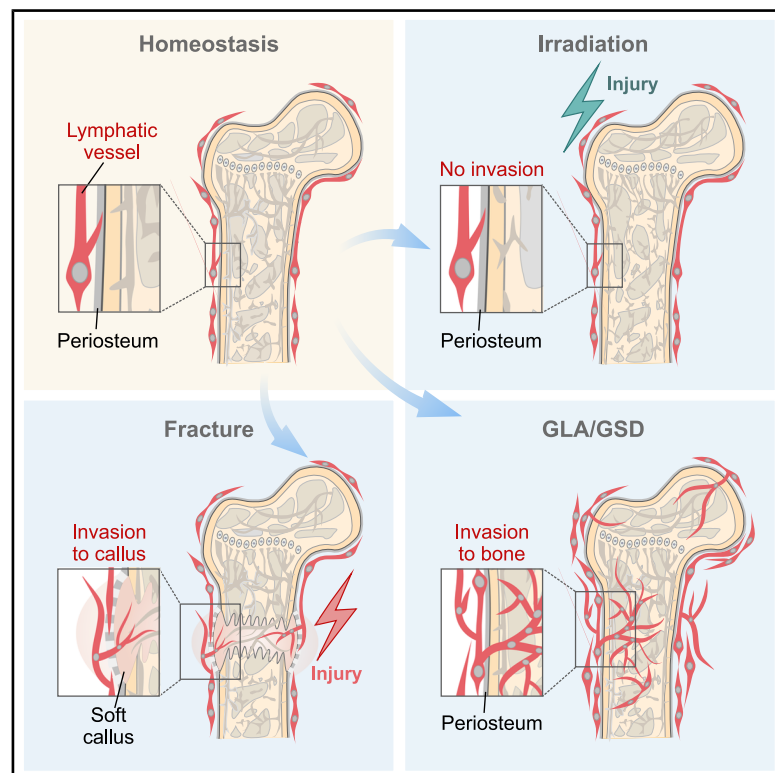


Lymphatic vessels invade bone in disease but are absent in health and regeneration

Graphical abstract



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In brief

Multiple lines of evidence argue against the presence of LECs inside bone during homeostasis or repair after injury, in contrast to a previous study published in *Cell* (Biswas et al. 2023). Instead, LECs actively invade bone in disease models of GLA/GSD.

Highlights

- Specific genetic tools reveal the absence of LECs in bone during homeostasis
- Bone regeneration after irradiation does not trigger LEC emergence inside the bone
- LECs expand within the callus but do not infiltrate the bone after fracture
- LECs invade bone under pathological conditions in GLA/GSD mouse models



Matters Arising

Lymphatic vessels invade bone in disease but are absent in health and regeneration

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SUMMARY

A recent study reported the existence of lymphatic vessels in normal bone and suggested their involvement in bone regeneration after injury. However, this conclusion was based on approaches that do not allow unequivocal identification of the spatial localization of lymphatic endothelial cells (LECs). Here, we employed a Prox1-based genetic tool and a dual-recombinase-mediated LEC-specific labeling system to trace lymphatic vessels with high specificity. We found that LECs are present in the connective tissues, including the periosteum surrounding the bone. However, they do not reside within the bone itself, nor do they penetrate the periosteum to facilitate bone regeneration after injury. By contrast, hyperplastic LECs on the bone surface breach the periosteum and invade bone tissue in mouse models of generalized lymphatic anomaly and Gorham-Stout disease. These data demonstrate that lymphatic vessels are absent from bone during homeostasis and regeneration after injury but invade bone during disease. This Matters Arising paper is in response to Biswas et al. (2023), published in *Cell*. See also the response by Yang et al. (2026), published in this issue.

INTRODUCTION

Lymphatic vessels are essential for immune surveillance and clearance of interstitial fluid and macromolecules, and they are found in various organs, including the skin, heart, liver, lung, mesentery, and intestine.^{1,2} Unlike blood vessels, which are present throughout the body except for a few avascular tissues, traditional views posit that lymphatic vessels are absent in the brain, eye, and bone.³ Recently, several research groups have identified an intermediate vessel type resembling lymphatic vessels, referred to as Schlemm's canal, in the eye. This canal ex-

presses lymphatic endothelial cell (LEC) markers such as prospero-related homeobox 1 (PROX1) and vascular endothelial growth factor receptor 3 (VEGFR3), but does not express lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) or podoplanin (PDPN).^{4–7} Additionally, recent studies have identified functional meningeal lymphatic vessels or cranial lymphatic vessels in the dural sinus, which express all molecular LEC markers and are involved in the clearance of cerebrospinal fluid.^{4,8} Furthermore, using 3D imaging with the immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO) tissue-clearing method, Jacob et al. reported



that the vertebral canal contains lymphatic vessels, which rapidly expand in response to injury and are thought to contribute to the immune surveillance of the central nervous system.⁹ Notably, meningeal and vertebral lymphatic vessels primarily localize to the dura mater and intervertebral ligaments or joints, respectively, effectively avoiding bone tissue.^{4,8,9}

Traditionally, it has been believed that lymphatic vessels are absent in the healthy skeletal system.^{10,11} However, patients with generalized lymphatic anomaly (GLA) or Gorham-Stout disease (GSD) and mouse models of these diseases have lymphatic vessels inside the bone. The presence of lymphatic vessels in bone is accompanied by massive osteolysis.^{11–15} Lineage tracing experiments have shown that these ectopic LECs in GLA or GSD mouse models originate from LECs outside the bone, which breach the periosteum before invading the bone itself.¹¹ However, a recent paper published in *Cell* has reported that lymphatic vessels exist in normal bone and can support the regeneration of bone and hematopoietic cells after injury.¹⁶ In that study, lymphatic vessels in bone were identified using light-sheet imaging and immunostaining with several LEC markers, including LYVE1, PDPN, and PROX1. However, these markers are not strictly confined to LECs, as they are expressed in other types of cells,^{17–22} raising concerns about the accuracy of the identification. Additionally, the study lacks robust evidence from *in vivo* specific genetic lineage tracing, a method that could provide more definitive insights into the origin and function of the cells in question.

Genetic LEC ablation experiments by expression of diphtheria toxin (DTA) under control of the *Prox1-CreERT2* line,²³ a key experiment in the study,¹⁶ are problematic because this strategy cannot discriminate between different sites of *Prox1* expression, which includes the whole lymphatic vasculature throughout the body but also numerous non-endothelial cell populations, such as hepatocytes in the liver^{24,25} and cardiomyocytes in the heart.²⁶ Indeed, an earlier study using *Prox1-CreERT2* for the ablation of hair cells in the postnatal cochlea has reported a low survival rate of DTA-expressing mutants,²⁷ which was due to the extra-lymphatic role of *Prox1*. A similar problem complicates the interpretation of other cell ablation experiments reported by Biswas et al.¹⁶ *Myh11-CreERT2* mouse, a line that is expressed by smooth muscle cells throughout the body,²⁸ was used for DTA-mediated cell ablation, which also compromised the recovery of irradiated bone.¹⁶ The authors concluded that the targeted *Myh11*⁺ cell population, described as mature pericytes in their study, is controlled by the chemokine Cxcl12 released by bone LECs.¹⁶ As for the *Prox1-CreERT2*-controlled ablation approach, no information about the general health status of the DTA-expressing animals and alterations outside the skeletal system is provided, but it should be noted that other studies involving a partial ablation of pericytes/vascular smooth cells have reported weight loss, changes in adipose tissue, as well as altered heart function and blood pressure.^{29,30} These issues raise concerns that the reported role of lymphatic vessels and LEC-derived signals in bone regeneration¹⁶ might be indirect, reflecting compromised animal health but not a primary function in the skeletal system.

There are also technical challenges associated with the imaging of lymphatic vessels in the context of bone. It is difficult to

completely remove connective tissues from the outer bone surface through physical stripping or collagenase treatment, particularly in the epiphysis, which is surrounded by tightly adherent connective tissues, including periosteum, synovium, muscle, and tendon/ligament.^{31–33} This can result in residual connective tissues that are known to contain lymphatic vessels,¹¹ complicating the interpretation of imaging results. Furthermore, light-sheet imaging and 3D reconstruction techniques may compromise the spatial resolution of intricate structures, such as the periosteum or cortical bone, making it challenging to determine whether fluorescence signals originate from the outer bone surface or the inside. Given these noticeable limitations, and the fact that lymphatic vessels in bones—if confirmed—could open new avenues for basic research and therapeutic applications, the existence of bone lymphatics needs to be validated rigorously.

In this study, we have employed a genetic lineage tracing tool and utilized a dual-recombinase approach (*Cre-loxP* and *Dre-rox*) with an LEC-specific *Cre*, termed *LEC-iCre*,³⁴ to thoroughly validate the absence of LECs in cortical bone, the endosteum, and bone marrow during homeostasis. Serial bone sections and whole-bone immunostaining demonstrate that LECs are located in the connective tissues, such as periosteum and synovium, but not within the bone itself. Furthermore, these LECs outside of the bone remain in place following irradiation and are unable to breach the periosteum to facilitate bone regeneration. The LECs undergo expansion in the periosteum and callus following fracture, but they remain outside the bone structure during repair. In a GLA/GSD disease model, we observed LECs invading bone, serving as a positive control. Our findings challenge the previous conclusion of Biswas et al. regarding the presence of lymphatic vessels in the bone that support regeneration after injury.

RESULTS

Prox1-based genetic tracing reveals the absence of LECs in bone during homeostasis

LYVE1, the key marker used in the previous study to label LECs,¹⁶ is also expressed in other cell types, such as macrophages, sinusoidal endothelial cells, and high endothelial venules,^{17–20,35} making it an unreliable marker for the identification of lymphatic vessels when used in isolation. Our immunostaining data show that numerous LYVE1⁺ cells located outside bone co-express the marker CD45, indicating that these LYVE1⁺ cells are likely immune cells rather than LECs (Figure S1A). Therefore, LYVE1 is not specific enough to differentiate lymphatic vessels from the abundant immune cells found in the bone marrow. Similarly, PDPN, another marker used by Biswas et al. for detecting LECs,¹⁶ is expressed by multiple other cell types, including stromal cells in bone marrow (Figure S1B).^{21,22} The transcription factor PROX1 is an important regulator of LEC differentiation, and the corresponding gene is frequently used for genetic experiments targeting the lymphatic vasculature.^{23,36,37} To detect the presence or absence of LECs in bone, we used the *Prox1-2A-CreER* mouse line,³⁴ in which the P2A-CreER cassette was inserted into the *Prox1* gene locus at 3' UTR (Figure 1A). This modification enables CreER expression under endogenous gene regulation while maintaining normal *Prox1* gene expression, allowing the efficient visualization of LECs.

