) after pIpC treatment (n = 5/each group). **(G and H)** Inmmunophenotypes, and distribution of *Mx1*-induced osteoprogenitors. **(G)** Mx1/$mGFP^+$ population in sagittal and coronal sutures of *Mx1-Cre+/mTomato-mGFP* dual reporter mice (Mx1/mTmG) was compared with GFP^+ population in that of *Col2.3*-GFP or *osteocalcin*-GFP mice. **(H)** FACs analysis for indicated markers in isolated osteoprogenitors ($CD45^-CD31^-Ter119^-CD140a^+CD105^+$).

Figure S4.

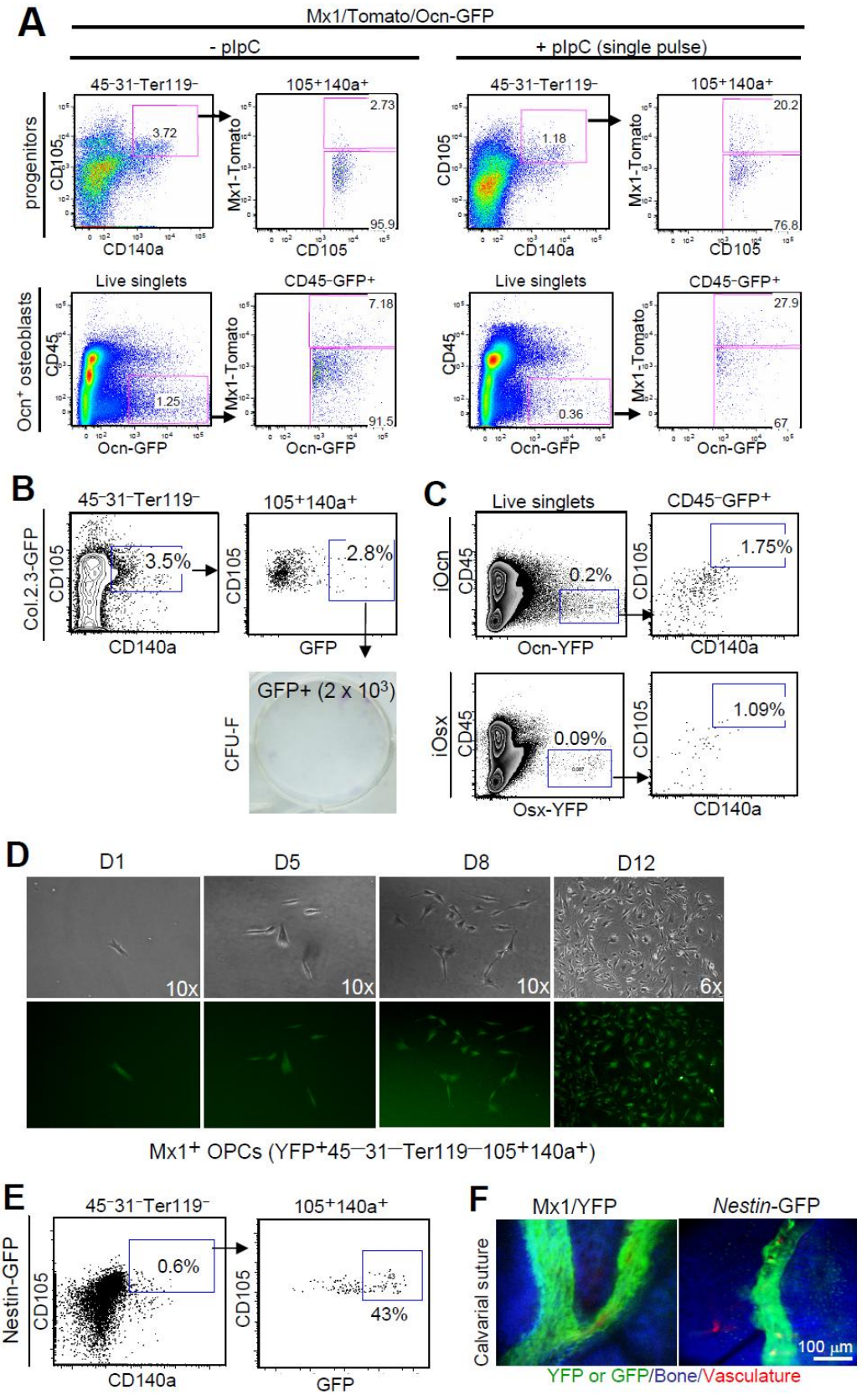
A
Mx1/Tomato/Ocn-GFP
- pIpC
+ pIpC (single pulse)
progenitors
45-31-Ter119-
105+140a+
CD105
CD140a
Mx1-Tomato
3.72
2.73
95.9
1.18
20.2
76.8
Ocn+ osteoblasts
Live singlets
CD45-GFP+
CD45
Ocn-GFP
1.25
7.18
91.5
0.36
27.9
67
B
Col.2.3-GFP
3.5%
2.8%
GFP
CFU-F
GFP+ (2 x 10³)
C
iOcn
Ocn-YFP
0.2%
1.75%
iOsx
Osx-YFP
0.09%
1.09%
D
D1
D5
D8
D12
10x
6x
Mx1+ OPCs (YFP+45−31−Ter119−105+140a+)
E
Nestin-GFP
0.6%
43%
F
Mx1/YFP
Nestin-GFP
Calvarial suture
100 μm
YFP or GFP/Bone/Vasculature

