学位論文

Macrophages promote bone regeneration through the activation of LepR (+) cells

Zhifeng He

Department of Hard Tissue Research, Graduate School of Oral Medicine

Academic Advisors:

Professor Yasuhiro Kobayashi and Professor Nobuyuki Udagawa

The thesis submitted to the Graduate School of Oral Medicine,

Matsumoto Dental University, for the degree Ph.D. (in Dentistry)

Macrophages promote bone regeneration through the activation of LepR (+) cells

マクロファージは LepR (+) 細胞を活性化し、骨再生を促進する

CONTENTS

ABBREVIATION

BM	Bone marrow
BV/TV	Bone volume over total volume
CB	Cortical bone
Col1	Type I Collagen α
DiI	Fluorescent DiI Liposome
FACS	Fluorescence-activated cell sorter
F4/80	cell surface glycoprotein F4/80
GFP	Green Fluorescent Protein
LepR	Leptin Receptor
MicroCT	Micro-computed tomography
PFA	Paraformaldehyde
Tb.N.	Trabecular number
Tb.Th.	Trabecular thickness
Tb.Sp.	Trabecular separation
Sp7	Sp7 transcription factor
SSPC	Skeletal stem and progenitor cell

1. ABSTRACT

Macrophages not only clean up damaged tissues but also promote the proliferation and differentiation of stem cells during the regeneration process in various tissues such as bone and muscle. However, the mechanisms by which macrophages promote bone regeneration has remained to be elucidated. We have shown here that macrophages promote the differentiation of an osteogenic progenitor Leptin Receptor (LepR) (+) lineage cells during the bone regeneration process through Wnt/β-catenin signals. The cortical bone of tibiae was punctured in LepR-Cre; Rosa26- tdTomato (tdTomato) mice and then the clodronate liposome (Clo-lip) or control liposome (Ctrl-lip) was intraperitoneally injected into those mice to deplete macrophages. F4/80 (+) macrophages was largely depleted in Clo-lip-treated mice. Micro CT analysis revealed that the newly formed bone volume in Clo-lip-treated mice was significantly lower than that in Ctrl-lip-treated mice in the bone injury site at 7 days post injury (dpi). LepR-Cre labeled bone marrow stromal cells were markedly increased in Ctrl-lip-treated but not in Clo-lip-treated mice in the bone injury site at 4 dpi and the percentage of proliferating Ki67 (+) LepR (+) cells is lower in Clo-lip-treated mice. Most of Sp7 (+) osteoblasts in the injury site at 4 and 7 dpi were labeled by LepR-Cre, suggesting that osteoblasts are differentiated from LepR-Cre-labeled lineage cells. To clarify the involvement of Wnt signals in the differentiation of LepR (+) cells into osteoblasts, the bone regeneration was examined using tamoxifen-treated Axin2-CreERT2; tdTomato mice in which cells with activated Wnt/β-catenin signals express red fluorescent protein tdTomato. Clo-lip administration decreased the percentage of Axin2-tomato (+) Sp7 (+) cells in the bone injury site at 7 dpi. Together, macrophages promote Wnt signals in LepR (+) cells and then facilitate bone regeneration.

2. INTRODUCTION

Bone is a highly regenerative tissue in our body. Bone repeats a cycle of bone resorption and formation to maintain its structure and function. The osteoblasts and osteoclasts are responsible for bone formation and resorption, respectively [1]. Osteoblasts originate from bone marrow stromal cells (BMSCs) and osteoclasts are from monocyte-macrophage lineage cells in bone marrows [2-5]. BMSCs are mesenchymal cells, which are frequently observed in perivascular areas with reticular morphology. They support hematopoietic stem cells by secreting cytokines such as CXCL12 (C–X–C motif chemokine 12) and SCF (stem cell factor, known as Kit ligand). Moreover, mouse BMSCs also express LepR (Leptin Receptor), Nestin, Prx1 (Paired-related homeobox 1), and PDGFRβ (Platelet-derived growth factor receptor beta), which were commonly used as BMSC marker genes for generating transgenic Cre mouse lines to reveal in vivo cell fates and their functions [4, 6, 7].

Musculoskeletal disorders are a major cause of disability in the world. In recent years, there are still a large number of patients suffer from bone fracture caused decreased productivity, low quality of daily life, high medical expenditure and other difficult issues [8-12]. With growing our understanding of bone regeneration process, a great variety of approaches using recombinant proteins, stem cells, and related genes that tried to be applied to help with fracture repair [13-16]. However, clinical limitations still exist and satisfactory healing has not yet been accomplished in all cases. To overcome those clinical limitations, our knowledge of cellular and molecular basis in bone regeneration need to be continuously expanded.

Bone healing process is composed of hematoma formation, inflammation, granulation tissue deposition, callus formation, and bone remodeling [17]. Several key factors in every step are important for the outcome of fracture

healing. A well-known factor affecting the outcome of bone healing is the degree of displacement between fractured bone ends. During the inflammatory phase, skeletal progenitor cells are recruited to the bone injury site and undergo osteogenesis according to guides of immune cells including macrophages that secrete growth factors and cytokines [18]. Thus, the callus formation is considered to depend on the immune cells and skeletal progenitor cells. Recently, macrophage has regarded as one of the most crucial immune cells because they are among the first cells to achieve to the bone injury site and present throughout the whole bone regeneration process [19, 20].

As phagocytes, macrophages remove cell fragments and eliminate pathogens in wound repair [21]. In bone healing process, various macrophage subpopulations not only play roles in damaged tissue clearance but also promote new bone formation. The depletion of macrophages at bone injury region by establishing a several experimental animal models lead to the failure in bone repair [22-25]. F4/80 (+) macrophage subsets that at least include F4/80 (+) Mac-2 (-/low) TRACP (-) osteomacs and F4/80 (+) Mac-2 (hi) TRACP (-) inflammatory cells have been demonstrated that they took part in the bone tissue regeneration during the whole healing process [23]. Besides characterized as classically activated macrophages (M1; pro-inflammatory) and alternatively activated macrophages (M2; anti-inflammatory), CD169 (+) macrophages have reportedly played important roles in bone healing, erythropoiesis and kidney disease [25, 26] These findings have suggested a possibility that different macrophage subpopulations are involved in each step during bone healing process. However, the molecular mechanism by which macrophages promote bone regeneration and what macrophage subpopulations involved in bone healing process in vivo remains largely unknown.

Nineteen Wnt proteins (Wnts) have been identified in mammals and exert two signaling pathways: the canonical Wnt/β-catenin pathway and the β-catenin-independent non-canonical Wnt pathway [27-30]. Generally, in Wnt/β -

catenin signal pathway, Wnt ligands bind to surface receptor Frizzled (FZD) and a co-receptor LRP5/6, which in turn, inhibiting the activity of β-catenin degradation complexes. This led to the cytosolic accumulation and the nuclear translocation of β-catenin. Consequently, transcriptional activators (TCF/LEF) together with β-catenin induces the expression of the target genes in certain cells [31-33]. Wnt/β-catenin signaling is involved in regulating bone metabolism and regenerative process [6, 34-36] as well as in the self-renewal of stem cells and the cell fate determinations [37].

Here we show that F4/80 (+) macrophage subset depleted by clodronate liposome administration leads to the impaired osteogenesis at bone injury site and the impaired activation of LepR (+) skeletal progenitor cells in the inflammatory phase. Furthermore, we have observed the depletion of F4/80 (+) macrophage caused limited number of Wnt activated osteoblastic lineage cells during bone healing, providing vital support to the macrophage subsets regulate the activation of skeletal progenitor cells in regenerative state directly or indirectly through the Wnt signaling pathway.

3. EXPERIMENTAL PROCEDURES

3.1 Experimental animals.

C57BL/6 mice were obtained from Japan SLC. B6.129-Leprtm2(cre)Rck/J (LepR-Cre) [38], Axin2Cre/ERT2 mice (Stock#018867) [39] and Rosa26tdTomato mice (Stock#007914) [40] were purchased from Jackson Laboratory (Bar Harbor, ME). Col1(2.3)-green fluorescent protein (GFP) mice were [41] kindly provided by Dr. K. Matsuo at Keio University. All animal studies were approved by the Animal Management Committee of Matsumoto Dental University and animal experiments were made to minimize suffering.

3.2 Antibodies and reagents.

FITC-conjugated anti-F4/80 rat monoclonal antibody [CI: A3-1] (ab105155), anti-Sp7/osterix rabbit polyclonal antibody (ab22552) and anti-beta-catenin rabbit monoclonal antibody (ab32572) were purchased from Abcam (Cambridge, UK). Anti-mouse osteocalcin monoclonal antibody (R21C-01A M188) was from TAKARA (Shiga, Japan). Rat IgG2b κ Isotype control antibody (RTK4530 40060) was from BioLegend (San Diego, CA, USA). Anti-leptin receptor goat antibody (AF497) and normal goat IgG control (AB-108-C) were from R&D Systems (Minneapolis, MN, USA). FITC-conjugated Ki-67 monoclonal antibody (SolA15) was from eBioscience (Waltham, MA, USA). FITC-conjugated Ki-67 monoclonal antibody (11-5698-82) from Invitrogen (Waltham, MA, USA).

The secondary antibodies used were FITC-conjugated donkey anti-rat IgG [AP189F; Sigma-Aldrich St. Louis, MO, USA] and Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (ab150073; Abcam). Alexa Fluor® 488-

conjugated donkey anti-goat IgG (A11055) and Alexa Fluor® 594-conjugated donkey anti-rat IgG (A21209) were from Invitrogen. For enhancing immunoreactions, Can Get Signal (TOYOBO CO, Osaka, Japan) was used. Nuclei were stained with TO-PRO-3 Iodide (642/661) (Molecular Probes, Waltham, MA, USA).

3.3 Mouse tibia intramembranous bone injury surgery.

Mice at 10 to 12 weeks old were used. Under general anesthesia, an incision was made in the skin over the tibia, and cortical bone was exposed. A hole (0.8 mm diameter) on the cortical bone was created using a 21-gauge needle.