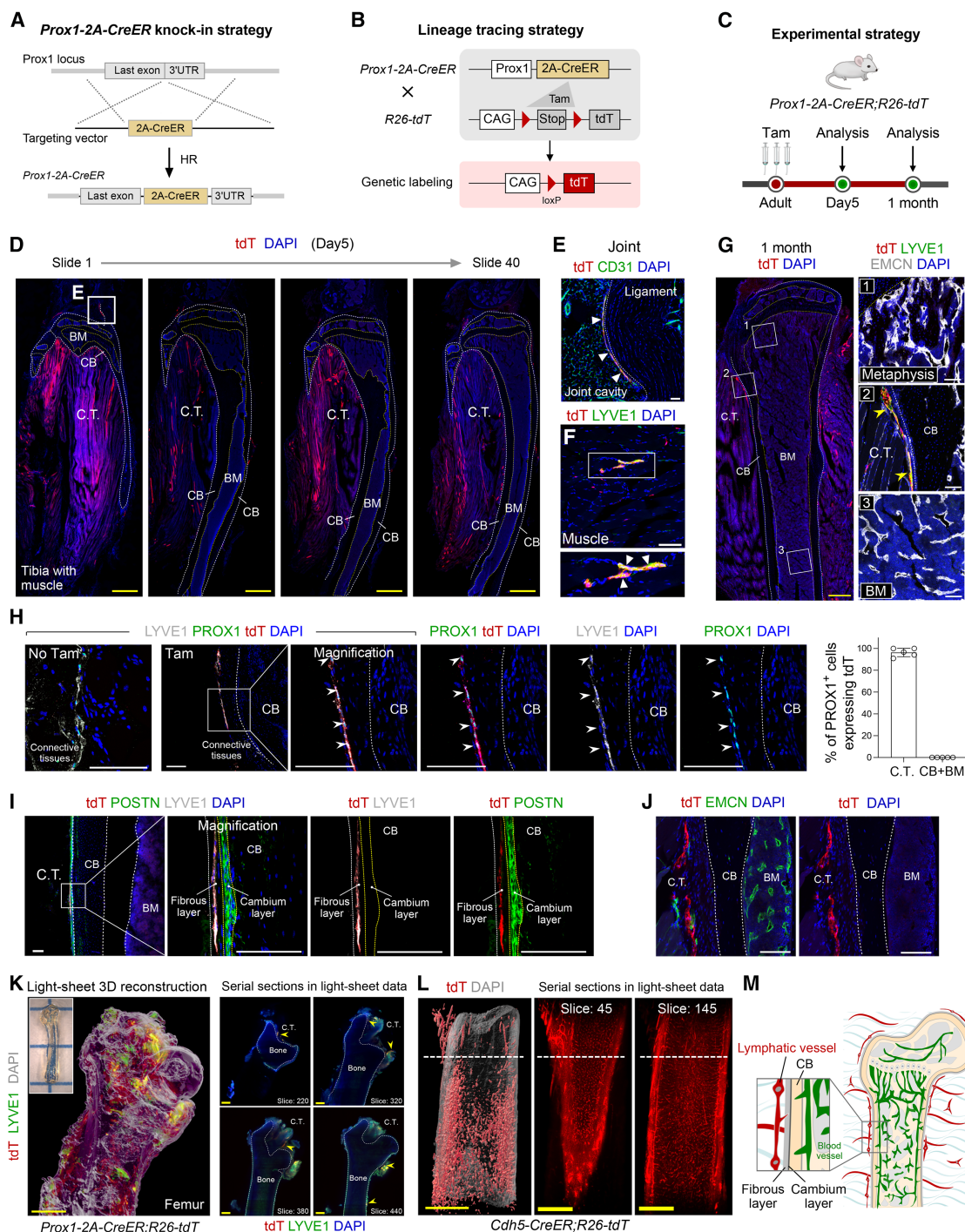


Figure 1. PROX1-based genetic lineage tracing in bone and connective tissues

(A) A schematic diagram illustrating the knockin strategy of the *Prox1*-2A-CreER line by homologous recombination using CRISPR-Cas9.
 (B) A cartoon image showing the genetic strategy for lineage tracing of PROX1⁺ cells.
 (C) A schematic diagram illustrating the experimental design for lineage tracing of PROX1⁺ cells.
 (D) Immunostaining for tdT on tibia serial sections (with muscle) from *Prox1*-2A-CreER;*R26*-tdT mice after Tam. The boxed region of the joint is magnified in (E).
 (E) Higher-magnification view of the joint region reveals tdT⁺ LECs (arrowheads).
 (F) Higher-magnification view of the muscle shows tdT⁺ LECs (arrowheads).
 (G) Immunostaining for tdT, LYVE1, and EMCN on bone sections collected at 1 month) from *Prox1*-2A-CreER;*R26*-tdT mice after Tam treatment. Arrowheads indicate tdT⁺ LECs on the bone surface. Higher-magnification view of the bone marrow reveals EMCN⁺ vascular structures without tdT⁺ LECs.

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We first crossed the *Prox1-2A-CreER* mice with the corresponding reporter, *R26-LSL-tdT* (*R26-tdT*), to generate *Prox1-2A-CreER;R26-tdT* mice (Figure 1B). 5 days or 1 month after tamoxifen (Tam) treatment, tissues from these mice were collected for analysis (Figure 1C). We analyzed approximately 60 serial bone sections from each of the 5 mice in this experiment. Immunostaining showed no tdT signal in these samples, including the cortical bone and bone marrow, across all sections examined (Figure 1D), consistent with previous studies showing that bones lack lymphatic vessels.¹¹ Nevertheless, the tdT signal was abundant in the surrounding tissues, which is likely to reflect the previously reported expression of PROX1 in muscle fibers and satellite cells³⁸ as well as in other cell lineages (Figures 1D and S1C). Additionally, tdT⁺ LECs were readily detected in the surrounding joint and muscle tissues (Figures 1E and 1F). Importantly, examination of samples collected one month after Tam administration confirmed that tdT⁺ LECs remained restricted to the surrounding connective tissue and were not observed within the bone itself (Figure 1G). Notably, some tdT⁺ cells co-expressing LYVE1 and PROX1 were detected in the connective tissue close to the bone surface (Figure 1H). Quantification data showed that tdT is expressed in $96.34\% \pm 3.92\%$ ($n = 5$) of PROX1⁺ LECs in connective tissues close to the bone (Figure 1H), indicating the high LEC labeling efficiency of the *Prox1-2A-CreER* mouse line. Further immunostaining for tdT and periostin (POSTN) showed that these tdT⁺LYVE1⁺ LECs were localized in the fibrous layer of the periosteum, whereas such cells were not present inside bone, including cortical bone and bone marrow (Figure 1I). Of note, the periosteal tdT⁺LYVE1⁺ cells were LECs expressing CD31 (Figure S1D), but they were not capillary vascular ECs, as indicated by the lack of endomucin (EMCN) expression (Figure 1J).

To further validate these findings, we performed immunostaining of whole bone using the tissue-clearing method reported by Biswas et al.¹⁶ In our whole-mount imaging data, convincing tdT⁺LYVE1⁺ signals were detected only on the outer bone surface (Figure 1K). While 3D light-sheet microscopic images appear to show some tdT signal within the bone, this is likely an artifact due to residual signals from the bone surface that remained visible in the 3D reconstruction. Accordingly, serial sections from the light-sheet data did not show any tdT signal inside the bone itself (Figure 1K). As a positive technical control, we analyzed femurs from Tam-treated *Cdh5-CreER;R26-tdT* mice using light-sheet microscopy and readily detected abundant tdT⁺ vascular ECs inside both cortical bone and bone marrow (Figures 1L and S1E). Taken together, these data demonstrate that tdT⁺ LECs are localized on the outer bone surface and within

the surrounding connective tissues, but not inside the bone during homeostasis, as revealed by the *Prox1*-based genetic lineage tracing (Figure 1M).

LEC-*iCre* sensitively and specifically detects LECs outside, but not within, the bone

Prox1-controlled genetic experiments are complicated by the expression of the transcription factor in multiple cell lineages, including neurons, muscle cells, satellite cells, hepatocytes, epithelium, cardiomyocytes (Figure S1C), and certain vascular ECs.³⁹ Accordingly, the tdT signal was also observed outside the lymphatic vasculature in our experiments (Figures 1D and S1). To more specifically visualize LECs without labeling other cell populations, we employed a dual-recombinase-mediated LEC-specific Cre, *LEC-iCre* (*Cdh5-Dre;Prox1-RSR-CreER*),³⁴ which utilizes Dre-mediated expression of CreER under the *Prox1* gene promoter (Figure 2A). Using the endothelial cell-specific tool *Cdh5-Dre*, CreER activity was confined to endothelial cell-derived PROX1⁺ LECs after Dre-rox-mediated removal of the transcriptional Stop sequence from the *Prox1-RSR-CreER* allele (Figure 2A). By crossing with an intersectional reporter *R26-RSR-LSL-tdT* (*R26-RL-tdT*),⁴⁰ EC-derived PROX1⁺ cells were labeled with tdT following Tam treatment, whereas non-endothelial PROX1⁺ cell lineages were not labeled due to the absence of *Cdh5* expression (Figure 2B).

We generated *Cdh5-Dre;Prox1-RSR-CreER;R26-RL-tdT* triple-positive mice to lineage trace LECs, and collected tissues for analysis following Tam treatment (Figure 2C). Whole-mount fluorescence images of individual organs, including the heart, stomach, mesentery, colon, and intestine, displayed specific lymphatic vascular patterns with robust tdT signals (Figure 2D). Immunostaining of sections from the liver, heart, or other organs showed that only PROX1⁺LYVE1⁺ or LYVE1⁺CD31⁺ LECs were labeled by tdT, whereas tdT was absent from hepatocytes, cardiomyocytes, or other cell types (Figures 2D and S2A). Thus, the dual-recombinase-mediated *LEC-iCre* allowed us to more clearly visualize tdT⁺ LECs without interference from other cell populations. We then used this highly sensitive and specific *LEC-iCre* tool to investigate whether tdT⁺ LECs were present in bone tissue during homeostasis. Light-sheet 3D reconstruction of the *LEC-iCre*-labeled bone revealed tdT⁺ signals near the bone surface (Figure 2E). Detailed analysis of each serial slice confirmed that these signals were located on the outer, periosteal bone surface but not inside the bone (Figures 2F and S2B). This spatial distribution was further confirmed in tissue samples collected one month after Tam treatment (Figure 2G),

(H) Immunostaining for tdT, PROX1, and LYVE1 on tibia sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice treated with or without Tam (left). Quantification of the percentage of PROX1⁺ LECs expressing tdT in connective tissues (right). White arrowheads, tdT⁺PROX1⁺LYVE1⁺ LECs.