Figure S4. (A) Both progenitors and mature osteoblasts are concurrently labeled by *Mx1.* Before (-pIpC) or twenty four hours after single pIpC pulse (+pIpC), collagenase-treated bone cells from Mx1/Tomato/Ocn-GFP mice were stained with CD45, CD31, Ter119, CD105, CD140a, and PI. Percentage of $Mx1^+$ ($Tomato^+$) cells within $CD45^-Ter119^-CD31^-CD105^+CD140a^+$ fraction (top) or $CD45^-Ocn\text{-}GFP^+$ fraction (bottom) was analyzed. **(B and C)** Mature osteoblasts are non-clonogenic and not in 105^+140a^+ fraction*.* **(B)** Collagenase-treated bone cells from *Col2.3*-GFP (mature osteoblasts) were stained with the indicated antibodies in Figure 3D. Percentage of GFP^+ cells within $CD105^+CD140a^+$ fraction was analyzed and used for CFU-F assay (right). **(C)** Distribution of Ocn/YFP^+ cells and Osx/YFP^+ cells within CD105 and CD140a fraction was tested. **(D)** After sorting $Mx1/YFP^+$ osteoprogenitor cells (OPCs), single cell-derived colonies (CFU-F) were tracked by fluorescent microscopy *in vitro***. (E and F)** *Nestin-GFP*$^+$ MSCs are enriched in $CD105^+140a^+$ fraction and calvarial sutures*.* **(E)** Collagenase-treated bone cells from *Nestin-GFP* mice were stained with CD45, CD31, Ter119, CD105, CD140a, and PI (not shown). Percentage of GFP cells within CD105 and CD140a fraction was analyzed. **(F)** Suture cells from Mx1/YFP and *Nestin-GFP* mice were imaged by intravital microscopy. All data represent at least 3 independent experiments.

Figure S5.