3.4 Depletion of macrophages during bone regeneration process by clodronate liposome administration.

Liposomes used in this study were purchased from Liposoma BV Co.ltd. (Science Park 408, 1098XH AMSTERDA, the Netherlands). Clodronate liposomes: a suspension of artificially prepared lipid vesicles encapsulating clodronate. The concentration of clodronate in the suspension is ca. 5 mg/ml. Control liposomes: a suspension of artificially prepared lipid vesicles encapsulating an aqueous PBS solution. All the mice were treated with intraperitoneal injections (10.0 μ l/g) of the clodronate liposome (Clo-lip) or Control liposome (Ctrl-lip) at the time of surgery and followed by every two days administrations until 1 day before tissue harvests.

3.5 Micro-computed tomography (micro-CT) analysis.

Micro-CT analysis (ScanXmate-A080, Comscan Tecno, Kanagawa, Japan) was performed in 10 to 12-week-old mice to evaluate the bone regeneration condition in the bone injury site 7 dpi. Calculation of trabecular

bones located at injury site on day 7 using image analysis software (TRI/3D-BON, Ratoc System Engineering, Tokyo, Japan).

3.6 Histological analysis.

Samples were fixed by 4% paraformaldehyde (PFA) overnight at 4 ºC. Then the samples were transferred into 20% sucrose in PBS to incubate at 4 ºC more than 24 h. O.C.T compound was used for embedding tissues. The sections were made at 10 μm thickness with Kawamoto's film method using Cryofilm type IIIC and a tungsten carbide knife (Section-Lab Co. Ltd. Hiroshima, Japan). The sections were incubated with primary antibody overnight at 4 ºC and were further incubated with secondary antibody at room temperature for 2 h. The sections were mounted using 30% glycerol, covered with coverslips, and sealed with nail polish. Fluorescence images were acquired using a confocal laser scanning system (LSM 510; CarlZeiss, Oberkochen, Germany).

For paraffin-embedded sections. Mouse tibias were fixed by 4% paraformaldehyde (PFA) and then hard tissues were decalcified in 10% EDTA for 1 week at 4 ºC. The sections were prepared at a thickness of 4 μm and stained with hematoxylin and eosin after deparaffinization.

3.7 Flow cytometry analysis.

Fluorescence-labeled antibodies, FITC-conjugated anti-F4/80 rat monoclonal [CI: A3-1] (ab105155) and isotype-matched control IgG were purchased from BioLegend. Fluorescent DiI Liposomes (a suspension of artificially prepared lipid vesicles labeled with DiI encapsulating an aqueous PBS solution) was purchased from Liposoma BV and used for detecting phagocytes according to the same protocol for the injections of Clo-lip. Flow cytometric analysis was performed using a flow cytometer (Cytomics FC500; Beckman

Coulter, Fullerton, CA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

3.8 RNA isolation and quantitative real-time PCR.

Samples of bone tissue from bone injury site of mouse tibiae and control samples of uninjured region from the contralateral tibiae were homogenized in TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) using TissueLyser II (Qiagen, Hilden, Germany). Total RNA was purified using RNA isolation kits (NucleoSpin RNA, Macherey-Nagel, Düren, Germany). cDNA was synthesized from 1 mg purified RNA with iScript gDNA Clear cDNA Synthesis Kit (BIO-RAD) according to the standard protocol and real-time RT-PCR was performed using Fast SYBR Green (Thermo Fisher Scientific) and the StepOnePlus system (Thermo Fisher Scientific). The PCR conditions were 95 ºC for 20 s for reverse transcription, followed by 40 cycles of 95 ºC for 3 s and 60 ºC for 30 s. Gene expression data was normalized to Gapdh. The sequences of other primers for each gene were as follows:

Gapdh, 5′ -TGTGTCCGTCGTGGATCTGA-3′ (forward) and 5′ -TTGCTGTTGAAGTCGCAGGAG-3′ (reverse);

Sp7, 5′ - CGCATCTGAAAGCCCACTTG-3′ (forward) and 5′ -CAGCTCGTCAGAGCGAGTGAA′ (reverse);

Axin2, 5′ - ATGTCCTGTCTGCCAGCGTTC-3′ (forward) and 5′ -CAAGCACTAGCCAGTGGGTCAA-3′ (reverse).

3.9 Induction of Cre-mediated recombination

Tamoxifen (T-5648; Sigma) was dissolved in corn oil followed by sonication for 15 to 30 min at 37 ºC. The tamoxifen solution (2 mg of tamoxifen per 30 g of body weight) was intraperitoneally administered into Axin2-Cre ERT2; Rosa26 tdTomato mice.

3.10 Statistics.

Data were evaluated by unpaired Student' s t-tests. Independent experiments were performed at least three times. Statistical analyses were performed with GraphPad Prism 7 (GraphPad Softwear Inc., La Jolla, CA). $P < 0.05$ was considered significant.

4. RESULTS

4.1 F4/80 (+) phagocytic macrophages are indispensable for bone regeneration.

The injection of Clo-lip is established as a macrophage depleting method in mice [23]. To deplete bone marrow macrophages during the bone regeneration process *in vivo*, Clo-lip was injected intraperitoneally every other day after exerting bone injury surgery until tissue harvest (Fig. 1A). The reduction of macrophages was confirmed by immunofluorescence analysis of F4/80 (Fig. 1B). Micro-CT analysis showed that the the formation of regenerated bone was suppressed in the macrophage-depleted mice compared with Ctrl-lip-treated mice. Clo-lip-treated mice exhibited the significant reduction of bone volume and trabecular bone number as well as the increase of trabecular bone separation at injury site (Fig. 1C). To confirm the incorporation of liposomes into macrophages, we injected DiI (red fluorochrome)-liposome, instead of Clo-lip into the mice. More than 50% of F4/80 (+) macrophages were DiI-positive (54.93±6.72%, n=3) in the injury site at 4 dpi (Fig. 1D). FACS analysis also showed that most of the DiI (+) cells highly expressed F4/80 (Fig. 1E). These findings suggest that Clo-lip is incorporated into F4/80 (+) macrophages and induces their cell death.

Hematoxylin and eosin (H&E) staining showed that most of newly formed bone matrices associated with osteoblasts on their surfaces exhibited dark pink color in the injury site at 7 dpi in Ctrl-lip-treated mice. In the Clo-lip-treated bone injury site, most of bone matrices exhibited pale pink with lesser cells on their surfaces and hypertrophic cartilage-like tissues were observed. This finding indicates that the macrophage depletion causes a delay of bone formation. (Fig. 2A).

We next examined whether the macrophage depletion affects osteoblast differentiation. Using Col1(2.3)-GFP mice [42, 43] in which osteoblasts express GFP and exhibit green fluorescent, we assessed localizations of osteoblasts in the callus of 7 dpi by detection of GFP (+) and osteocalcin (+) immunofluorecent signals in those mice treated with Clo-lip (Fig. 2B, C). In the macrophage-depleted mice, the number of osteoblasts markedly decreased in the injury site compared to the controls. These results suggest that F4/80 (+) macrophage subsets are the targeted cells of clodronate liposome, which are indispensable for osteogenesis in the bone injury site to create the callus.

4.2 F4/80 (+) macrophages promote bone regeneration via stimulating LepR (+) skeletal progenitor cell proliferation.

Leptin receptor (+) cells largely contribute to newly differentiated osteoblasts in the fracture region 2 weeks after injury, but not in uninjured bone [4]. In our bone injury model, most of the osteocalcin (+) osteoblasts (97.10±1.51%) in callus 1 week after injury are Tom (+) in LepR-Cre; Rosa26 tdTomato (R26-Tomato) mice (data not shown). From 1 to 7 dpi, the LepR-Cre labeled cells gradually expanded and moved to the central area of injury site. These cells were extremely abundant in the injury site at 4 dpi. In addition, we noted that LepR (+) cells synchronously expanded with the F4/80 (+) macrophage expansion (data not shown). Furthermore, F4/80 (+) signals (green) were observed to be partially overlapped with LepR (+) signals (red), indicating that macrophages were closely associated with LepR (+) cells during healing process. Therefore, we examined whether the ablating of macrophages affected on the expansion of LepR (+) cells. As a result, the number of LepR (+) cells significantly decreased in the injury site at 4 dpi after F4/80 (+) macrophages depletion (Fig. 3A-C). Immuno-fluorescent staining by LepR and Sp7 antibodies further revealed the LepR-Cre labeled cells express LepR protein and Sp7 in the injury sites at 4 dpi in Ctrl-lip-treated mice. In contrast, LepR-

Cre-labeled LepR (+) cells and –labeled Sp7(+) cells are markedly decreased in the injury sites in Clo-lip-treated mice (Fig. 3. D, E). These findings suggest that F4/80 (+) macrophages support the expansion of osteoblastic bone marrow stromal cells.

Next, we examined whether the proliferation of LepR (+) cells was impacted by the absence of F4/80 (+) macrophages during healing processes. The ablation of macrophages markedly reduced the percentage of proliferating LepR (+) cells (yellow cells) in injured site, but not in uninjured region (Fig. 4 A, B), implying that LepR (+) cells receive mitogenic signals from F4/80 (+) macrophages due to the specific interactions between them during bone regeneration.

4.3 F4/80(+) macrophages depletion caused limited number of Wnt activated osteoblastic lineage cells.

Wnt/ β-catenin signals are involved in cell proliferation [44] and the expression of Runx2 and Sp7 which regulates osteoblastic differentiation [45]. We therefore examined whether the macrophage ablation had impact on the activation of Wnt/β-catenin signals of LepR (+) cells during bone healing process. The expression of Sp7 and a Wnt target gene Axin2 mRNA was upregulated at 4 dpi in Ctrl-lip-treated mice but not in Clo-lip-treated mice (Fig. 5A, B). Moreover, the percentage of Wnt/β-catenin signals activated β-catenin (+) LepR-Cre labeled cells significantly decreased in the injury site at 4 dpi in Clo-lip-treated mice (Fig. 5C). These data indicated the Wnt/β-catenin signaling might be suppressed in LepR-Cre labeled osteoblastic lineage cells after the depletion of macrophages.