(I) Immunostaining for tdT, periostin (POSTN), and LYVE1 on tibia sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice.

(J) Immunostaining of tdT and EMCN on tibia sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice.

(K) 3D reconstruction images of tibia immunolabeled with tdT and LYVE1 from *Prox1-2A-CreER;R26-tdT* mice treated with Tam (left). Images of serial sections in light-sheet data with immunostaining for tdT and LYVE1 (right). Yellow arrowheads, tdT⁺ LECs.

(L) 3D reconstruction images of tdT-immunolabeled tibia from *Cdh5-CreER;R26-tdT* mice treated with Tam. The right panel displays corresponding serial sections from the light-sheet dataset, confirming the presence of tdT⁺ vessels in regions indicated by dotted lines in the 3D reconstruction.

(M) A cartoon image showing that lymphatic vessels were localized in the fibrous layer of the periosteum but were not present within the bone.

Data are mean $\pm$ SD; $n = 5$ mice. Yellow bars in (D) and (G), 1 mm; yellow bars in (K) and (L), 500 μ m; white bars, 100 μ m. CB, cortical bone; BM, bone marrow; C.T., connective tissues.

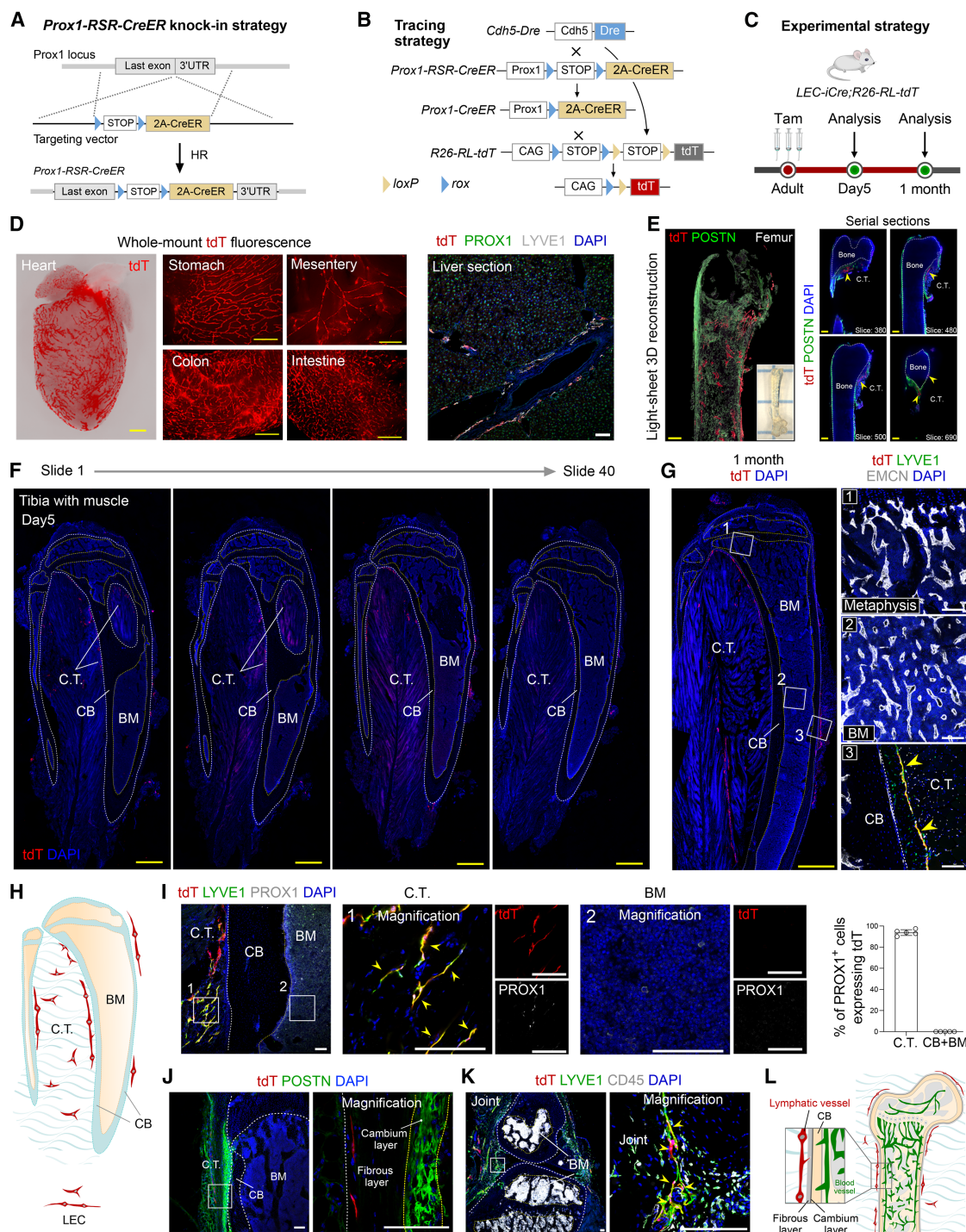


Figure 2. *LEC-iCre* sensitively and specifically detects LECs outside, but not within, the bone

(A) A schematic diagram illustrating the knockin strategy of the *Prox1*-RSR-CreER line by homologous recombination using CRISPR-Cas9.
 (B) A cartoon image showing the intersectional genetic strategy for lineage tracing of *Cdh5*-derived *PROX1*⁺ LECs.
 (C) A schematic diagram illustrating the experimental design for lineage tracing of *Cdh5*-derived *PROX1*⁺ LECs.
 (D) Whole-mount fluorescence images of heart, stomach, mesentery, colon, and intestine (left). Immunostaining for *tdT*, *PROX1*, *LYVE1* on liver section from *LEC-iCre;R26-RL-tdT* mice (right).
 (E) 3D reconstruction images of humerus immunolabeled with *tdT* and *POSTN* from *LEC-iCre;R26-RL-tdT* mice treated with Tam (left). Images in each slice showing *tdT* and *POSTN* immunostaining (right). Yellow arrowheads, *tdT*⁺ LECs.

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demonstrating the consistent absence of intraosseous LECs over time (Figure 2H).

High-resolution imaging of sections co-stained for tdT, LYVE1, and PROX1 revealed efficient labeling of PROX1⁺ LECs (93.82% ± 2.76%) specifically within connective tissue, with no evidence of their presence in bone structures (Figure 2I). Co-staining for tdT and POSTN on bone sections further localized these tdT⁺ LECs to the fibrous layer of the periosteum, but not within the bone itself (Figure 2J). These tdT⁺LYVE1⁺ cells were LECs expressing CD31 (Figure S2C). Additionally, tdT⁺LYVE1⁺ LECs were readily detected in joint tissues (Figure 2K), validating our methodology's sensitivity in identifying lymphatics where they are known to exist. Together, these results establish that LECs are present on the bone surface and surrounding connective tissues, but do not reside within bone during homeostasis (Figure 2L).

Lymphatic vessels do not emerge inside the bone during bone regeneration after irradiation

As Biswas et al. reported an expansion of bone lymphatic vessels after irradiation, suggesting that increased lymphangiogenesis supports hematopoietic regeneration,¹⁶ we also addressed whether LECs can be detected inside bone under these conditions. For this purpose, we first treated *Prox1-2A-CreER;R26-tdT* mice with Tam, followed by irradiation and bone marrow transplantation (BMT) after a 3-week washout period, as previously described¹⁶ (Figure 3A). 7 days post-injury, we observed typical changes in the bone vasculature associated with irradiation in C57BL/6 mice,⁴¹ namely the strong dilation of EMCN⁺ sinusoidal vessels and the loss of CD31⁺EMCN⁺ hematopoietic cells (Figures 3B, S3A, and S3B), indicating successful induction of injury. We then collected the bone tissues at the same time points post-injury as Biswas et al.,¹⁶ and examined the presence of tdT⁺ LECs in the bone (Figure 3A). Immunostaining for tdT on serial bone sections (~80 sections from each of the 5 mice) revealed tdT⁺ cells in the muscle and connective tissues on the bone surface but not within the bone, including the cortical bone and bone marrow (Figures 3C, 3D, and S3C).

Immunostaining for tdT and POSTN showed that the tdT⁺ LECs were confined to the periosteum and did not invade the bone following irradiation (Figures 3E and 3F). Quantification of bone sections obtained 15 days post-injury showed that 97.28% ± 1.37% (*n* = 5) of LYVE1⁺CD31⁺ LECs expressed tdT in connective tissues, and no tdT⁺ LECs were found inside the bone (Figures 3G and 3H). We also failed to detect tdT⁺ LECs in the bone during different stages (up to 66 days) of bone

marrow recovery after transplantation, with each time point assessed from 5 mice (Figure 3I). Furthermore, periosteal tdT⁺ LECs remained EMCN-negative, indicating that tdT⁺ LECs do not adopt a blood vessel EC fate after irradiation injury (Figure 3J). We also noticed that the connective tissues close to the bone contained some tdT⁺ cells expressing LYVE1⁺, which corresponded to LYVE1⁺CD45⁺ immune cells, further emphasizing that LYVE1 is not a reliable marker for the identification of LECs (Figures 3K and 3L). Together, these results provide no genetic evidence for the presence of LECs in the bone after irradiation (Figure 3).