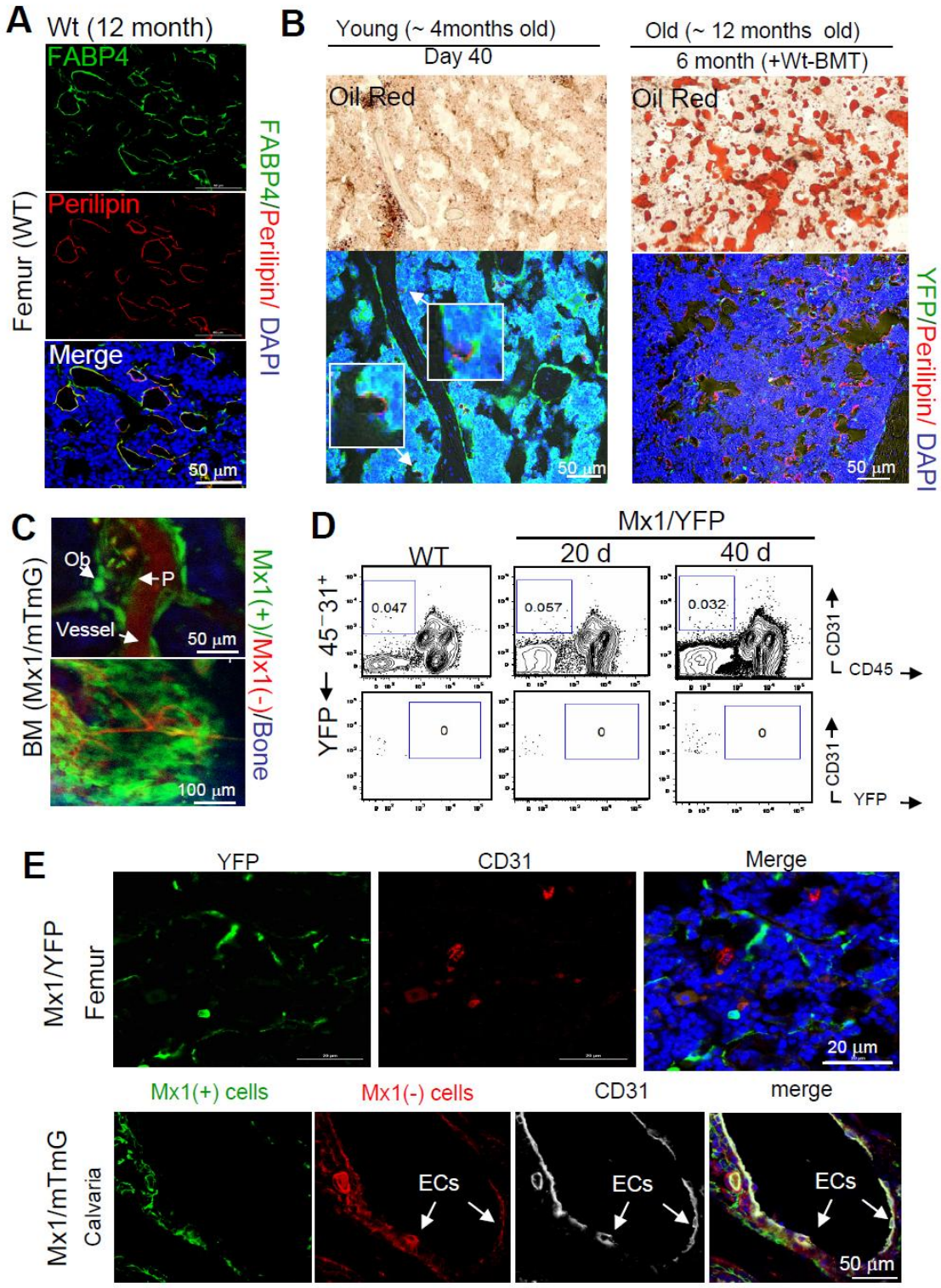
A
Wt (12 month)
FABP4
Perilipin
Merge
Femur (WT)
FABP4/Perilipin/ DAPI
50 μm
B
Young (~ 4months old)
Day 40
Old (~ 12 months old)
6 month (+Wt-BMT)
Oil Red
Oil Red
50 μm
50 μm
YFP/Perilipin/ DAPI
C
Ob
P
Vessel
50 μm
100 μm
BM (Mx1/mTmG)
Mx1(+)/Mx1(-)/Bone
D
Mx1/YFP
WT
20 d
40 d
0.047
0.057
0.032
45⁻31⁺
CD31
CD45
YFP
0
0
0
CD31
YFP
E
YFP
CD31
Merge
Mx1/YFP
Femur
20 μm
Mx1(+) cells
Mx1(-) cells
CD31
merge
Mx1/mTmG
Calvaria
ECs
ECs
ECs
50 μm

Figure S5. *Mx1*-induced cells are distinct from adipocytes and vascular endothelial cells. **(A)** Bone marrow adipocytes were stained by anti-FABP4 (green) and anti-Perilipin (red) antibodies. Blue, DAPI. **(B)** Contribution of *Mx1*-induced cells to adipocyte differentiation was analyzed in femurs from *Mx1* mice 40 days after pIpC treatment (~ 4 months old) or 6 months after pIpC treatment following wild-type marrow transplantation (~ 12 months old) using Oil-red O staining or anti-GFP/YFP and anti-Perilipin antibodies. **(C)** Vessels (Red) and *Mx1*-labeled cells (Green) in Mx1/mTmG mouse bone marrow were imaged by intravital microscopy. Green, *Mx1*-induced cells; Red, *Mx1*-noninduced cells; Blue, bone. **(D)** Bone marrow cells used in Figure S3D were stained with CD31-PE and CD45-Pacific blue. YFP^+ cells within $CD31^+CD45^-$ fraction were analyzed. **(E)** Femur sections from Figure 3A (bottom) or calvaria sections from Figure 3G were stained with anti-CD31 antibody for simultaneously visualizing *Mx1*-induced cells (top green, YFP; bottom green, $Mx1^+$), *Mx1*-noninduced cells (bottom red, $Mx1^-$), and $CD31^+$ endothelial cells (ECs) (top, red; bottom, white). Data represent three (C, E) or five (D) independent experiments with comparable results.