We next examined the bone healing processes of Axin2-CreERT2 tdTomato mice since Axin2 was known as an established target gene of Wnt/β-catenin signals [46-49]. Axin2-Cre–labeled Tomato (+) cells were observed

during bone healing phases by tamoxifen injections. The number of Axin2-Cre–labeled Tomato (+) cells located at bone injury site at 4, 5 and 7 dpi were lower in Clo-lip -treated mice compared with Ctrl-lip-treated mice (Fig. 5D). Furthermore, we examined the expression of LepR and Sp7 proteins in Axin2-Cre-labelled cells because Sp7 is continuously expressed during the transition of osteoblastic lineage cells from reticular-osteoblast hybrid state to Wnt/β-catenin active skeletal stem cell-like state while the expression of LepR is gradually decreased with osteogenesis [6]. The percentage of both LepR (+) and Sp7 (+) Axin2-Cre-labeled cells significantly decreased at 5 dpi in mice treated with Clo-lip (Fig. 5E-I). Similar results have been obtained at 7 dpi (Fig.5 J-M). These findings indicated that the macrophages depletion decreased the number of Wnt activated osteoblastic lineage cells and implied that F4/80 (+) macrophages played a critical role that promoted the proliferation and differentiation of LepR (+) cells through the direct or indirect activation of Wnt/β-catenin signals in bone repair (Fig. 6).

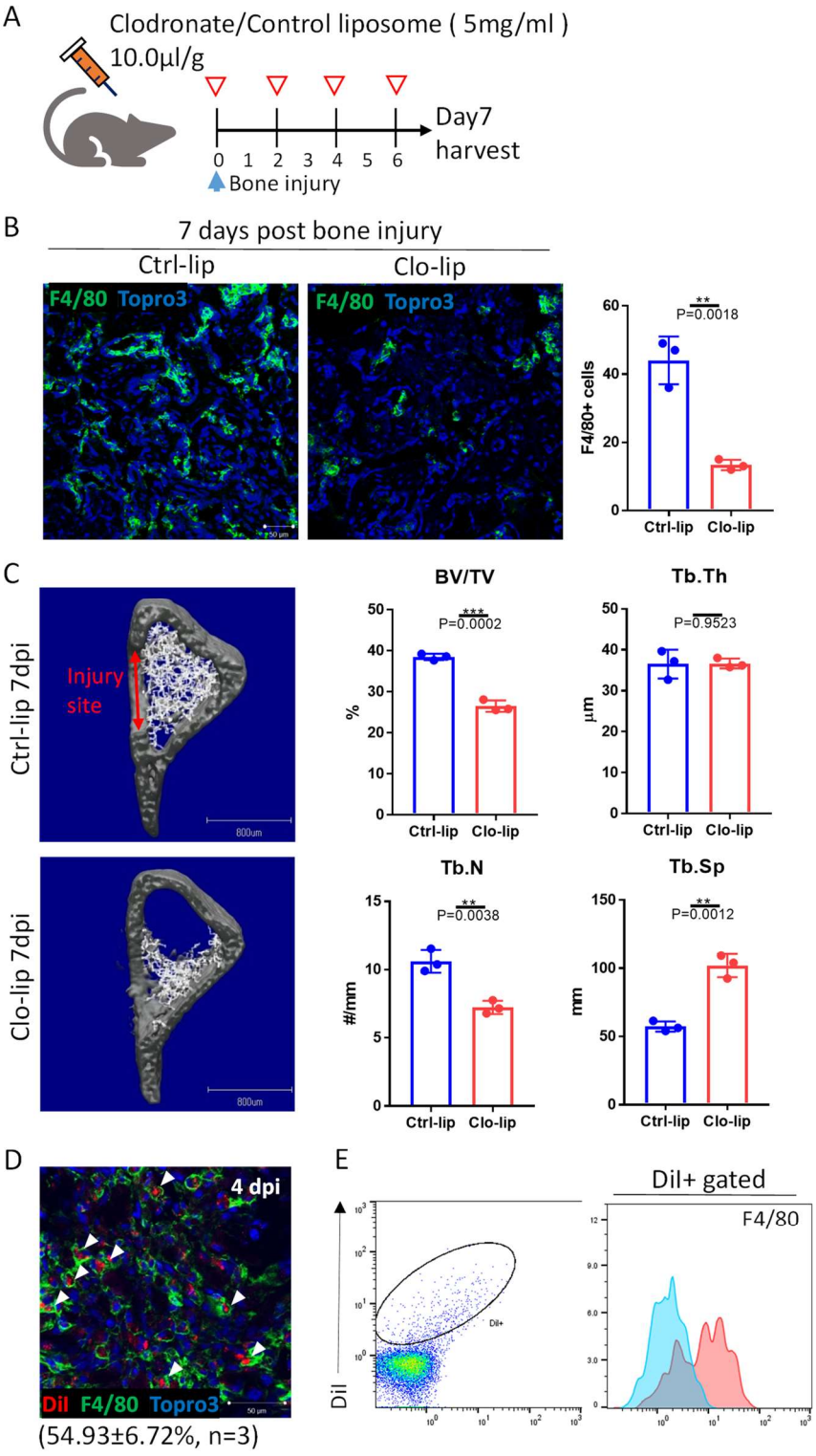

Fig. 1 F4/80 (+) phagocytic macrophages are indispensable for bone regeneration.

Fig. 1 F4/80 (+) phagocytic macrophages are indispensable for bone regeneration.

A. Experimental protocol for injections of clodronate liposome (Clo-lip) or Control liposome (Ctrl-lip).
B. Immuno-fluorescent analysis of F4/80 (+) cells in the bone injury site at 7 dpi. Nuclei were stained with TO-PRO-3 Iodide. Statistical analysis was performed using unpaired Student' s t-tests. Scale bar = 50 μm.
C. Micro CT analysis of the bone injury site on 7 dpi during bone regeneration. Bone volume / tissue volume (BV/TV), trabecular bone thickness (Tb. Th), trabecular bone number (Tb. N) and trabecular bone separation (Tb. Sp) of the injured region. Statistical analysis was performed using unpaired Student' s t-tests. Scale bar = 800 μm.
D. Immuno-fluorescent analysis of F4/80 (+) and DiI (+) cells in the bone injury site at 4 dpi. % of DiI (+); F4/80 (+) cells/F4/80 (+) cells (mean ± s.d, n=3 mice). Scale bar = 50 μm.
E. Flow cytometry analysis of F4/80 expression in DiI (+) cells (n=3 mice).

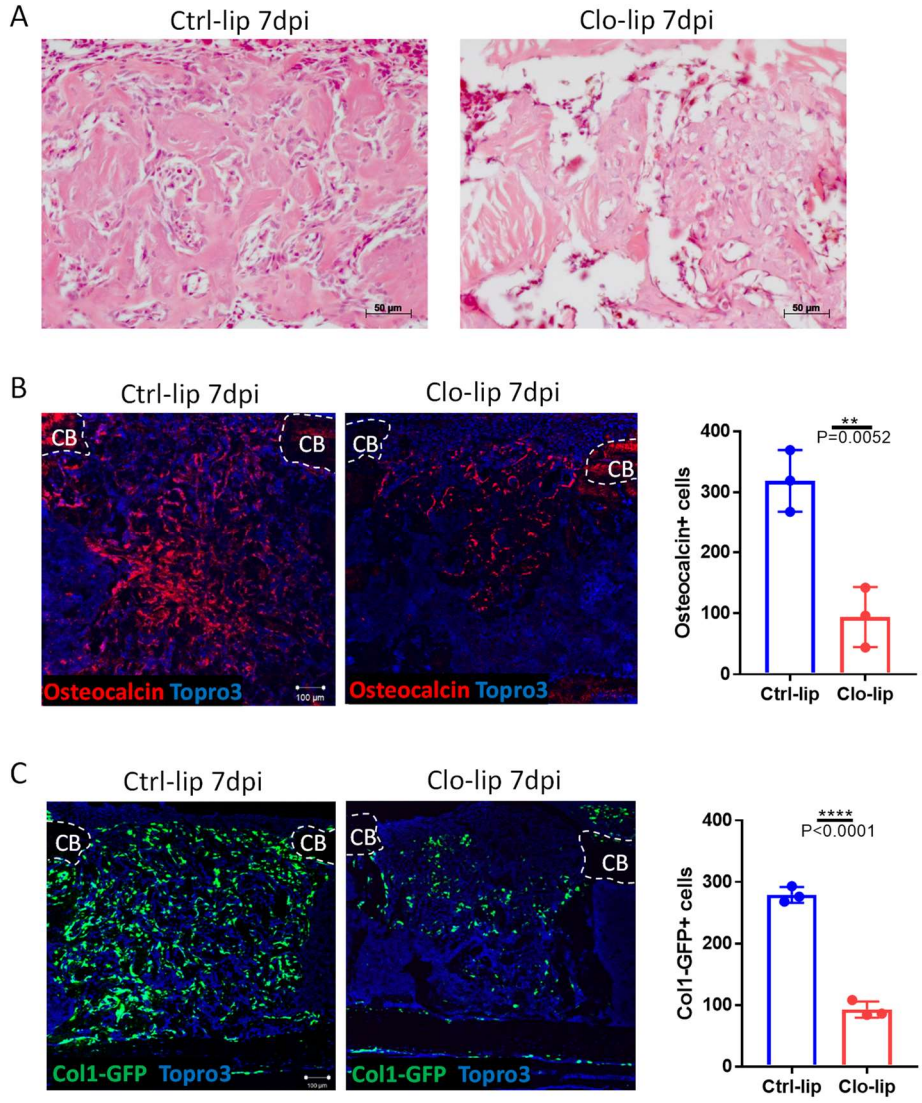

Fig. 2 F4/80 (+) phagocytic macrophages which are depleted by clodronate liposome regulates osteogenesis at bone injury site.

Fig. 2 F4/80 (+) phagocytic macrophages which are depleted by clodronate liposome regulates osteogenesis at bone injury site.