To further support this conclusion, we performed irradiation injury experiments on *LEC-iCre* mice, benefiting from the high specificity of the intersectional genetic approach. 3 weeks after the Tam injection, the *LEC-iCre;R26-RL-tdT* mice were irradiated and received BMT. Long bones were collected at specific time points (Figure 4A). 3D light-sheet microscopy of bones from both sham and injury groups revealed tdT⁺ signals in connective tissue and on the bone surface, but no signals within the bone itself (Figure 4B). Similar to homeostatic analysis, some tdT⁺ signals appearing inside the bone after 3D reconstruction are likely artifacts, potentially due to residual signals from the bone surface that were visible from certain angles in the cleared tissue samples. Accordingly, these tdT⁺ signals were not detected in the serial sections of the light-sheet microscopy data (Figure 4B). Immunostaining for tdT on serial bone sections (~80 sections from each of the 5 mice) showed tdT⁺ cells localized to the outer bone surface and connective tissue but not within the bone itself (Figures 4C and 4D). Higher magnification of the stained sections confirmed that tdT⁺ cells were restricted to the periosteum and did not invade the cortical bone or bone marrow (Figures 4E and 4F). Additionally, immunostaining for tdT, LYVE1, and CD31 on bone sections showed that 93.91% ± 2.34% (*n* = 5) of LYVE1⁺CD31⁺ LECs expressed tdT in connective tissue and the periosteum, with no tdT signal detected in the bone (Figures 4G and 4H). Moreover, no CD31⁺LYVE1⁺ cells expressing tdT were detected in the bone during the long-term repair period, with each time point assessed from 5 mice (Figure 4I). Additionally, tdT⁺ LECs did not acquire EMCN expression (Figure 4J). Collectively, these data confirm that LECs do not emerge within the bone during the repair process following injury (Figure 4K).

Lymphatic vessels expand in the callus but do not invade bone following fracture

Certain cell lineages residing in the fibrous layer can invade the cambium layer of the periosteum and subsequently enter the

(F) Immunostaining for tdT on tibia serial sections (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice treated with Tam.

(G) Immunostaining for tdT, LYVE1, and EMCN on tibia sections from *LEC-iCre;R26-RL-tdT* mice collected 1 month after Tam treatment. Arrowheads indicate tdT⁺LYVE1⁺ LECs localized to the bone surface. Higher-magnification view of the bone marrow shows EMCN⁺ vascular structures without tdT⁺ LECs.

(H) Cartoon image showing labeled tdT⁺ cells in bone (with connective tissues) of *LEC-iCre;R26-RL-tdT* mice.

(I) Immunostaining for tdT, LYVE1, and PROX1 on tibia sections (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice (left). High-magnification views show tdT⁺PROX1⁺ LECs in connective tissue (C.T., magnification 1) but not within bone marrow (BM, magnification 2). The right panel shows the quantification of the percentage of PROX1⁺ LECs expressing tdT in C.T., CB, and BM on bone sections from *LEC-iCre;R26-tdT* mice. Yellow arrowheads, tdT⁺PROX1⁺LYVE1⁺ LECs.

(J) Immunostaining for tdT and POSTN on tibia sections (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice.

(K) Immunostaining for tdT, LYVE1, and CD45 on joint sections from *LEC-iCre;R26-RL-tdT* mice. Yellow arrowheads, tdT⁺ LYVE1⁺ LECs.

(L) A cartoon image showing that lymphatic vessels were localized in the fibrous layer of the periosteum but were absent within the bone.

Data are mean ± SD; *n* = 5 mice. Yellow bars in (D), (F), and (G), 1 mm; yellow bars in (E), 500 μm; white bars, 100 μm. CB, cortical bone; BM, bone marrow; C.T., connective tissues.

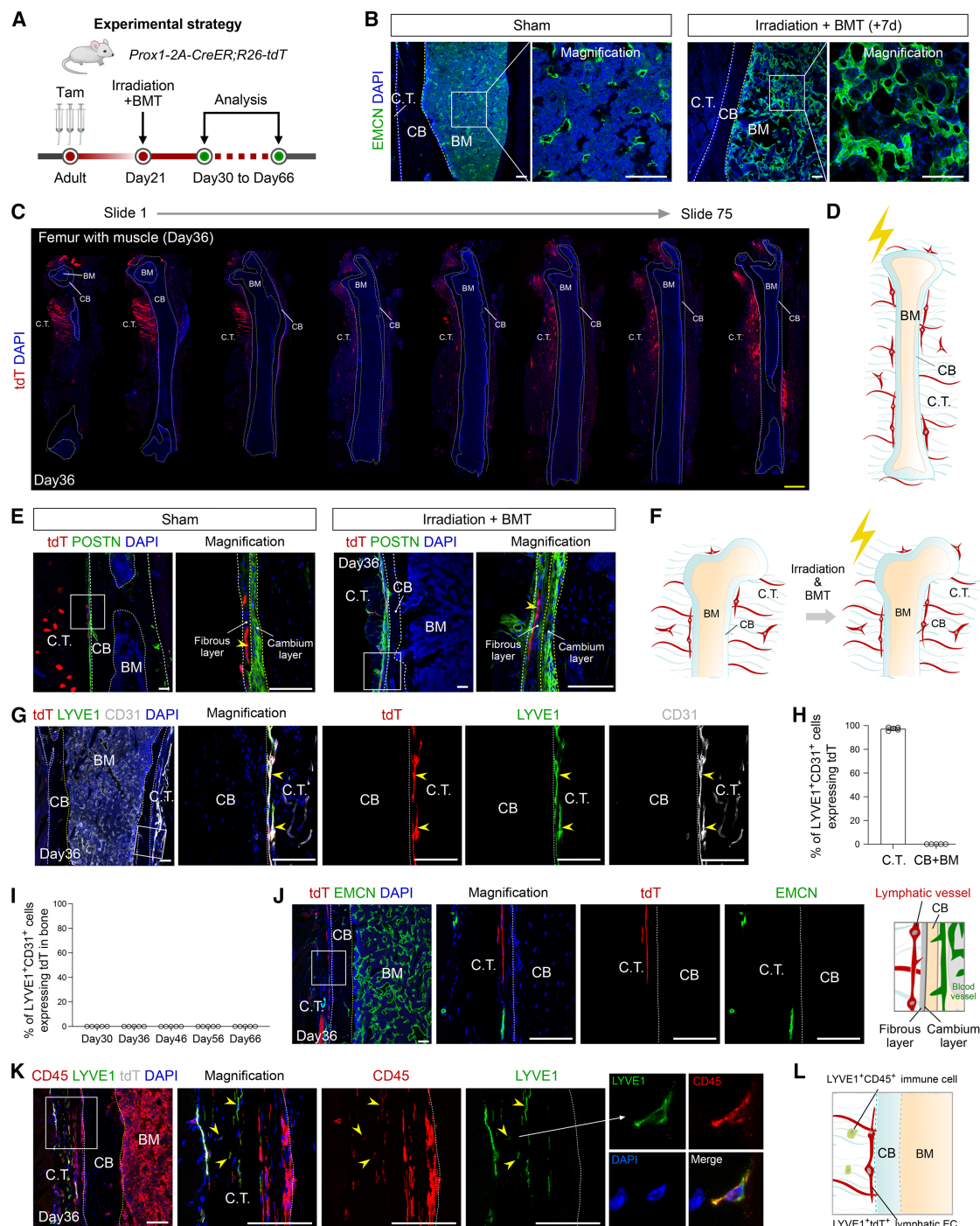


Figure 3. Lymphatic vessels do not emerge during bone regeneration after irradiation injury

(A) A schematic diagram illustrating the experimental design.

(B) Immunostaining for EMCN on femur sections from control or irradiated C57BL/6 mice.

(C) Immunostaining for tdT on femur serial sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice after irradiation.

(D) Cartoon image showing labeled tdT⁺ cells in the femur (with connective tissues) of *Prox1-2A-CreER;R26-tdT* mice after irradiation.

(E) Immunostaining of tdT and POSTN on tibia sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice after irradiation. Yellow arrowheads, tdT⁺ LECs.

(F) Cartoon image showing tdT⁺ cells remaining in muscle and connective tissues and on the bone surface after irradiation in *Prox1-2A-CreER;R26-tdT* mice.

(G) Immunostaining for tdT, LYVE1, and CD31 on tibia sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice after irradiation. Yellow arrowheads, tdT⁺ LECs.

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bone marrow, playing a critical role in fracture repair.^{42,43} Furthermore, bone fracture induces the dilation of draining lymphatic vessels and enlargement of lymph nodes, which, in turn, can influence inflammation and subsequent stages of the healing process.^{44–46} To investigate whether mechanical damage disrupting the periosteum could trigger the ingrowth of lymphatic vessels into bone, we employed a femur fracture model⁴⁷ in *LEC-iCre;R26-RL-tdT* mice. After Tam treatment and a subsequent 3-week washout period, the *LEC-iCre;R26-RL-tdT* mice underwent surgical fracture with internal fixation of the femur. Bones were then collected at various stages post-fracture for further analysis (Figure 5A). After the initial hematoma formation, which is characterized by inflammatory processes, blood clotting, and the onset of revascularization, staining of bone sections isolated during the first week post-fracture revealed the expected formation of a cartilaginous (soft) callus at the fracture site (Figures 5B–5E). Immunostaining of these sections demonstrated a significant expansion of tdT⁺ LECs compared with normal bone (Figures 5B and 5E). Additionally, safranin O/fast green staining, which highlights cartilage in orange/red and the surrounding callus in cyan, along with immunostaining of consecutive bone sections, showed that tdT⁺ LECs were present within the callus as well as in remote (undamaged) femoral areas (Figures 5D and 5E).

After 2 weeks of fracture healing, both the size of the callus and the area of cartilage decreased, reflecting the ongoing mineralization and conversion into a fibrous or hard callus. This process involved the reduction of cartilage and increased mineralization of the callus due to active osteogenesis (Figures 5C, 5F, and 5G).⁴⁸ Higher-magnification images revealed a significant increase of tdT⁺ LECs as fracture repair progressed, particularly within the regions of the fibrous callus enriched with LYVE1⁺CD31⁺ blood vasculature (Figure 5G and 5H). We also detected tdT⁺LYVE1⁺CD45⁺ immune cells within connective tissues and inside the bone marrow post-fracture, similar to what was described above for both homeostasis and irradiation injury (Figures S1A and 3K). LYVE1⁺CD45⁺ immune cells did not express the EC marker CD31 (Figures S4A–S4C). Some tdT⁺LYVE1^{low}CD31⁺ cells were detected within the callus at this stage, which also expressed EMCN and were therefore likely to represent capillary blood vessels (Figures S4D and S4E). These results emphasize the notion that, without co-staining with additional markers such as PROX1, LYVE1 is of limited value for identifying lymphatic vessels (Figures S4A and S4E).