Figure S6.

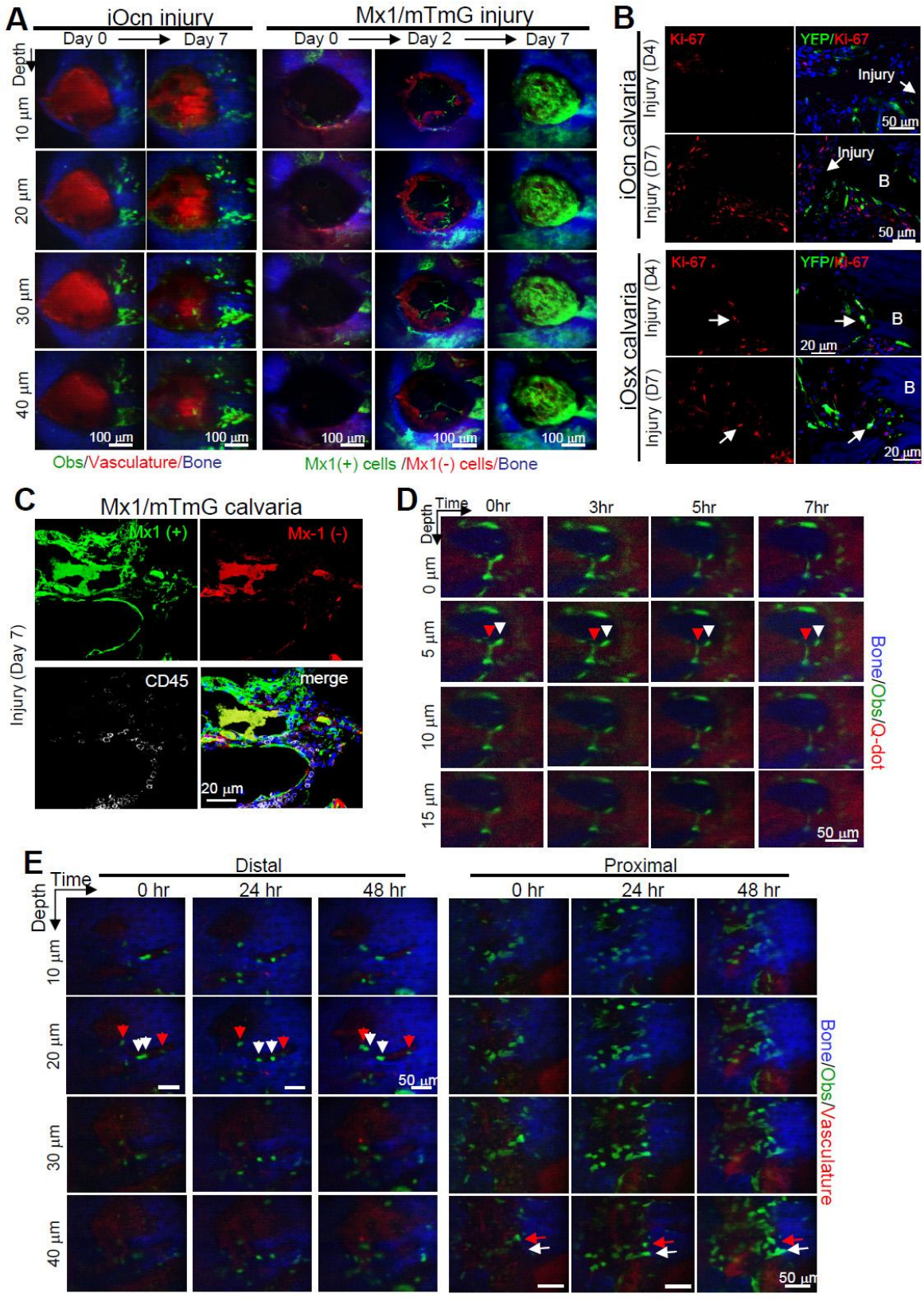
A
iOcn injury
Day 0
Day 7
Mx1/mTmG injury
Day 0
Day 2
Day 7
Depth
10 μm
20 μm
30 μm
40 μm
100 μm
Obs/Vasculature/Bone
Mx1(+) cells /Mx1(-) cells/Bone
B
iOcn calvaria
Injury (D4)
Injury (D7)
Ki-67
YFP/Ki-67
Injury
B
50 μm
iOsx calvaria
20 μm
C
Mx1/mTmG calvaria
Injury (Day 7)
Mx1 (+)
Mx-1 (-)
CD45
merge
20 μm
D
Time
0hr
3hr
5hr
7hr
Depth
0 μm
5 μm
10 μm
15 μm
50 μm
Bone/Obs/Q-dot
E
Time
Distal
Proximal
0 hr
24 hr
48 hr
Depth
10 μm
20 μm
30 μm
40 μm
50 μm
Bone/Obs/Vasculature