A. Representative images of hematoxylin and eosin (H&E) staining in the bone injury site at 7 dpi (n=3 mice). Scale bar = 50 μm.
B. Immuno-fluorescent analysis of osteocalcin (+) cells in the bone injury site at 7 dpi. Nuclei were stained with TO-PRO-3 Iodide. Statistical analysis was performed using unpaired Student' s t-tests. Scale bar = 100 μm.
C. Immuno-fluorescent analysis of Col1-GFP (+) cells in the bone injury site at 7 dpi. Nuclei were stained with TO-PRO-3 Iodide. Statistical analysis was performed using unpaired Student' s t-tests. Scale bar = 100 μm.

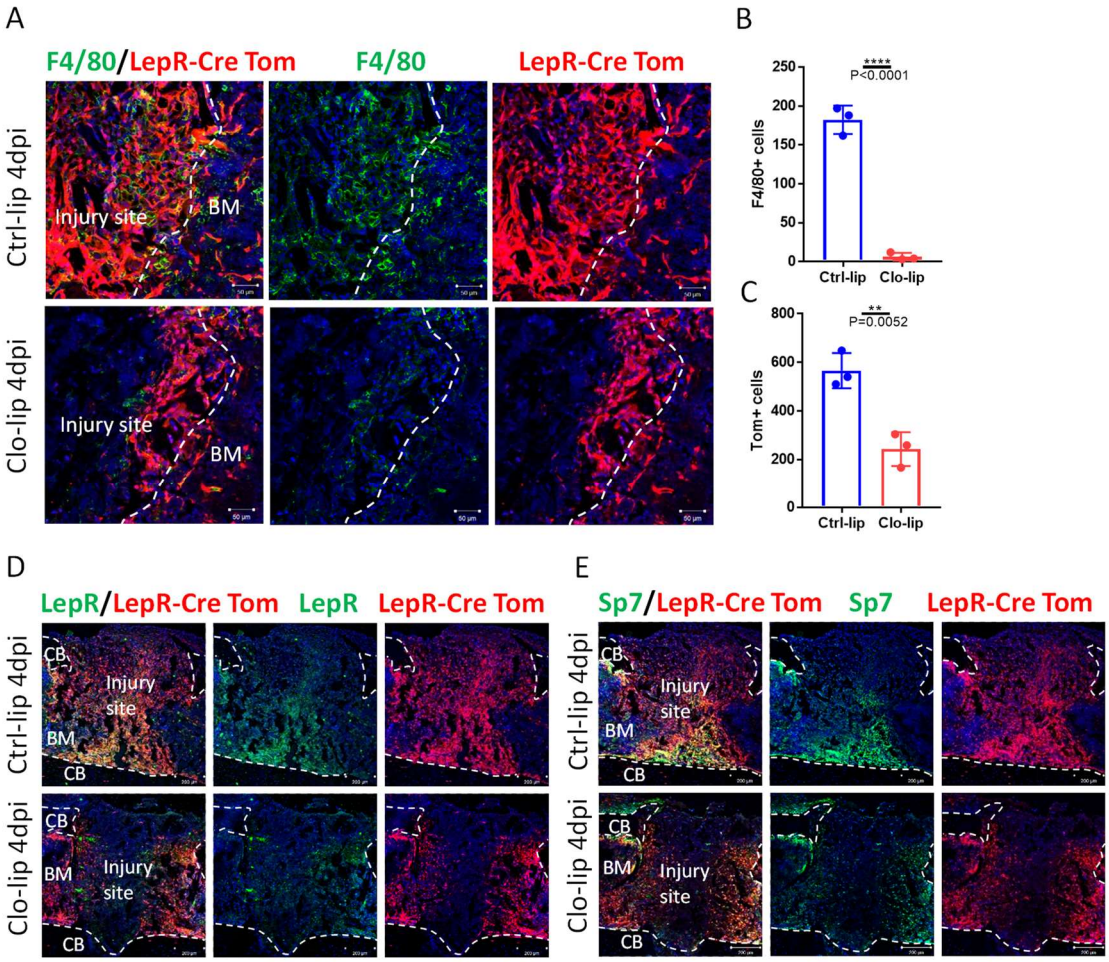

Fig. 3 Macrophages induce LepR (+) cells activation during bone regeneration.

Fig. 3 Macrophages induce LepR (+) cells activation during bone regeneration.

A. Immuno-fluorescent analysis of F4/80 (+) cells in LepR-Cre tdTomato mice in the bone injury site at 4 dpi. Nuclei were stained with TO-PRO-3 Iodide. Scale bar = 50 μm.
B. Quantitative data of F4/80 (+) cells mice in the bone injury site at 4 dpi. Statistical analysis was performed using unpaired Student' s t-tests.
C. Quantitative data of LepR-Cre labeled Tomato (+) cells mice in the bone injury site at 4 dpi. Statistical analysis was performed using unpaired Student' s t-tests.
D. Immuno-fluorescent analysis of LepR (+) cells in LepR-Cre tdTomato mice in the bone injury site at 4 dpi (n=3 mice). Nuclei were stained with TO-PRO-3. Iodide. Scale bar = 200 μm.
E. Immuno-fluorescent analysis Sp7 (+) cells in LepR-Cre tdTomato mice in the bone injury site at 4 dpi (n=3 mice). Nuclei were stained with TO-PRO-3. Iodide. Scale bar = 200 μm.

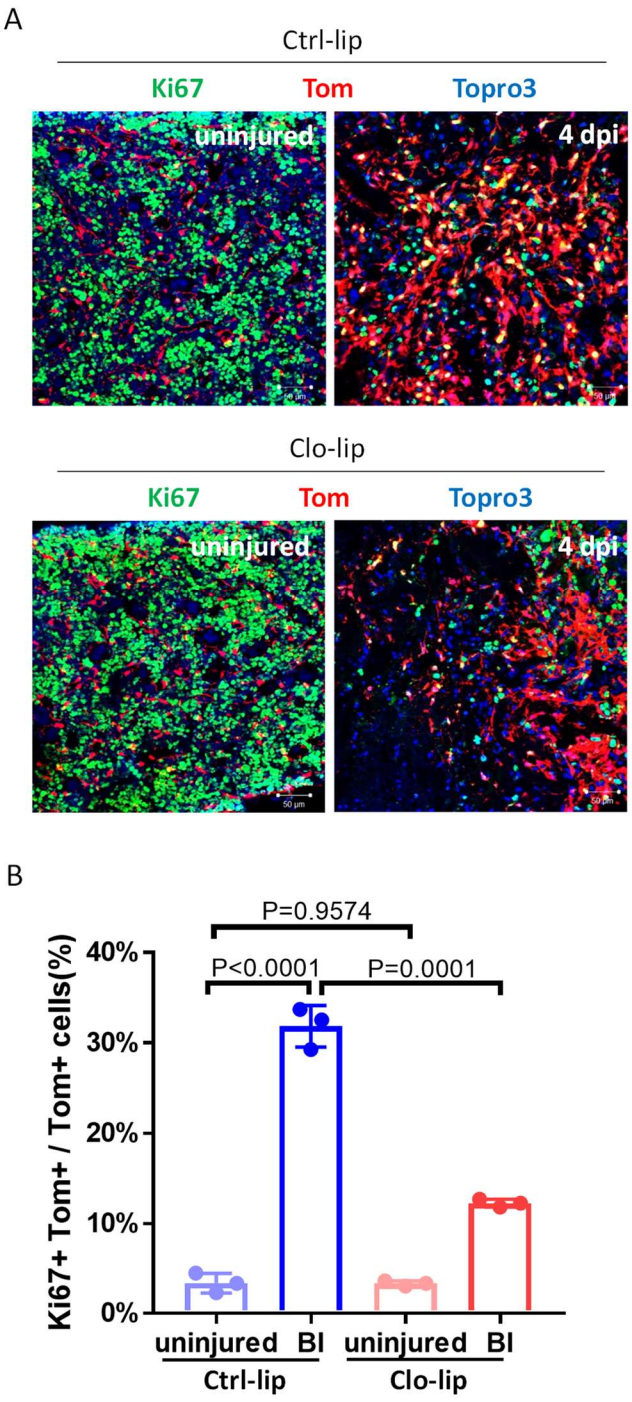

Fig. 4 Macrophage depletion decreased the proliferation rate of the LepR+ SSPCs in the bone injury site at 4 dpi.

Fig. 4 Macrophage depletion decreased the proliferation rate of the LepR+ SSPCs in the bone injury site at 4 dpi.

A. Immuno-fluorescent analysis of Ki67 (+) cells in LepR-Cre tdTomato mice in the bone injury site at 4 dpi and uninjured bone marrow area of diaphysis. Nuclei were stained with TO-PRO-3 Iodide. Scale bar = 50 μm.
B. The percentage of Ki67 (+) LepR-Cre labeled Tomato (+) in all Tomato (+) cells in the bone injury site at 4 dpi. Statistical analysis was performed using unpaired Student' s t-tests.