Similar to the findings from 1-week post-fracture bone, tdT⁺ LECs were localized within connective tissues, including the periosteum and muscle in remote regions, as well as within the fibrous callus at the injury site. Quantitative analysis of bone sections showed that 96.52% ± 2.44% and 94.92% ± 3.33% of

LYVE1⁺CD31⁺EMCN⁺ LECs expressed tdT in the periosteum and callus after 1 and 2 weeks post-fracture, respectively, whereas no tdT⁺ LECs were observed within the bone (Figures 5I and 5J). Additionally, fracture injury was also induced in *Prox1-2A-CreER;R26-tdT* mice (Figure S4F). Immunostaining of bone sections from these mice 2 weeks post-fracture revealed widespread tdT expression inside muscle and connective tissues (Figure S4G), as PROX1 is expressed in multiple cell lineages (Figure S1C). A sparse tdT signal was also detected in CD31⁺LYVE1⁺ chondrocytes and newly formed bone (Figures S4H and S4I). Consistent with data from *LEC-iCre* lineage tracing, we could not detect any tdT⁺LYVE1⁺CD31⁺ LECs in the disrupted bone, callus cartilage, or newly formed bone using the *Prox1-2A-CreER* tool.

Collectively, these findings demonstrated that our lineage tracing tools effectively captured the significant expansion of LECs following fracture, which was confined to the periosteum and fibrous regions of the callus. Notably, our analysis did not uncover evidence that lymphatic vessels invade bone structures during fracture repair (Figure 5C).

Analysis of LECs in human bone biopsies

Single-cell RNA sequencing (scRNA-seq) is a powerful approach enabling the identification and detailed characterization of cell populations within organs. Biswas et al. have provided human scRNA-seq data, obtained from bone biopsies taken from two cancer patients, and reported that this data contains different endothelial subsets, including a cluster of LECs.¹⁶ Independent reanalysis and UMAP (uniform manifold approximation and projection) reduction of this dataset, however, revealed that it contains a very small number (<60 cells) of genuine ECs expressing the markers *CDH5* (encoding VE-cadherin) and *PECAM1/CD31* (Figure 6A). Some level of *PECAM1* expression was detectable in the majority of subclusters, but these cells lacked detectable *CDH5* transcript and expressed markers of various hematopoietic cell populations, most abundantly *PTPRC* (encoding the immune cell marker *CD45*), agreeing with data of low *Pecam1* expression in mouse hematopoietic cells (Figures 6A and 6B, and [immgen.org](#)). The data also includes a small population of *PDGFRA*⁺ fibroblasts, which represent another non-endothelial contamination (Figure 6B). Most importantly, the data contained only very few *PROX1*⁺ cells, and the majority of these cells lacked *CDH5* expression. Given the very small number of total ECs, further analysis of endothelial heterogeneity in the dataset was not sensible, but we noted that the majority of *CDH5*⁺ cells express *EPHB4*, *NR2F2*, and *FLT4*, which are markers for venous endothelium but also sinusoidal ECs from the bone marrow (Figure 6B). In conclusion, the human scRNA-seq data provided by Biswas et al.¹⁶ represent mostly contaminating cells

(H) Quantification of the percentage of LYVE1⁺CD31⁺ LECs expressing tdT in C.T., CB, and BM on bone sections from *Prox1-2A-CreER;R26-tdT* mice.

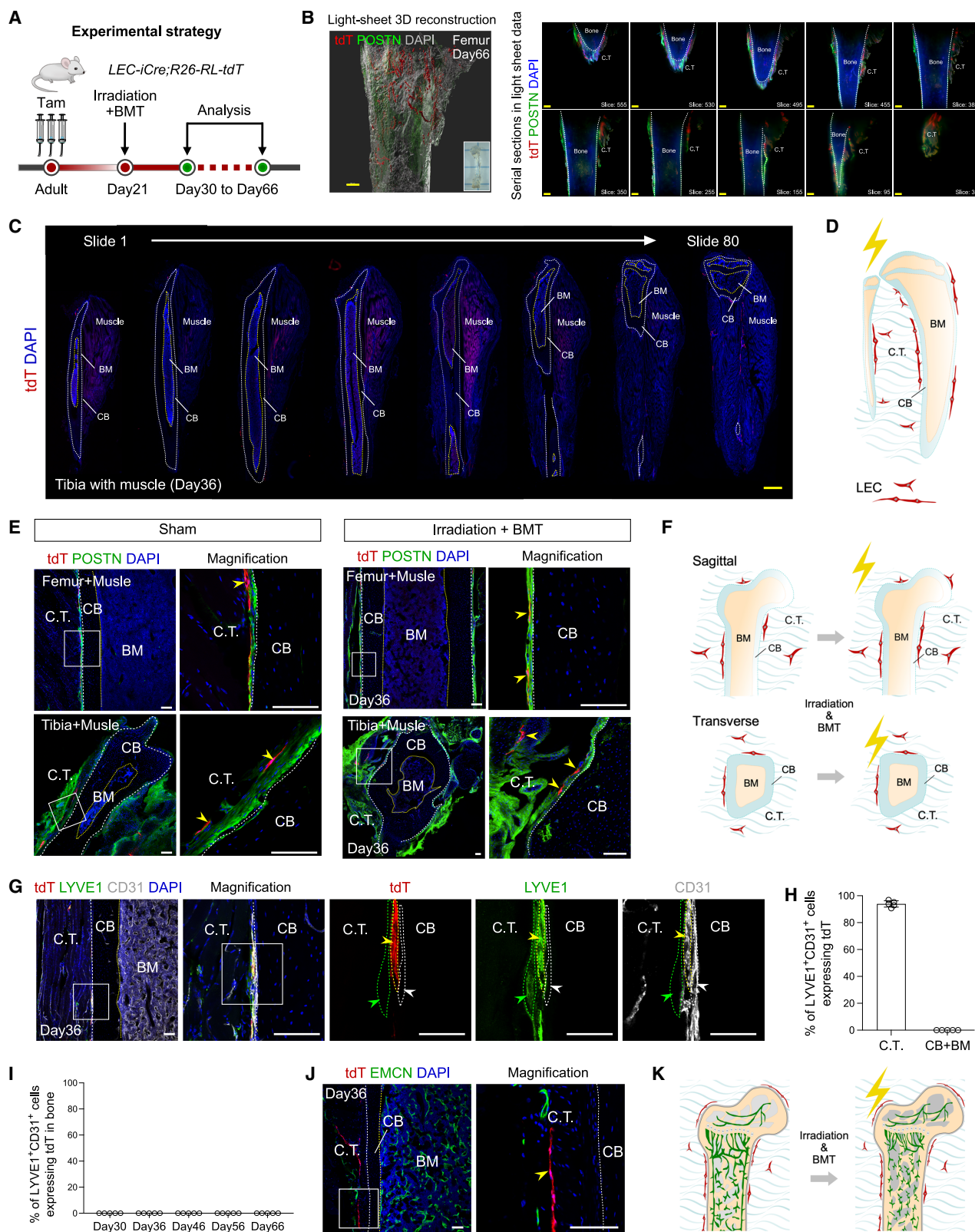
(I) Quantification of the percentage of LYVE1⁺CD31⁺ LECs expressing tdT in the bone from *Prox1-2A-CreER;R26-tdT* mice.

(J) Immunostaining for tdT and EMCN on bone sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice after irradiation (left). A cartoon image showing that LECs were localized in the fibrous layer of the periosteum (right).

(K) Immunostaining of tdT, LYVE1, and CD45 on femur sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice after irradiation. Yellow arrowheads, LYVE1⁺CD45⁺tdT⁺ immune cells.

(L) A cartoon image showing LYVE1⁺CD45⁺tdT⁺ immune cells in the connective tissues.

Data are mean ± SD; n = 5 mice. Yellow bars, 1 mm; white bars, 100 μm. CB, cortical bone; BM, bone marrow; C.T., connective tissues.



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and do not support the presence of LECs in human bone. It is also not clear whether any precautions were taken to prevent the inclusion of cell populations from the surrounding tissue and, most importantly, the LEC-containing periosteum during the biopsy procedure. While Biswas et al.¹⁶ also presented images of cleared and immunostained human bone samples, the approach showed LYVE1⁺ cells but fails to reveal the presence of lumen-containing lymphatic vessels, lymphatic valves, or a monolayer of LECs with nuclear PROX1 staining inside the bone.

Genetic tracing of lymphatic vessels in bone using a GLA/GSD model

Previous studies have established that *Pik3ca*^{H1047R} overexpression in LECs drives lymphatic vessel formation in bone, modeling human GLA/GSD.^{11,14} To specifically trace and visualize LEC dynamics in bone, we used a dual recombinase-mediated genetic strategy by generating *Cdh5-DreER;Prox1-RSR-CreER;R26-Pik3ca-tdT* mutant mice (Figures 7A and 7B). Tam was administered starting at 3 weeks, with bone collection at 7, 9, and 11 weeks (Figure 7C). While control mice (*Cdh5-DreER;Prox1-RSR-CreER;R26-tdT*) showed tdT⁺ LECs restricted to the periosteum and connective tissues (Figure 7D), mutant mice exhibited tdT⁺ LECs within bone (Figure 7E). Time-course analysis showed tdT⁺LYVE1⁺CD31⁺ LECs progressively migrating toward cortical bone during early expansion (Figure 7E), appearing within cortical bone by intermediate stages, and ultimately infiltrating bone marrow as GLA progressed (Figures 7E–7G). These results demonstrate that *Pik3ca*^{H1047R}-induced hyperplastic LECs breach the periosteum and invade bone tissue (Figure 7H), providing a critical positive control for intraosseous LEC detection and highlighting the pathogenic potential of bone-invading lymphatics.