Figure S6. (A) Injury sites on iOcn mice or Mx1/mTmG mice were imaged immediately after injury (day 0) and the same positions were reimaged at day 2, day 4 (Fig. 5a, bottom) and day 7. Selected Z stack images (10 μm intervals) are shown. **(B)** The proliferation of YFP tagged *Osx*$^+$ preosteoblasts or *Ocn*$^+$ mature osteoblasts was examined by Ki-67 staining four (D4) or seven (D7) days after injury (imposed 14 days after 4-OHT administration) in iOcn or iOsx mice as indicated. Arrows indicate rare proliferating *Osx*$^+$ cells. **(C)** Immunohistochemical analysis of *Mx1*-induced cells (green), *Mx1*-noninduced cells (red), CD45$^+$ cells (top, white), and fibroblasts (ER-TR7; bottom, white) at the sites of injury. Data represent at least five independent experiments. **(D and E)** Sequential imaging of migrating osteoblasts around injury. **(D)** Continuous tracking of osteoblasts near injury at single cell level *in vivo*. Ocn/YFP$^+$ osteoblasts in a single position of mouse calvaria were sequentially imaged every hour for 7 hours with Z stacks. Selected Z stack images at the indicated time points are shown. **(E)** At 14 days after 4-OHT treatment, representative YFP$^+$ osteoblasts in a single position (proximal: < 400 μm from injury and distal: > 400 μm away from injury) were imaged immediately after injury (0 hr), and the same position was re-imaged at 24 hr and 48 hr after injury. Each image was recorded with Z-stacks (2 μm intervals) and selected images taken at the indicated depth are shown. Arrows indicate stationary (red) and migrating (white) osteoblasts (D and E).

Figure S7.