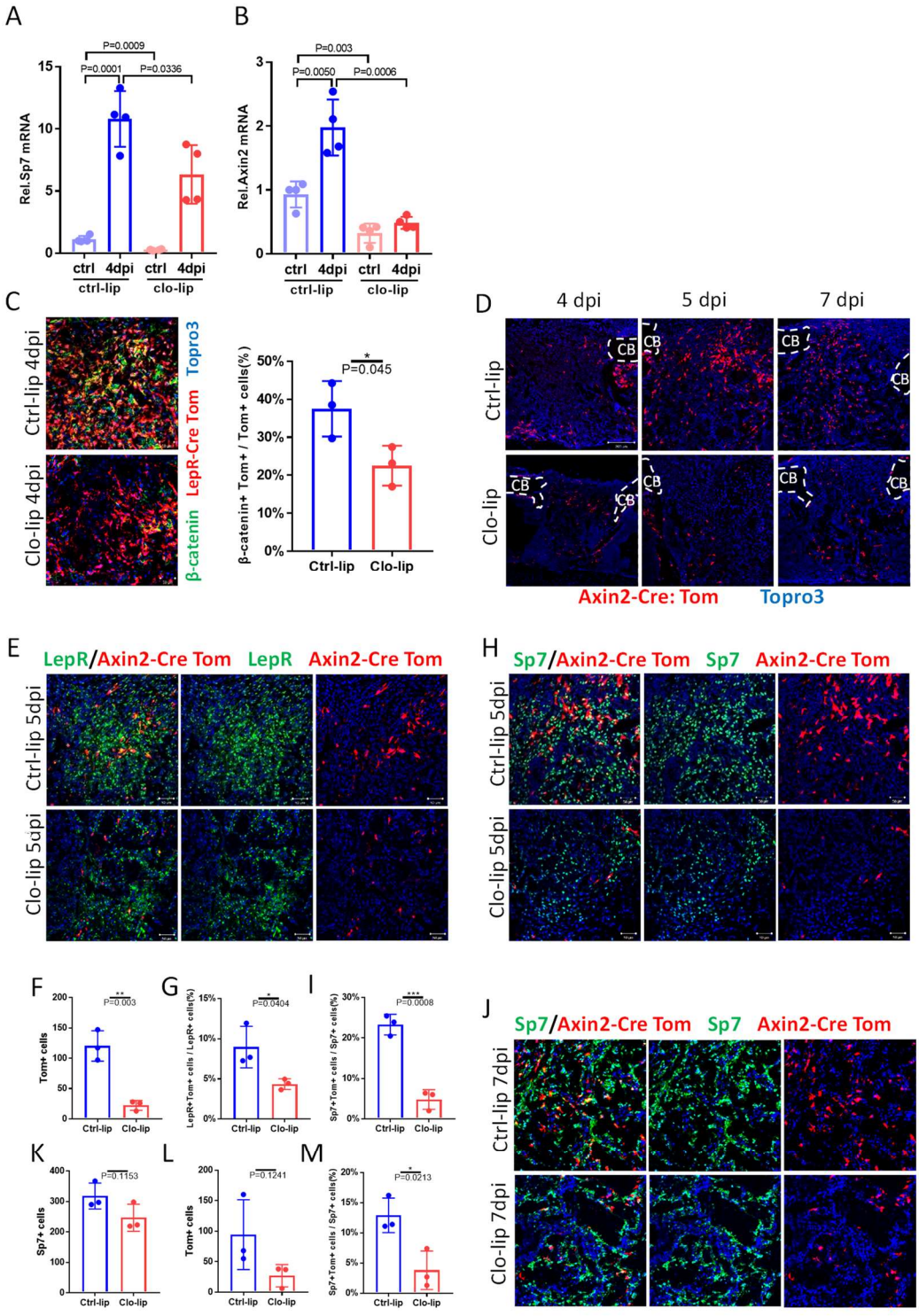

Fig. 5 Macrophages promote osteogenesis via canonical wnt signaling.

Fig. 5 Macrophages promote osteogenesis via canonical wnt signaling.

A. Quantitative RT-PCR analyses of Sp7 mRNA expression level. Samples of bone tissue from bone injury site of mouse tibiae and control samples of uninjured region from the contralateral tibiae were collected. Gene expression data was normalized to Gapdh. Statistical analysis was performed using unpaired Student' s t-tests.
B. Quantitative RT-PCR analyses of Axin2 mRNA expression level. Gene expression data was normalized to Gapdh. Statistical analysis was performed using unpaired Student' s t-tests.
C. Immuno-fluorescent analysis of β-catenin (+) cells in LepR-Cre tdTomato mice in the bone injury site at 4dpi. Nuclei were stained with TO-PRO-3 Iodide. Statistical analysis was performed using unpaired Student' s t-tests. Scale bar = 50 μm.
D. Immuno-fluorescent analysis of Tomato (+) cells in the bone injury site at 4, 5 and 7 dpi in Axin2-Cre ERT2 tdTomato mice (n=3 mice). Cre recombination was induced by i. p. injection of Tamoxifen for three consecutive days before tissue harvest. Nuclei were stained with TO-PRO-3 Iodide. Scale bar = 200 μm.
E. Immuno-fluorescent analysis of LepR (+) cells in Axin2-Cre ERT2 tdTomato mice in the bone injury site at 5dpi. Nuclei were stained with TO-PRO-3 Iodide. Scale bar = 50 μm.
F. Quantitative data of Axin2-Cre labeled Tomato (+) cells mice in the bone injury site at 5 dpi. Statistical analysis was performed using unpaired Student' s t-tests.
G. The percentage of LepR (+) Axin2-Cre labeled Tomato (+) in all Tomato (+) cells in the bone injury site at 5dpi. Statistical analysis was performed using unpaired Student' s t-tests.

H. Immuno-fluorescent analysis of Sp7 (+) cells in Axin2-Cre ERT2 tdTomato mice in the bone injury site at 5 dpi. Nuclei were stained with TO-PRO-3 Iodide. Scale bar = 50 μm.

I. The percentage of Sp7 (+) Axin2-Cre labeled Tomato (+) in all Tomato (+) cells in the bone injury site at 5 dpi. Statistical analysis was performed using unpaired Student' s t-tests.

J. Immuno-fluorescent analysis of Sp7 (+) cells in Axin2-Cre ERT2 tdTomato mice in the bone injury site at 7dpi. Nuclei were stained with TO-PRO-3 Iodide. Scale bar = 50 μm.

K. Quantitative data of Sp7 (+) cells mice in the bone injury site at 7dpi. Statistical analysis was performed using unpaired Student' s t-tests.

L. Quantitative data of Axin2-Cre labeled Tomato (+) cells mice in the bone injury site at 7 dpi. Statistical analysis was performed using unpaired Student' s t-tests.

M. The percentage of Sp7 (+) Axin2-Cre labeled Tomato (+) in all Tomato (+) cells in the bone injury site at 7 dpi. Statistical analysis was performed using unpaired Student' s t-tests.

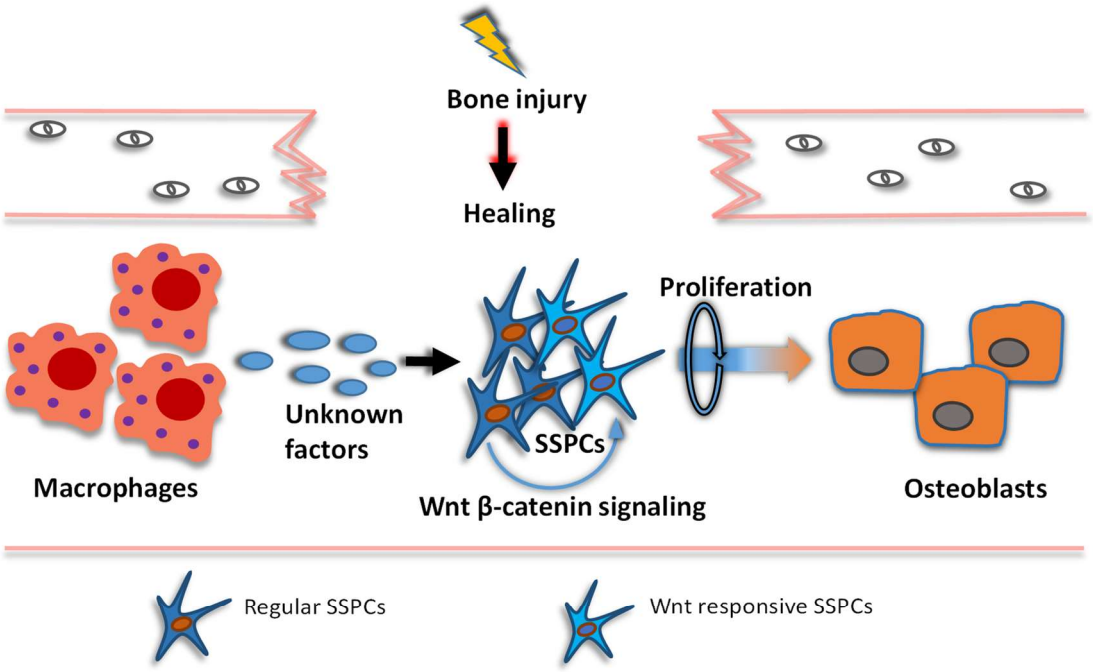

Fig. 6 Macrophages regulate the activation of skeletal stem and progenitor cells in regenerative state through Wnt signaling pathway.

Fig. 6 Macrophages regulate the activation of skeletal stem and progenitor cells in regenerative state through Wnt signaling pathway.

5. DISCUSSION

Here, we demonstrated that F4/80 (+) macrophages promotes bone regeneration through the activation of LepR (+) bone marrow stromal cells. The proliferation and differentiation of LepR (+) bone stromal cells are largely dependent on the recruitment of F4/80 (+) macrophages into bone injury sites. F4/80 (+) macrophages are involved in the activation of Wnt/β-catenin signals in LepR (+) cells, which in turn promoting osteogenesis during bone healing processes (Fig. 6).

Macrophages are indispensable in various tissue homeostasis and regeneration. Recently, they are considered to be composed of heterogeneous subpopulations due to our deeper understanding of their origin, maturation, and development in multiple tissue micro environments [50]. During embryonic stage, macrophages are derived from yolk sac and fetal liver erythro-myeloid progenitors (EMPs). Some of these macrophage populations enter into tissues and maintain themselves by self-renewal [51-53]. Moreover, bone marrow hematopoietic stem cells (HSCs)-derived circulating monocytes are able to replenish tissue resident macrophages in adult tissues except brain [3, 51]. Tissue resident macrophages secrete several cytokines and bioactive molecules such as PDGF and NAMPT and regulate tissue-specific cells in each organ under a pathological state-dependent manner [54-56]. Our present study has showed that F4/80 (+) macrophages directly or indirectly activate Wnt/β-catenin signals in osteoblast precursors in the bone injury sites although the origin (HSC or fetal EMPs) of F4/80 (+) macrophages has still been unclear.