DISCUSSION

In this study, we present robust *in vivo* lineage tracing evidence that challenges the conclusions drawn by Biswas et al. regarding the existence of bone lymphatic vessels and their role in bone regeneration after injury.¹⁶ Our data highlight the risks of using LEC markers such as LYVE1 without accounting for their broad

expression in non-EC cell types. Most importantly, our *Prox1*-based lineage tracing strategy demonstrated that LECs are absent in bone during homeostasis and repair following irradiation or fracture injury. However, some non-endothelial labeling was observed due to PROX1 expression in other cell lineages. Utilizing a dual-recombinase-mediated genetic approach (*Cre-loxP* and *Dre-rox*), we employed the LEC-specific *Cre*, *LEC-iCre*, to precisely trace LECs. Whole-bone immunostaining and 3D reconstruction demonstrate that whereas LECs are present on the bone surface, they do not reside within the bone itself. Serial bone sections provided higher spatial resolution, revealing that these LECs are localized within the connective tissues and the fibrous layer of the periosteum, rather than inside the bone, as was previously reported.¹⁶ Moreover, we found no evidence that LECs invade the bone to contribute to regeneration after irradiation, indicating that the stress induced by irradiation is insufficient to prompt LECs to breach the periosteum. To further investigate whether an injury model involving mechanical damage that disrupts the periosteum could trigger LEC invasion, we performed a fracture injury model. While LECs expanded significantly after fracture, they remained localized outside the bone, including the disrupted and newly formed bone structures. LECs were confined to periosteum- or soft tissue-derived regions at 2 weeks post-fracture, consistent with their established location during homeostasis. Of note, our genetic lineage tracing tools robustly detect lymphatic vessels invading the bone in a GLA/GSD model, providing a critical positive control that validates our system's detection capability.

The primary evidence for the presence of lymphatic vessels in bone provided by Biswas et al. includes light-sheet imaging of whole-bone immunostaining with the markers LYVE1, PDPN, and PROX1, imaging of bone from *LYVE1-EGFP* reporter mice, injection of Evans blue dye to map lymphatic vessels, and quantitative PCR and flow cytometry analyses of whole bone or the outer surface and cortical bones separated by scraping with a scalpel blade.¹⁶ However, there are several limitations associated with these methods. First, the immunodetection of single LEC markers is insufficient to confirm the lymphatic endothelial identity. For example, LYVE1 is also expressed in macrophages, sinusoidal endothelial cells, and high endothelial venules,

Figure 4. *LEC-iCre* detects LECs outside, but not within, the bone during regeneration

- (A) A schematic diagram illustrating the experimental design.
(B) 3D reconstruction images of femur immunolabeled with tdT and POSTN from *LEC-iCre;R26-RL-tdT* mice after irradiation (left). Images in each slice showing tdT and POSTN immunostaining (right).
(C) Immunostaining for tdT on tibia serial sections (with muscle) from *LEC-iCre;R26-RL-tdT* mice after irradiation.
(D) Cartoon image showing labeled tdT⁺ cells in the tibia (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice after irradiation.
(E) Immunostaining of tdT and POSTN on femur or tibia sections (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice after irradiation (or sham). Yellow arrowheads, tdT⁺ LECs.
(F) Cartoon image showing tdT⁺ cells remaining in muscle and connective tissues and on the bone surface of *LEC-iCre;R26-RL-tdT* mice after irradiation.
(G) Immunostaining for tdT, LYVE1, and CD31 on femur sections (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice after irradiation. Yellow arrowheads, tdT⁺LYVE1⁺CD31⁺ LECs; white arrowheads, tdT⁺LYVE1⁺CD31⁺ ECs; green arrowheads, tdT⁺LYVE1⁺CD31⁺ non-ECs.
(H) Quantification of the percentage of LYVE1⁺CD31⁺ LECs expressing tdT in C.T., CB, and BM on bone sections from *LEC-iCre;R26-tdT* mice after irradiation.
(I) Quantification of the percentage of LYVE1⁺CD31⁺ LECs expressing tdT in the bone from *LEC-iCre;R26-RL-tdT* mice after irradiation.
(J) Immunostaining of tdT and EMCN on femur sections (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice after irradiation. Yellow arrowheads, tdT⁺ LECs.
(K) A cartoon image showing that tdT⁺ LECs remain in connective tissues and on the bone surface after irradiation.
Data are mean ± SD; n = 5 mice. Yellow bars in (B), 300 μm; Yellow bars in (C), 1 mm; white bars, 100 μm. CB, cortical bone; BM, bone marrow; C.T., connective tissues.

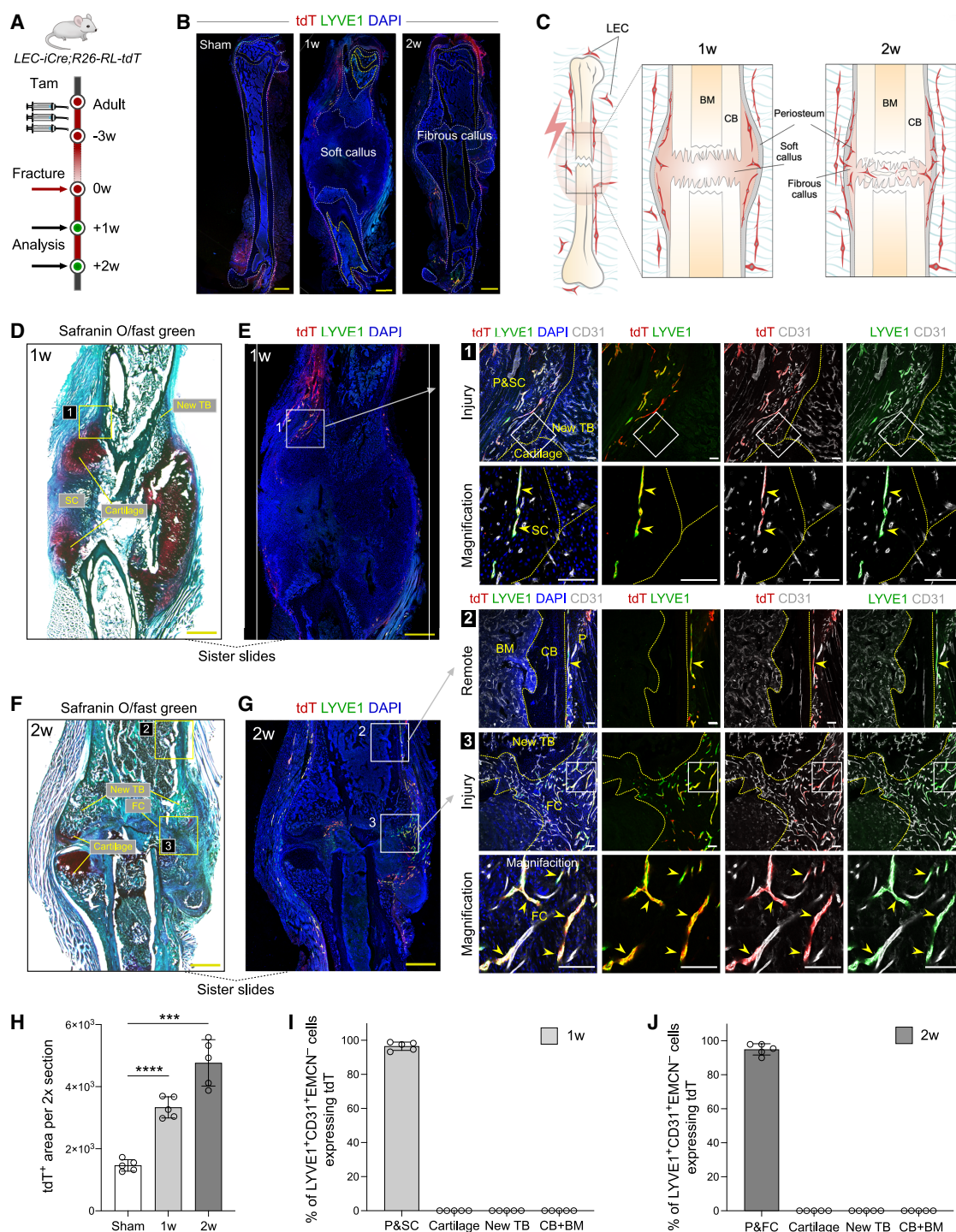


Figure 5. Lymphatic vessels expand in the callus but do not invade the bone after fracture

(A) A schematic diagram illustrating the experimental design.

(B) Immunostaining for tdT and LYVE1 on sham or fractured femur sections (with muscle) from LEC-iCre;R26-RL-tdT mice.

(C) Cartoon images showing labeled tdT⁺ cells in the femur (with connective tissues) from LEC-iCre;R26-RL-tdT mice after fracture.

(D and F) Safranin O/fast green staining of femur sections (with connective tissues) from 1w (D) or 2w (F) post-fracture LEC-iCre;R26-RL-tdT mice.

(E and G) Immunostaining of tdT, LYVE1, and CD31 on femur sections (with connective tissues) from 1 week (E) or 2 weeks (G) post-fracture LEC-iCre;R26-RL-tdT mice. Yellow arrowheads, tdT⁺LYVE1⁺CD31⁺ LECs.

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whereas PDPN is expressed in mesenchymal cells.^{17–22} Similarly, the *LYVE1-EGFP* genetic tool does not achieve precise LEC labeling due to interference from *LYVE1*⁺ non-LEC lineages. Although Biswas et al. utilized the *Prox1-CreER* transgenic tool, their study lacks robust *Prox1*-traced immunostaining to confirm the presence of LECs within the bone. Additionally, Biswas et al. reported accumulation of Evans blue in tibial bones after subcutaneous injection of the dye into the inner leg, evaluated after 4–6 h post-injection.¹⁶ Lymphatic vessels serve as unidirectional conduits for the transport of interstitial fluid from peripheral tissues toward lymph nodes and, ultimately, into the venous circulation. This process necessitates the prevention of backflow by intraluminal lymphatic valves.³ This transport is comparably rapid so that the uptake of dye into the lymphatic network and transport in collecting ducts is observable within minutes.⁴⁹ By contrast, the transfer of Evan's blue from subcutaneous tissue into bone described by Biswas et al.¹⁶ occurs over the period of hours and, together with the complete absence of evidence for lymphatic valves, does not fulfill the established criteria of lymphatic drainage.

qPCR and flow cytometry analyses sacrifice spatial information, making it impractical to completely separate the connective tissues, particularly the periosteum, from the bone surface through methods such as physical stripping or collagenase treatment, where LECs are abundant. This limitation may lead to false-positive signals in whole-mount imaging, where lymphatic vessels on the bone surface could be mistakenly identified as structures within the bone or bone marrow. Furthermore, the scRNA-seq data of human bone samples provided by Biswas et al.¹⁶ fail to support the existence of lymphatic vessels within the bone. This dataset is heavily contaminated by non-endothelial populations and contains only a single, small cluster of *CDH5*⁺ ECs. In this context, it is puzzling that the proposed LEC cluster (cluster 6) reported by Biswas et al.¹⁶ appears to be identical to a cluster of *CLEC14A*⁺ angiogenic ECs presented in a recent preprint from the same group.⁵⁰