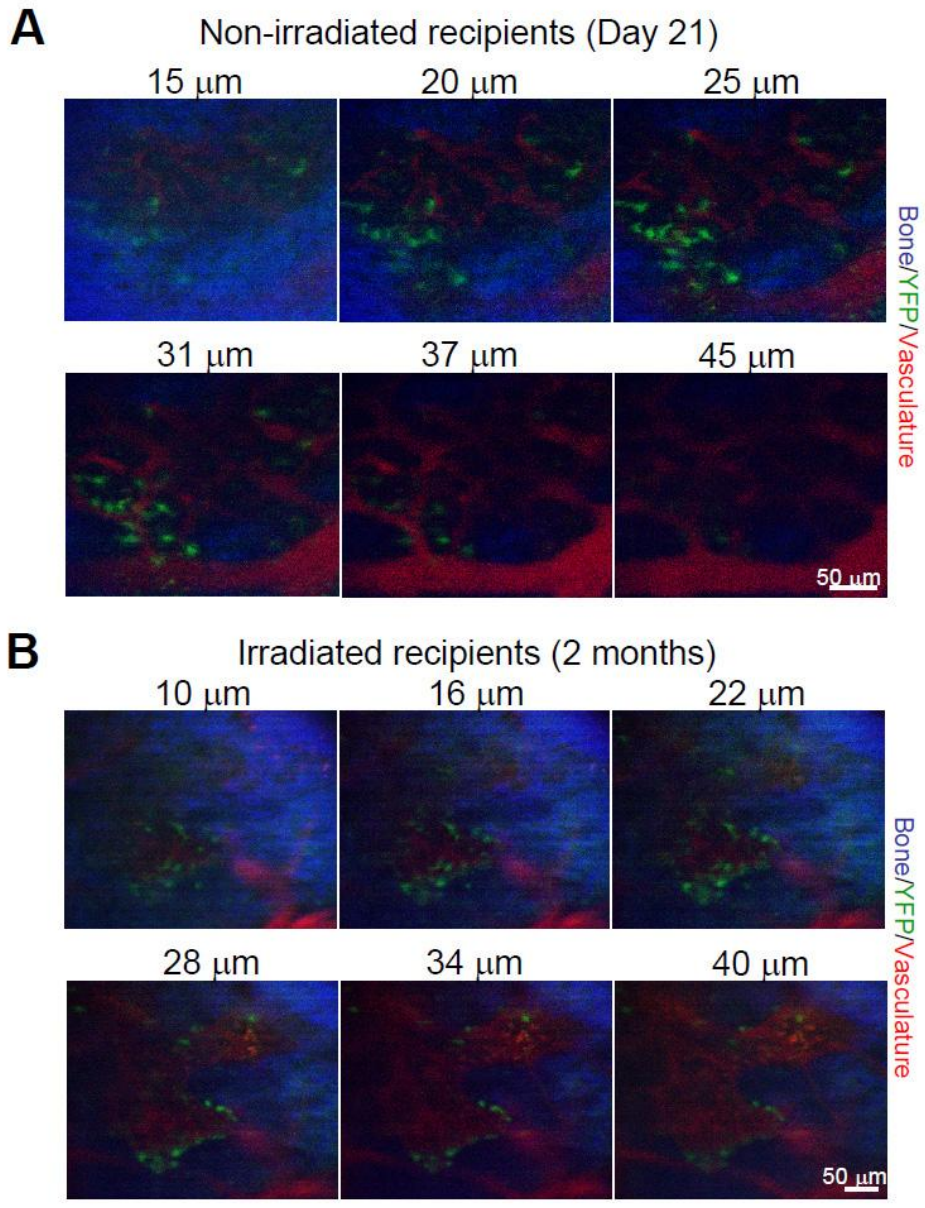

Figure S7. Repopulation of *Mx1*-induced cells in recipient mouse bone after transplantation. Z stack images of repopulated Mx1/YFP-labeled osteoprogenitors in endosteal surface of 3 weeks (A) or 2 months (B) of recipient mice after transplantation. Numbers indicate selected Z stack images.

Supplementary Methods

Genotyping, diphtheria toxin and pIpC induction. Genotyping of all *Cre*-transgenic mice was performed by PCR using primers detecting the *Cre* sequence for *Mx1-Cre* mice (Jackson laboratory protocols). Genotyping of the Rosa locus was performed according to Jackson laboratory protocols. To induce the YFP labeling of osteoblasts in iOcn reporter mice, two doses of 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich) were injected intraperitoneally in two day intervals as 1 mg in 100 μl of corn oil per dose. For inducible ablation of iOcn/iDTR^{+} osteoblasts, mice were injected intraperitoneally for three days with 100 μl of diphtheria toxin (1 mg/ml PBS) once per day. For *Mx1-Cre* induction, mice were injected intraperitoneally with 25 mg/kg of pIpC (Sigma) every other day for 10 days. For long-term tracing of *Mx1*-induced osteogenic cells, Mx1/YFP mice were lethally irradiated with 9.5 Gy one day before intravenous transplantation of 10^6 whole bone marrow mononuclear cells (WT-BMT) from wild type C57BL/6 mice. Eight weeks later, mice were injected with five doses of pIpC (25 mg/kg) every other day to induce *Mx1* activity.

Intravital microscopy. Before imaging, all mice except Mx1/mTmG dual reporter mice and recipient mice for homing experiments were injected retro-orbitally with 20 μl of non-targeted Q-dot 705 (Invitrogen) diluted in 80 μl of PBS. After the mouse was mounted on a 3 axis motorized stage (Suter MP385), the calvarial surface was scanned for second harmonic generation (SHG by femto-second titanium:sapphire laser pulses: 880 nm) from bones and Q-dot signals (633 nm excitation, 650–760 nm detection) from vasculature to identify the injury sites and the intersection of sagittal and coronal sutures. YFP- expressing osteoblasts (492 nm excitation, 520 nm detection) or Tomato-expressing osteoprogenitors (532 nm excitation, 590 nm detection) were simultaneously imaged by confocal microscopy. All images were recorded with their distance to the intersection of the sagittal and coronal sutures, to define their precise location. A PCI-based image capture board (Snapper, Active Silicon) was used to acquire up to three channels simultaneously using iPhoton software. After *in vivo* imaging, the scalp was re-closed using a VICRYL plus suture (Ethicon), and post-operative care was provided as described (Lo Celso et al., 2009).