F4/80 (+) macrophages were abundant in inflammatory phases (4 dpi) and able to phagocytose liposomes because DiI-liposomes were incorporated into those cells and Clo-lip depleted them with a high efficiency. The importance of F4/80 (+) macrophages in osteoblastogenesis are highlighted by the findings

that 1) the increase in LepR (+) stromal cells was synchronized by the recruitment of F4/80 (+) macrophages throughout the bone healing process; 2) F4/80 (+) macrophages were in the close proximity of LepR (+) stromal cells; 3) the depletion of F4/80 (+) macrophages decreased the proliferation of LepR(+) stromal cells and the expression of Sp7 in those cells. Since F4/80, a pan marker for macrophages, is widely expressed in macrophage populations, those F4/80 (+) macrophages were likely to consist of different subtypes of macrophages such as M1 and M2 macrophages during bone repair. We tried to detect Arginase-1, a M2 macrophage marker using immuno-fluorescent techniques but failed it due to the unavailability of Arginase 1 antibodies suitable for immuno-fluorescent techniques. We didn't further distinguish subsets included in F4/80 (+) macrophages using subset-specific markers. Further studies are needed to find out a specific macrophage subset involved in osteoblastogenesis during bone healing.

Several macrophage depletion methods have been reported previously. van Rooijen and van Nieuwmegen reported in 1984 the eliminate of phagocytic cells in spleen by intravenous injections of liposome-encapsulated clodronate [57]. Since then, this method had been widely used. AFS98 a neutralizing antibody against Colony-stimulating factor1 receptor (Csf1r; also called c-fms) was also effective for the depleting macrophages [58, 59]. Recently, with the development of mouse genetics, various lines of Cre mice are generated to express Cre recombinases under the control of macrophage lineage cell-specific gene promotor including Csf1r and Cx3cr1. In addition, the inducible and cell-specific ablation model has been developed using the Cre-recombinase-mediated expression of human diphtheria toxin receptors (DTR) induced cell death in cells treated with diphtheria toxin (DT) in Cre-inducible DTR transgenic (iDTR) mice [60]. To deplete Csf1r (+) cell lineage, Csf1r-CreERT2; iDTR mice were treated with tamoxifen to inducibly express DTR in macrophage lineage cells and the bone injury was induced in those mice treated with DT.

The injections of DT had no effects on regenerative bone mass in those mice even though the number of DTR (+) cells were significantly depleted (data not shown). These findings suggested that macrophages expresseing Csf1r marked by DTR during healing process were dispensable in bone regeneration.

The *Csf1r* gene has been extensively used to generate reporter mice as well as *Csf1r*-deficient mice to examine macrophage functions and lineage traces *in vivo*. The *Csf1r* gene is however expressed not only in typical monocyte-macrophages lineage cells but also neutrophils, dendritic cells and their precursors with a low expression level [61, 62]. Therefore, we cannot rule out a possibility that various types of cells expressing Csf1r may deplete in Csf1r-CreERT2; iDTR mice. Clo-lip has also experimental limitations known for the inhibitory effects on bone resorption [63, 64]. However, the obvious reduction of newly formed bone mass had been observed in mice treated with Clo-lip in the present study. This finding indicated that effects of the macrophage depletion by Clo-lip on bone formation was more obvious than the inhibitory effects of Clo-lip on osteoclastic bone resorption in the bone injury model we used.

Successful bone regeneration is required for the coordinated interactions between mesenchymal stem cells (MSCs) and macrophages [20]. Our current data demonstrated that macrophages activated Wnt/β-catenin signals in LepR (+) MSCs *in vivo*. Previous *in vitro* experiments have suggested that macrophages-derive cytokines such as Oncostatin M (OsM) and Bone Morphogenetic Protein-2 (BMP2) to positively regulate osteoblastic differentiation of MSCs [65, 66]. Monocytes reportedly induced the activation of STAT3 in human MSCs, which in turn facilitating their osteoblastic differentiation [67]. These findings indicated that Wnt ligands cooperated with OsM and BMP2 to promote the differentiation of BMSCs into osteoblasts. Further studies are needed to clarify mechanism by which Wnt signals promote osteoblastic differentiation.

Macrophages reportedly secrete Wnt ligands to regulate tissue specific stem cells and their niches (68, 69). Therefore, the expression of Wnt ligands was searched on Tabula Muris transcriptome data base. No Wnt ligand expressed specifically was found in bone marrow macrophages. In addition, the expression levels of most Wnt ligands is lower in bone macrophages compared with BMSCs. We performed a real time PCR analysis of bone injury sites at 4 dpi and found the expression of several Wnt ligands was increased (data not shown). These findings suggest that Wnt ligands certainly expressed in bone injury sites activate Wnt/β-catenin signals in LepR (+) MSCs. Further studies are needed to clarify which Wnt ligand is dominantly involved in bone healing and which type of cells secrete the Wnt ligands.

The present study demonstrated that F4/80 (+) macrophage play an important role in bone regeneration. These macrophages activate Wnt/β-catenin signals in MSCs, which in turn promoting their proliferation and differentiation into osteoblasts. The identification of master regulators in these processes enable us to develop new methods using recombinant proteins, cDNA, and mRNA of targets for treatments of bone fractures including facial bones and jaw surgeries.

6. REFERENCES

1. T Katagiri and N Takahashi (2002) Regulatory mechanisms of osteoblast and osteoclast differentiation. Oral Diseases 8,147–159.
2. Patricia Ducy, Rui Zhang, Valérie Geoffroy, Amy L Ridall and Gérard Karsenty (1997) Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell, 89, 747–754.
3. Yasuhito Yahara, Tomasa Barrientos, Yuning J. Tang, Vijitha Puviindran, Puviindran Nadesan. Hongyuan Zhang, Jason R. Gibson, Simon G. Gregory, Yarui Diao, Yu Xiang, Yawar J. Qadri, Tomokazu Souma, Mari L. Shinohara and Benjamin A. Alman (2020) Erythromyeloid progenitors give rise to a population of osteoclasts that contribute to bone homeostasis and repair. Nature Cell Biology volume 22, 49–59.
4. Bo O. Zhou, Rui Yue, Malea M. Murphy, James G. Peyer, and Sean J. Morrison (2014) Leptin-Receptor-Expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. Cell Stem Cell 15, 154-168.
5. Tetsuo Hasegawa, Junichi Kikuta, Takao Sudo, Yoshinobu Matsuura Takahiro Matsui, Szandor Simmons, Kosuke Ebina, Makoto Hirao, Daisuke Okuzaki, Yuichi Yoshida, Atsushi Hirao, Vladimir V. Kalinichenko, Kunihiro Yamaoka, Tsutomu Takeuchi and Masaru Ishii (2019) Identification of a novel arthritis-associated osteoclast precursor macrophage regulated by FoxM1. Nature Immunology volume 20,1631–1643.
6. Yuki Matsushita, Mizuki Nagata, Kenneth M. Kozloff, Joshua D. Welch, Koji Mizuhashi, Nicha Tokavanich, Shawn A. Hallett, Daniel C. Link, Takashi Nagasawa, Wanida Ono, Noriaki Ono (2020) A Wnt-mediated transformation of the bone marrow stromal cell identity orchestrates skeletal regeneration. Nature Communication 11:332.

7. Anna-Marei Böhm, Naomi Dirckx, Robert J Tower, Nicolas Peredo, Sebastiaan Vanuytven, Koen Theunis, Elena Nefyodova, Ruben Cardoen, Volkhard Lindner, Thierry Voet, Matthias Van Hul, Christa Maes (2019) Activation of skeletal stem and progenitor cells for bone regeneration is driven by PDGFRβ signaling. Developmental Cell 51, 236–254.
8. S.S. Lim, T. Vos, A.D. Flaxman, G. Danaei, K. Shibuya, H. Adair-Rohani, M. Amann, H.R. Anderson, K.G. Andrews, M. Aryee, C. Atkinson, L.J. Bacchus, A.N. Bahalim, K.Balakrishnan, J. Balmes, S. Barker-Collo, A. Baxter, M.L. Bell, J.D. Blore, F. Blyth, C. Bonner, G. Borges, R. Bourne, M. Boussinesq, M. Brauer, P. Brooks, N.G. Bruce, B. Brunekreef, C. Bryan-Hancock, C. Bucello, R. Buchbinder, F. Bull, R.T. Burnett, T.E. Byers, B. Calabria, J. Carapetis, E. Carnahan, Z. Chafe, F. Charlson, H. Chen, J.S. Chen, A.T. Cheng, J.C. Child, A. Cohen, K.E. Colson, B.C. Cowie, S. Darby, S. Darling, A. Davis, L. Degenhardt, F. Dentener, D.C. Des Jarlais, K. Devries, M. Dherani, E.L. Ding, E.R. Dorsey, T. Driscoll, K. Edmond, S.E. Ali, R.E. Engell, P.J. Erwin, S. Fahimi, G. Falder, F. Farzadfar, A. Ferrari, M.M. Finucane, S. Flaxman, F.G. Fowkes, G. Freedman, M.K. Freeman, E. Gakidou, S. Ghosh, E. Giovannucci, G. Gmel, K. Graham, R. Grainger, B. Grant, D. Gunnell, H.R. Gutierrez, W. Hall, H.W. Hoek, A. Hogan, H.D. Hosgood III, D. Hoy, H. Hu, B.J. Hubbell, S.J. Hutchings, S.E. Ibeanusi, G.L. Jacklyn, R. Jasrasaria, J.B. Jonas, H. Kan, J.A. Kanis, N. Kassebaum, N. Kawakami, Y.H. Khang, S. Khatibzadeh, J.P. Khoo, C. Kok, F. Laden, R. Lalloo, Q. Lan, T. Lathlean, J.L. Leasher, J. Leigh, Y. Li, J.K. Lin, S.E. Lipshultz, S. London, R. Lozano, Y. Lu, J. Mak, R. Malekzadeh, L. Mallinger, W. Marcenes, L. March, R. Marks, R. Martin, P. McGale, J. McGrath, S. Mehta, G.A. Mensah, T.R. Merriman, R. Micha, C. Michaud, V. Mishra, K. Mohd Hanafiah, A.A. Mokdad, L. Morawska, D. Mozaffarian, T. Murphy, M. Naghavi, B. Neal, P.K. Nelson, J.M. Nolla, R. Norman, C. Olives, S.B. Omer, J. Orchard, R. Osborne, B. Ostro, A. Page, K.D. Pandey, C.D.