Our lineage tracing data indicate that LECs are predominantly localized in the fibrous layer of the periosteum, both during homeostasis and following injuries. This localization suggests that lymphatic vessels emerging from within the bone may primarily be associated with abnormal bone physiology or pathological conditions, such as GLA or GSD, as previously reported.⁵¹ While there is a noticeable expansion of lymphatic vessels coupled with angiogenesis after femur fracture, this increase in LECs remained localized and confined to periosteum- or soft tissue-derived regions outside the bone structure in short-term fracture healing. However, the fate of these hyperplastic LECs in long-term fracture healing remains unclear as callus resorption and vascular regression occur in subsequent bone remodeling.^{45,48,52} These LECs may undergo apoptosis or transdifferentiate into ECs to contribute to new blood vessels within the bone,

or, though less likely, retain their LEC identity. Therefore, long-term monitoring of LEC fate post-fracture is warranted to explore these dynamics. Additionally, the functional role of these hyperplastic LECs following fracture is currently unclear. Previous studies have investigated the role of lymphatic vessels and lymph nodes during fracture healing.^{44–46,53} For instance, using a rat fracture model, researchers demonstrated that bone injury induces the enlargement of draining lymph nodes.⁴⁴ Near-infrared indocyanine green lymphatic imaging revealed that lymphatic platelet thrombosis blocks the draining lymphoid sinus and lymphatic vessels at the upstream lymph nodes, thereby inhibiting fracture healing in male mice through complex immunomodulation.⁴⁶ However, bone development and fracture healing appear normal in *Vegfr3*^{wt/Chy} mice, which have a defect in lymphatic system development. This suggests that lymphatics may be dispensable for normal bone development or repair.⁵³

In summary, our study provides compelling evidence that LECs are confined to the fibrous layer of the periosteum and do not invade bone during homeostasis or regeneration. These findings challenge the conclusions of Biswas et al. regarding the presence of functional lymphatics within bone and their proposed role in bone repair.¹⁶ While LECs expand following fracture, they remain restricted to periosteal and callus regions without penetrating bone tissue. The persistence and fate of these hyperplastic LECs during long-term remodeling warrant further investigation. Notably, under pathological conditions such as GLA/GSD, LECs acquire invasive capacity and penetrate bone. This stark distinction between physiological containment and pathological invasion underscores the necessity of context in evaluating lymphatic function and definitively clarifies the spatial boundaries of lymphatic vessels in bone biology.

Limitations of the study

While this study utilizes multiple genetic lineage tracing technologies and does not detect LECs inside bone during homeostasis or after injury, several limitations should be acknowledged. First, our lineage tracing experiments were performed using inducible CreER systems, which do not achieve 100% labeling efficiency in LECs. While it remains theoretically possible that a small number of LECs inside bone were not labeled by our inducible system, we consider this very unlikely. We believe the high labeling efficiency (>95%) achieved with both genetic approaches is unbiased and makes it unlikely that we missed LECs within bone. Second, while we observed no lymphatic vessel invasion into bone following irradiation or fracture, our injury models are limited to these two contexts. It remains possible that other types of bone injury or pathological conditions could elicit lymphatic invasion, and further studies using additional models are needed to determine the generality of our findings. Third, the mechanisms that restrict LECs to the periosteum rather than permitting their invasion into bone during homeostasis and after injury

(H) Quantification of the area of *tdT*⁺ LECs of sham or fractured femur sections (with connective tissues) from *LEC-iCre;R26-RL-tdT* mice. ****p* = 0.0004, *****p* < 0.0001; *n* = 5.

(I and J) Quantification of the percentage of *LYVE1*⁺*CD31*⁺*EMCN*[−] LECs expressing *tdT* in femur sections (with connective tissues) from 1 week (I) or 2 weeks (J) post-fracture *LEC-iCre;R26-RL-tdT* mice.

Data are mean ± SD; *n* = 5 mice. Yellow bars, 1 mm; white bars, 100 μm. Tam, tamoxifen; CB, cortical bone; BM, bone marrow; P, periosteum; SC, soft callus; FC, fibrous callus; P&SC, periosteum and soft callus; P&FC, periosteum and fibrous callus; New TB, new trabecular.

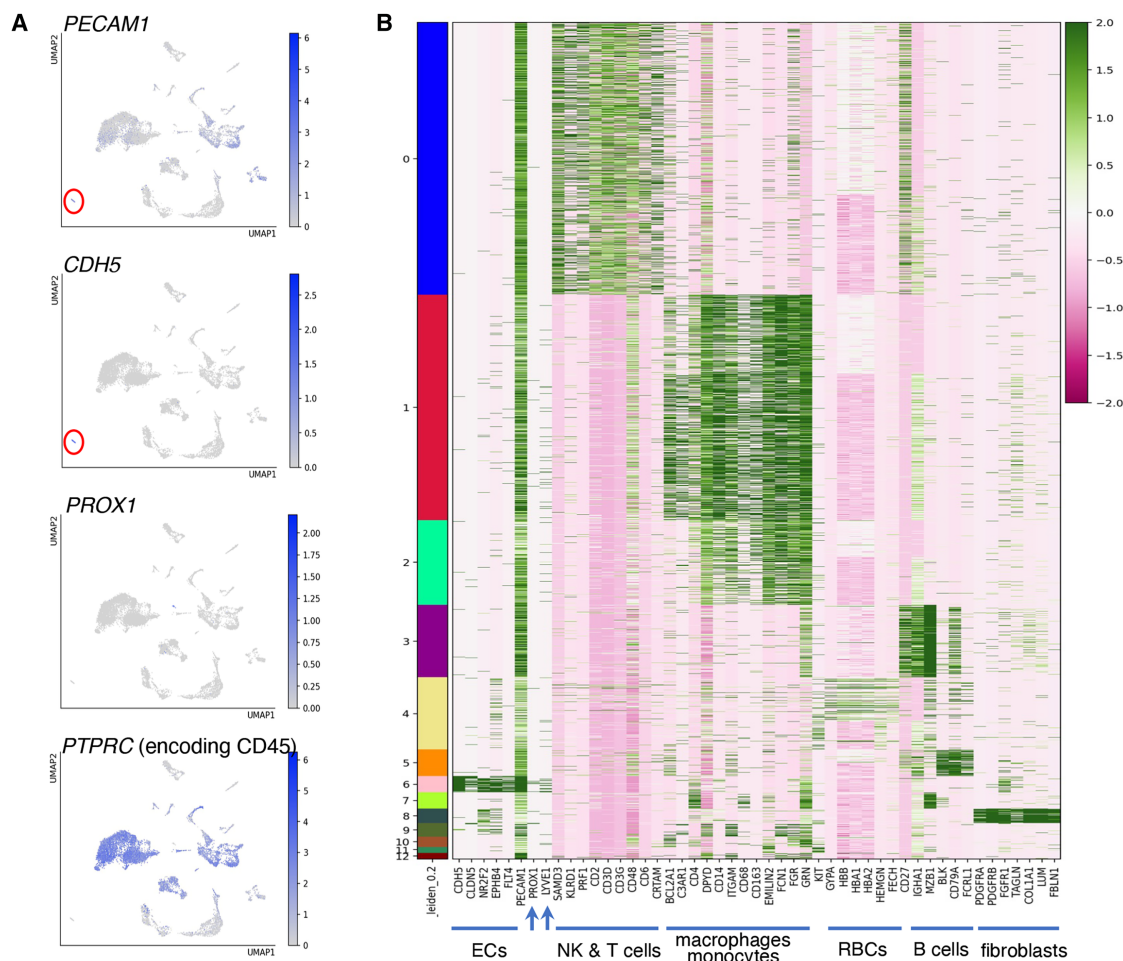


Figure 6. Reanalysis of scRNA-seq data from human bone

(A) UMAP plots showing the expression of *PECAM1*, *CDH5*, *PROX1*, and *PTPRC* (CD45) in the reanalyzed scRNA-seq dataset from human bone. Red circles indicate a small cluster of cells expressing both *PECAM1* and *CDH5*. Note that many clusters express *PECAM1* together with *PTPRC*, indicating hematopoietic cells, whereas there is no distinct *PROX1*⁺ cluster.

(B) Heatmap showing the expression of the indicated genes in the different subclusters. Note that *CDH5* and other EC markers are confined to the small cluster 6, marked in pink. Whereas the majority of clusters show expression of *PECAM1* together with different sets of markers for hematopoietic cell populations, there is no distinct cluster characterized by expression of *PROX1* and *LYVE1*.

remain incompletely understood and require further investigation. Similarly, the mechanisms underlying and the functional significance of hyperplastic LEC invasion in conditions such as GLA/GSD have yet to be elucidated. It remains unclear how LECs breach the periosteum and whether specific proteases or molecules secreted by aberrant LECs or other cell types mediate this process.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bin Zhou (zhoubin@sibs.ac.cn).

Materials availability

The materials generated in this study are available upon reasonable request by contacting the corresponding authors.