Isolation and flow cytometry analysis of bone marrow osteoprogenitor cells. To isolate cells from bones, dissected femurs, tibias and pelvis from mice were briefly cracked by a pestle and rinsed 3 times to remove bone marrow cells. To isolate donor cells from injury sites, the

sites were trimmed by surgical scissor. Remaining bones were fragmented by a scalpel and then incubated with 0.1 % collagenase and 1% FBS in PBS at 37°C for 45 min. Dissociated cells were washed with PBS, filtered with a 40-μm strainer and re-suspended at ~ 1 x 10^7 cells/ml. To analyze or isolate osteogenic progenitors, cells were stained with CD105-PE, CD140a-APC, CD45-pacific blue, Sca-1 pe-Cy7, Ter119-Alexa 750, and CD31-APC or CD31-Biotin followed by streptavidin pacific orange in combination with CD29-FITC, CD90-Alexa 488, CD133-FITC, and CD73-PE. Antibodies were purchased from eBioscience unless otherwise stated. Propidium iodide (PI) was used for viable cell gating. Flow cytometric experiments and sorting were performed using LSRII and FACS-Aria (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (TreeStar, Ashland, OR) and represented as histograms, contour, or dot plots of fluorescence intensity.

CFU-F and *ex vivo* multi-lineage differentiation assay. For the CFU-F assay, sorted cells were plated on 6 well plates at a level of 10^2 ~ 10^4 cells/well in MesenCult MSC medium (STEMCELL Technologies) for 14 days. The presence of more than 50 cells in a cluster was counted as a colony. To assess osteogenic differentiation, expanded cells from single cell-derived colonies were plated at 1 x 10^4 cells/well in a 24-well plate (BD Biosciences) at 37°C. The next day, medium was replaced with osteogenic induction medium: αMEM with 20% FBS modified with glycerol 2-phosphate (2.16 mg/ml), 2-phospho-l-ascorbic acid (0.05 mg/ml), and dexamethasone (10 nM) (Sigma-Aldrich, G6251, 49752 and D1756, respectively). After 4 weeks of differentiation, Alizarin red S staining was carried out to evaluate calcium-rich deposits by cells. For adipogenic differentiation, 1 x 10^4 cells/well were cultured with adipogenic medium containing DMEM with 1% antibiotic/antimycotic, 10% FBS, 5 μg/ml insulin, 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, and 60 μM indomethacin (all reagents purchased from Sigma-Aldrich). After 4 weeks, cells were washed, fixed in formalin, and stained with oil red O (according to the manufacturer's protocol) to detect lipid. For chondrogenic differentiation, the medium was carefully aspirated and replaced with DMEM containing 1% antibiotic/antimycotic, 10% FBS, 1 μM dexamethasone, 20 ng/ml TGF-β3 (R&D Systems), and 200 μM ascorbic acid. After 4 weeks, cells were stained with Toluidine blue (according to the manufacturer's protocol). In all experiments, NIH3T3 fibroblasts were used as control cells and control medium was DMEM containing 10% FBS with 1% antibiotic/antimycotic.

Immunofluorescence. Frozen sections from 4% paraformaldehyde fixed and decalcified bones were stained with goat or rabbit polyclonal anti-GFP/YFP antibody (Abcam), rabbit anti-

Osteocalcin (Abcam), rabbit anti-Osterix (Abcam), rabbit anti-Aggrecan (Santa Cruz), rabbit anti-collagen type II (Abcam), rabbit anti-Ki-67 (Leica), mouse monoclonal anti-nestin (Millipore, clone rat-401), rabbit anti-perilipin (Sigma) and anti-CD31 antibody (BD Pharmingen) according to the manufacturer's instructions. Anti-goat or anti-rabbit Alexa Fluor 488, and anti-rabbit Alexa Flour 595 or 647 were used for secondary antibodies (Invitrogen). Vectashield (Vector Labs) containing DAPI nuclear counter-stain was used to mount the sections. Images were acquired with a Nikon Eclipse Ti epifluorescence microscope equipped with a Q-imaging Micropublisher digital CCD color camera. Images were processed with Nikon NID-elements software.