Parry, E. Passmore, J. Patra, N. Pearce, P.M. Pelizzari, M. Petzold, M.R. Phillips, D. Pope, C.A. Pope III, J. Powles, M. Rao, H. Razavi, E.A. Rehfuess, J.T. Rehm, B. Ritz, F.P. Rivara, T. Roberts, C. Robinson, J.A. Rodriguez-Portales, I. Romieu, R. Room, L.C. Rosenfeld, A. Roy, L. Rushton, J.A. Salomon, U. Sampson, L. Sanchez-Riera, E. Sanman, A. Sapkota, S. Seedat, P. Shi, K. Shield, R. Shivakoti, G.M. Singh, D.A. Sleet, E. Smith, K.R. Smith, N.J. Stapelberg, K. Steenland, H. Stockl, L.J. Stovner, K. Straif, L. Straney, G.D. Thurston, J.H. Tran, R. Van Dingenen, A. van Donkelaar, J.L. Veerman, L. Vijayakumar, R. Weintraub, M.M. Weissman, R.A. White, H. Whiteford, S.T. Wiersma, J.D. Wilkinson, H.C. Williams, W. Williams, N. Wilson, A.D. Woolf, P. Yip, J.M. Zielinski, A.D. Lopez, C.J. Murray, M. Ezzati, M.A. AlMazroa, Z.A. Memish (2012) A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380, 2224–2260.

9. Pike C, Birnbaum HG, Schiller M, Sharma H, Burge R, Edgell ET (2010) Direct and indirect costs of non-vertebral fracture patients with osteoporosis in the US. PharmacoEconomics 28: 395–409.
10. Fredrik Borgström, Linda Karlsson, Gustav Ortsäter, Nicolas Norton, Philippe Halbout, Cyrus Cooper, Mattias Lorentzon, Eugene V McCloskey, Nicholas C Harvey, Muhamamd K Javaid, John A Kanis, International Osteoporosis Foundation (2020) Fragility fractures in Europe: burden, management and opportunities. Arch Osteoporos 15: 59.
11. Gemma Tatangelo, Jennifer Watts, Karen Lim, Catherine Connaughton, Julie Abimanyi-Ochom, Fredrik Borgström, Geoff C Nicholson, Catherine Shore-Lorenti, Amanda L Stuart, Sandra Iuliano-Burns, Ego Seeman, Richard Prince, Lyn March, Marita Cross, Tania Winzenberg, Laura L Laslett, Gustavo Duque, Peter R Ebeling, Kerrie M Sanders (2019) The

cost of osteoporosis, osteopenia, and associated fractures in Australia in 2017. J Bone Miner Res 2019 34: 616–25.
12. GBD 2019 Fracture Collaborators (2021) Global, regional, and national burden of bone fractures in 204 countries and territories, 1990–2019: a systematic analysis from the Global Burden of Disease Study 2019. Lancet Public Health 6: e907–18.
13. Rozalia Dimitriou, Elena Jones, Dennis McGonagle and Peter V Giannoudis (2011) Bone regeneration: current concepts and future directions. BMC Medicine 9:66.
14. Mattias Lorentzon (2019) Treating osteoporosis to prevent fractures: current concepts and future developments. Journal of Internal Medicine 285; 381–394.
15. Hiang Boon Tan, Peter V Giannoudis, Sally A Boxall (2015) The systemic influence of platelet-derived growth factors on bone marrow mesenchymal stem cells in fracture patients. BMC Medicine 13:6.
16. Ippokratis Pountos, Michalis Panteli, Anastasios Lampropoulos, Elena Jones, Giorgio Maria Calori and Peter V. Giannoudis (2016) The role of peptides in bone healing and regeneration: a systematic review. BMC Medicine 14:103.
17. Florence Loi, Luis A Córdova, Jukka Pajarinen, Tzu-hua Lin, Zhenyu Yao, Stuart B Goodman (2016) Inflammation, fracture and bone repair. Bone 86: 119-130.
18. Gurpreet S. Baht, Linda Vi4 and Benjamin A. Alman (2018) The role of the immune cells in fracture healing. Current Osteoporosis Reports 16:138–145.
19. Christian Guder, Sascha Gravius, Christof Burger, Dieter C. Wirtz and Frank A. Schildberg (2020) Osteoimmunology: a current update of the interplay between bone and the immune system. Front. Immunol. 11:58.
20. Jukka Pajarinen, Tzuhua Lin, Emmanuel Gibon, Yusuke Kohno, Masahiro Maruyama, Karthik Nathan, Laura Lu, Zhenyu Yao, Stuart B. Goodman

(2019) Mesenchymal stem cell-macrophage crosstalk and bone healing. Biomaterials 196: 80-89.

21. S.J. Leibovich, R. Ross (1975) The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. Am. J. Pathol. 78: 71–100.
22. Ming K. Chang, Liza-Jane Raggatt, Kylie A. Alexander, Julia S. Kuliwaba, Nicola L. Fazzalari, Kate Schroder, Erin R. Maylin, Vera M. Ripoll, David A. Hume and Allison R. Pettit (2008)
23. Kylie A Alexander, Ming K Chang, Erin R Maylin, Thomas Kohler, Ralph Müller, Andy C Wu, Nico Van Rooijen, Matthew J Sweet, David A Hume, Liza J Raggatt, and Allison R Pettit (2011) Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model. J Bone Miner Res 26(7):1517-32.
24. Claudia Schlundt, Thaqif El Khassawna, Alessandro Serra, Anke Dienelt, Sebastian Wendler, Hanna Schell, Nico van Rooijen, Andreas Radbruch, Richard Lucius, Susanne Hartmann, Georg N. Duda, Katharina Schmidt-Bleek (2018) Macrophages in bone fracture healing: Their essential role in endochondral ossification. Bone 106: 78–89.
25. Lena Batoon, Susan Marie Millard, Martin Eduard Wullschleger, Corina Preda, Andy Chiu-Ku Wu, Simranpreet Kaur, Hsu-Wen Tseng, David Arthur Hume, Jean-Pierre Levesque, Liza Jane Raggatt, Allison Robyn Pettit (2019) CD169^{+} macrophages are critical for osteoblast maintenance and promote intramembranous and endochondral ossification during bone repair. Biomaterials 196: 51-66.
26. Leslie Chávez-Galán, Maria L. Olleros, Dominique Vesin and Irene Garcia (2015) Much more than M1 and M2 macrophages, there are also CD169+ and TCR+ macrophages. Front. Immunol 6:263.
27. Bienz, M. & Clevers, H. (2000) Linking colorectal cancer to Wnt signaling. Cell 103, 311–320.
28. Miller, J.R. (2001) The Wnts. Genome Biol. 3, 3001.1–3001.15.

29. Logan, C.Y. & Nusse, R. (2004) The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. 20, 781–810.
30. Kazuhiro Maeda, Yasuhiro Kobayashi, Nobuyuki Udagawa, Shunsuke Uehara, Akihiro Ishihara, Toshihide Mizoguchi, Yuichiro Kikuchi, Ichiro Takada, Shigeaki Kato, Shuichi Kani, Michiru Nishita, Keishi Marumo, T John Martin, Yasuhiro Minami & Naoyuki Takahashi (2012) Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. Nature Medicine 18, 405–412.
31. Timothy F. Day, Xizhi Guo, Lisa Garrett-Beal, Yingzi Yang (2005) Wnt/-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Developmental Cell, 8, 739–750.
32. Jürgen Behrens, Jens P. von Kries, Michael Kühl, Laurakay Bruhn, Doris Wedlich, Rudolf Grosschedl & Walter Birchmeier (1996) Functional interaction of β-catenin with the transcription factor LEF-1. Nature 382, 638–642.
33. Miranda Molenaar, Marcvan de Wetering, Mariette Oosterwegel, Josi Peterson-Maduro, SusanGodsave, Vladimir Korinek, Jeroen Roose, Olivier Destrée, HansClevers (1996) XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos. Cell, 86, 391–399.
34. Theo P. Hill, Daniela Später, Makoto M. Taketo, Walter Birchmeier, Christine Hartmann (2005) Canonical Wnt/β-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Developmental Cell, 8, 727–73.
35. Baron, R. & Rawadi, G. (2007) Targeting the Wnt/β-catenin pathway to regulate bone formation in the adult skeleton. Endocrinology 148, 2635–2643.
36. Philipp Leucht, Sooyeon Lee, Nury Yim (2019) Wnt signaling and bone regeneration: Can't have one without the other. Biomaterials 196, 46-50.

37. Staal, F.J., Luis, T.C. & Tiemessen, M.M. (2008) WNT signaling in the immune system: WNT is spreading its wings. Nat. Rev. Immunol. 8, 581–593.
38. J DeFalco, M Tomishima, H Liu, C Zhao, X Cai, J D Marth, L Enquist, J M Friedman (2001) Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. Science 291(5513):2608-13.
39. Barbara Lustig, Boris Jerchow, Martin Sachs, Sigrid Weiler, Torsten Pietsch, Uwe Karsten, Marc van de Wetering, Hans Clevers, Peter M Schlag, Walter Birchmeier, Jürgen Behrens (2002) Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol 22(4):1184-93.
40. Linda Madisen, Theresa A Zwingman, Susan M Sunkin, Seung Wook Oh, Hatim A Zariwala, Hong Gu, Lydia L Ng, Richard D Palmiter, Michael J Hawrylycz, Allan R Jones, Ed S Lein, Hongkui Zeng (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 13(1):133-40.
41. Koichi Matsuo, Yukiko Kuroda, Nobuhito Nango, Kouji Shimoda, Yoshiaki Kubota, Masatsugu Ema, Latifa Bakiri, Erwin F. Wagner, Yoshihiro Takeda, Wataru Yashiro, Atsushi Momose (2015) Osteogenic capillaries orchestrate growth plate-independent ossification of the malleus. Development 142 (22): 3912–3920.
42. K. Matsuo, Y. Kuroda, N. Nango, K. Shimoda, Y. Kubota, M. Ema, L. Bakiri, E, F. Wagner, Y. Takeda, W. Yashiro, A. Momose (2015) Osteogenic capillaries orchestrate growth plate-independent ossification of the malleus. Development 142: 3912–3920.
43. J. Rossert, H. Eberspaecher, B. de Crombrugghe (1995) Separate cis-acting DNA elements of the mouse pro-alpha 1(I) collagen promoter direct expression of reporter genes to different type I collagen-producing cells in transgenic mice. J. Cell Biol. 129: 1421–1432.

44. Sachin Gopalkrishna Pai, Benedito A. Carneiro, Jose Mauricio Mota, Ricardo Costa, Caio Abner Leite, Romualdo Barroso-Sousa, Jason Benjamin Kaplan, Young Kwang Chae and Francis Joseph Giles (2017) Wnt/beta-catenin pathway: modulating anticancer immune response. Journal of Hematology & Oncology 10:101.
45. Hill TP, Später D, Taketo MM, Birchmeier W, Hartmann C (2005) Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev Cell 8(5):727-38.
46. Eek-hoon Jho, Tong Zhang, Claire Domon, Choun-Ki Joo, Jean-Noel Freund, Frank Costantini (2002) Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell Biol. 22, 1172–1183.
47. Janet Y. Leung, Frank T. Kollig, Rong Wu, Yali Zhai, Rork Kuick, Samir Hanash, Kathleen R. Cho, Eric R. Fearon (2002) Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J. Biol. Chem. 277, 21657–21665.
48. van Amerongen, R., Bowman, A. N. & Nusse, R. (2012) Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. Cell stem cell 11, 387–400.
49. R. C. Ransom, D. J. Hunter, S. Hyman, G. Singh, S. C. Ransom, E. Z. Shen, K. C. Perez, M. Gillette, J. Li, B. Liu, J. B. Brunski & J. A. Helms (2016) Axin2-expressing cells execute regeneration after skeletal injury. Scientific Reports 6:36524.
50. Siamon Gordon and Annette Plüddemann (2017) Tissue macrophages: heterogeneity and functions. BMC Biology 15:53.
51. Ginhoux F, Guilliams M. (2016) Tissue-resident macrophage ontogeny and homeostasis. Immunity 44(3):439–49.
52. Daigo Hashimoto, Andrew Chow, Clara Noizat, Pearline Teo, Mary Beth Beasley, Marylene Leboeuf, Christian D Becker, Peter See, Jeremy Price,

Daniel Lucas, Melanie Greter, Arthur Mortha, Scott W Boyer, E Camilla Forsberg, Masato Tanaka, Nico van Rooijen, Adolfo García-Sastre, E Richard Stanley, Florent Ginhoux, Paul S Frenette, Miriam Merad (2013) Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity. 38(4):792–804.

53. Guillaume Hoeffel, Jinmiao Chen, Yonit Lavin, Donovan Low, Francisca F Almeida, Peter See, Anna E Beaudin, Josephine Lum, Ivy Low, E Camilla Forsberg, Michael Poidinger, Francesca Zolezzi, Anis Larbi, Lai Guan Ng, Jerry K Y Chan, Melanie Greter, Burkhard Becher, Igor M Samokhvalov, Miriam Merad, Florent Ginhoux (2015) C-Myb (+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. Immunity 21;42(4):665-78.
54. Nehemiah Cox, Lucile Crozet, Inge R. Holtman, Pierre-Louis Loyher, Tomi Lazarov, Jessica B. White, Elvira Mass, E. Richard Stanley, Olivier Elemento, Christopher K. Glass, Frederic Geissmann (2021) Diet-regulated production of PDGFcc by macrophages controls energy storage. Science 373, 74.
55. Kazuyuki Takata, Tatsuya Kozaki, Christopher Zhe Wei Lee, Morgane Sonia Thion, Masayuki Otsuka, Shawn Lim, Kagistia Hana Utami, Kerem Fidan, Dong Shin Park, Benoit Malleret, Svetoslav Chakarov, Peter See, Donovan Low, Gillian Low, Marta Garcia-Miralles, Ruizhu Zeng, Jinqiu Zhang, Chi Ching Goh, Ahmet Gul, Sandra Hubert, Bernett Lee, Jinmiao Chen, Ivy Low, Nurhidaya Binte Shadan, Josephine Lum, Tay Seok Wei, Esther Mok, Shohei Kawanishi, Yoshihisa Kitamura, Anis Larbi, Michael Poidinger, Laurent Renia, Lai Guan Ng, Yochai Wolf, Steffen Jung, Tamer Önder, Evan Newell, Tara Huber, Eishi Ashihara, Sonia Garel, Mahmoud A Pouladi, Florent Ginhoux (2017) Induced-pluripotent-stem-cell-derived primitive macrophages provide a platform for modeling

tissue-resident macrophage differentiation and function. Immunity 47, 183–198.

56. Dhanushika Ratnayake, Phong D. Nguyen, Fernando J. Rossello, Verena C. Wimmer, Jean L. Tan, Laura A. Galvis, Ziad Julier, Alasdair J. Wood, Thomas Boudier, Abdulsalam I. Isiaku, Silke Berger, Viola Oorschot, Carmen Sonntag, Kelly L. Rogers, Christophe Marcelle, Graham J. Lieschke, Mikaël M. Martino, Jeroen Bakkers & Peter D. Currie (2021) Macrophages provide a transient muscle stem cell niche via NAMPT secretion. Nature 591, 281–287.
57. van Rooijen N, van Nieuwmegen R (1984) Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. An enzyme-histochemical study. Cell Tissue Res. 238(2):355-8.
58. . Jose MD, Le Meur Y, Atkins RC, Chadban SJ (2003) Blockade of macrophage colony-stimulating factor reduces macrophage proliferation and accumulation in renal allograft rejection. Am J Transplant 3(3):294-300.
59. Masashi Segawa, So-ichiro Fukada, Yukiko Yamamoto, Hiroshi Yahagi, Masanori Kanematsu, Masaki Sato, Takahito Ito, Akiyoshi Uezumi, Shin'ichi Hayashi, Yuko Miyagoe-Suzuki, Shin'ichi Takeda, Kazutake Tsujikawa, Hiroshi Yamamoto (2008) Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. Exp Cell Res 314(17):3232-3244.
60. Buch T, Heppner FL, Tertilt C, Heinen TJ, Kremer M, Wunderlich FT, Jung S, Waisman A (2005) A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. Nat Methods 2(6):419-26.
61. Sandra H. Burnett, Edward J. Kershen, Jiayou Zhang, Li Zeng, Susan C. Straley, Alan M. Kaplan,and Donald A. Cohen (2004) Conditional

macrophage ablation in transgenic mice expressing a Fas-based suicide gene. J. Leukoc. Biol. 75: 612– 623.

62. Kathleen Grabert, Anuj Sehgal, Katharine M. Irvine, Evi Wollscheid-Lengeling, Derya D. Ozdemir, Jennifer Stables, Garry A. Luke, Martin D. Ryan, Antony Adamson, Neil E. Humphreys, Cheyenne J. Sandrock, Rocio Rojo, Veera A. Verkasalo, Werner Mueller, Peter Hohenstein, Allison R. Pettit, Clare Pridans, and David A. Hume (2020) A transgenic line that reports CSF1R protein expression provides a definitive marker for the mouse mononuclear phagocyte system. The Journal of Immunology, 2020, 205: 000–000.
63. Lin HN, O'Connor JP. (2017) Osteoclast depletion with clodronate liposomes delays fracture healing in mice. J Orthop Res 35:1699-706.
64. K.N. Bailey, B.D. Furman, J. Zeitlin, K.A. Kimmerling, C.-L. Wu, F. Guilak, S.A. Olson (2020) Intra-articular depletion of macrophages increases acute synovitis and alters macrophage polarity in the injured mouse knee. Osteoarthritis and Cartilage 28: 626-638.
65. Y. Zhang, T. Bose, R.E. Unger, J.A. Jansen, C.J. Kirkpatrick, J.J. van den Beucken (2017) Macrophage type modulates osteogenic differentiation of adipose tissue MSCs. Cell Tissue Res. 369 (2) 273-286.
66. Yang Zhang, Thomas Böse, Ronald E. Unger, John A. Jansen, Charles James Kirkpatrick & Jeroen J. J. P. van den Beucken (2017) Macrophage type modulates osteogenic differentiation of adipose tissue MSCs. Cell Tissue Res 369:273–286.

67. Vicky Nicolaidou, Mei Mei Wong, Andia N. Redpath, Adel Ersek, Dilair F. Baban, Lynn M. Williams, Andrew P. Cope, Nicole J. Horwood (2012) Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation. PLoS ONE 7(7): e39871.

68. Luke Boulter, Olivier Govaere, Tom G Bird, Sorina Radulescu, Prakash Ramachandran, Antonella Pellicoro, Rachel A Ridgway, Sang Soo Seo,

Bart Spee, Nico Van Rooijen, Owen J Sansom, John P Iredale, Sally Lowell, Tania Roskams & Stuart J Forbes (2012) Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 18(4):572-9.

69. Rumela Chakrabarti, Toni Celià-Terrassa, Sushil Kumar, Xiang Hang, Yong Wei, Abrar Choudhury, Julie Hwang, Jia Peng, Briana Nixon, John J. Grady, Christina DeCoste, Jie Gao, Johan H. van Es, Ming O. Li, Iannis Aifantis, Hans Clevers, Yibin Kang (2018) Notch ligand Dll1 mediates cross-talk between mammary stem cells and the macrophageal niche. Science 360, 1421.

7. ACKNOWLEDGMENTS

I would like to express my gratitude sincerely to Dr. Y. Kobayashi and Dr. N. Udagawa for always providing guidance and encouragements throughout the course in this project. Thanks to Dr. T. Mizoguchi from Tokyo Dental university for supporting our experiment by offering Col1(2.3)-GFP mice.

I would specially thank to Dr. N. Takahashi for constantly motivating in scientific field and giving me a lot of support in my own life.

I am also very grateful to Dr. S. Uehara, Dr. Y. Nakamichi, Dr. T. Yamashita and Dr. M. Koide in Koba 's team who support me with academic instructions and comments.

At the end, I would like to thank to all the teachers and colleagues from Division of Hard Tissue Research of MDU for their enormous help in this project and unconditional love from my family members.