Data and code availability

- scRNA-seq data are available at ArrayExpress: E-MTAB-11560.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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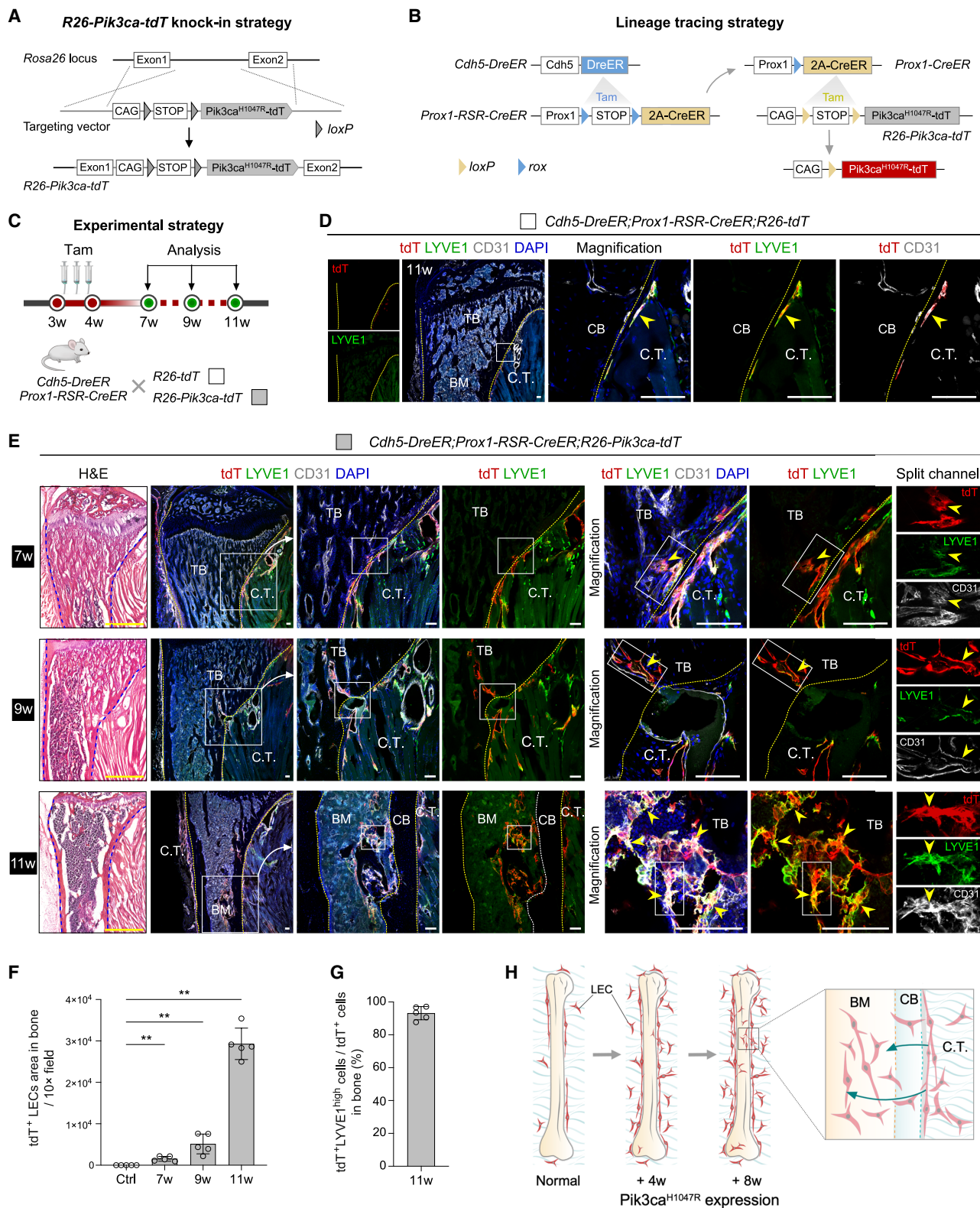


Figure 7. Lymphatic vessels invade the bone after *Pik3ca*^{H1047R} overexpression
(A) A schematic illustrating the knockin strategy of *R26-Pik3ca-tdT* mice.
(B) A schematic illustrating the strategy for tracing and *Pik3ca*^{H1047R} overexpression of LECs.

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AUTHOR CONTRIBUTIONS

X. Meng, K.L., M.T., W.W., Z.Z., X. Huang, Z.H., X. Han, R. Sun, R. Shen, and X. Ma bred mice, performed mouse experiments, and analyzed the data; T.V.P., T.M., and M.T.D. provided intellectual input and analyzed data; K.K. analyzed scRNA-seq data; R.H.A. and B.Z. conceived the project, interpreted the data, and drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used DeepSeek in order to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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 - Irradiation and bone marrow transplantation
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SUPPLEMENTAL INFORMATION

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(C) A schematic diagram illustrating the experimental design.

(D) Immunostaining of tdT, LYVE1, and CD31 on tibia sections (with connective tissues) from *Cdh5-DreER;Prox1-RSR-CreER;R26-tdT* mice. Yellow arrowheads, tdT⁺LYVE1⁺CD31⁺ LECs.

(E) H&E staining of tibia sections (with connective tissues) from 7, 9, and 11 weeks *Cdh5-DreER;Prox1-RSR-CreER;R26-Pik3ca-tdT* mice (left). Immunostaining of tdT, LYVE1, and CD31 on tibia sections (with connective tissues) from 7, 9, 11 weeks *Cdh5-DreER;Prox1-RSR-CreER;R26-Pik3ca-tdT* mice (right). Yellow arrowheads, tdT⁺LYVE1⁺CD31⁺ LECs.

(F) Quantification of tdT⁺ LECs area in bone per 10× section in the control mice (Ctrl, *Cdh5-DreER;Prox1-RSR-CreER;R26-tdT*) and the *Cdh5-DreER;Prox1-RSR-CreER;R26-Pik3ca-tdT* mice at the stages of 7, 9, and 11 weeks. ***p* = 0.0079 (adjusted *p* = 0.0237, Bonferroni correction); *n* = 5.

(G) Quantification of the percentage of tdT⁺LYVE1^{hi} cells in tdT⁺ cells on bone sections from 11 weeks *Cdh5-DreER;Prox1-RSR-CreER;R26-Pik3ca-tdT* mice.

(H) Cartoon image showing labeled tdT⁺ Pik3ca^{H1047R} mutant LECs expand and invade the bone after long-term Pik3ca^{H1047R} overexpression.

Data are mean ± SD; *n* = 5 mice. Yellow bars, 1 mm; white bars, 100 μm. Tam, tamoxifen. CB, cortical bone. TB, trabecular bone. BM, bone marrow. C.T., connective tissues. H&E, hematoxylin and eosin.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-tdTomato	Rockland	Cat#600-401-379; RRID: AB_2209751
Goat anti-tdTomato	Rockland	Cat#200-101-379; RRID: AB_2744552
Rat anti-tdTomato	Proteintech	Cat#5F8; RRID: AB_2336064
Goat anti-E-cadherin	R&D system	Cat#AF748; RRID: AB_355568
Syrian Hamster anti-PDPN/T1a	DSHB	Cat# 8.1.1; RRID: AB_531893
Goat anti-Troponin I	Abcam	Cat#ab56357; RRID: AB_880622
Goat anti-mPeriostin	R&D system	Cat#AF2955; RRID: AB_664123
Goat anti-CD31/PECAM	R&D system	Cat#AF3628; RRID: AB_2161028
Rabbit anti-LYVE1	Abcam	Cat#ab14917; RRID: AB_301509
Rabbit anti-PROX1	Abcam	Cat#ab101851; RRID: AB_10712211
Goat anti-PROX1	R&D	Cat#AF2727; RRID: AB_2170716
Mouse anti-NeuN	Merck	Cat#MAB377X; RRID: AB_2149209
Rabbit anti-NeuN	Abcam	Cat#ab177487; RRID: AB_2532109
Rat anti-EMCN	Santa Cruz	Cat#sc-65495; RRID: AB_2100037
Mouse anti-HNF4α	Abcam	Cat#ab41898; RRID: AB_732976
Rat anti-CD45-FITC	eBioscience	Cat#11-0451-82; RRID: AB_465050
Rat anti-CD45-APC	eBioscience	Cat#17-0451-82; RRID: AB_469393
Rat anti-LYVE1-FITC	eBioscience	Cat#53-0443-80; RRID: AB_1633417
Alexa donkey anti-rabbit 488	Invitrogen	Cat#A21206; RRID: AB_2535792
Alexa donkey anti-rabbit 555	Invitrogen	Cat#A31572; RRID: AB_162543
Alexa donkey anti-rabbit 647	Invitrogen	Cat#A31573; RRID: AB_2536183
Alexa donkey a-mouse 647	Invitrogen	Cat#A31571; RRID: AB_162542
Alexa donkey anti-rat 647	Abcam	Cat#ab150155; RRID: AB_2813835
Alexa donkey anti-rat 488	Invitrogen	Cat#a21208; RRID: AB_2535794
Alexa donkey anti-goat 488	Invitrogen	Cat#A11055; RRID: AB_2534102
Alexa donkey anti-goat 555	Invitrogen	Cat#A21432; RRID: AB_2535853
Alexa donkey anti-goat 647	Invitrogen	Cat#A21447; RRID: AB_141844
Alexa goat anti-hamster 488	Invitrogen	Cat#A21110; RRID: AB_141509
Chemicals, peptides, and recombinant proteins		
Tamoxifen	Sigma-Aldrich	Cat#T5648
PBS	Invitrogen	Cat#C10010500BT
HBSS	Invitrogen	Cat#14175103
UltraPure Distilled Water	Invitrogen	Cat#10977023
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat#P6148-1KG
Sucrose	Sigma-Aldrich	Cat#V900116
Gelatin	Solarbio	Cat#G8061
O.C.T.	Sakura	Cat#4583
Donkey serum	Abcam	Cat#ab7475
Triton X-100	Sigma-Aldrich	Cat#Sigma-X-100
DAPI	Sigma-Aldrich	Cat#D21490
EDTA	Sigma-Aldrich	Cat#798681-500G
PVP360	Sigma-Aldrich	Cat#PVP360-100G
Fast Green solution	Sigma-Aldrich	Cat#F7252

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Acetic acid	Sangon	Cat#A501931
Safranin O solution	Sigma-Aldrich	Cat#S2255
Glutaraldehyde	Sigma-Aldrich	Cat#G5882
Urea	Sigma-Aldrich	Cat#U5378
Glycerol	Sigma-Aldrich	Cat#G5516
Collagenase A	Roche	Cat#10103586001
FBS	Thermo Fisher	Cat#10099141c
DMSO	Sigma-Aldrich	Cat#D5879
Ethyl cinnamate (ECi)	Sigma-Aldrich	Cat#112372-100g
Polyethylene glycol (PEG)	Sigma-Aldrich	Cat#409529
Methanol	Sigma-Aldrich	Cat# 34860-2L-R
Ethanol	Sigma-Aldrich	Cat#E7023
Tamoxifen	Sigma-Aldrich	Cat#T5648

Deposited data

Single cell RNA-seq	Biswas et al. ¹⁶	ArrayExpress (E-MTAB-11560)
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Experimental models: Organisms/strains

Mouse: <i>Prox1-2A-CreER</i>	Han et al. ³⁴	N/A
Mouse: <i>Prox1-RSR-CreER</i>	Han et al. ³⁴	N/A
Mouse: <i>Cdh5-Dre</i>	Pu et al. ⁵⁴	N/A
Mouse: <i>Cdh5-DreER</i>	Pu et al. ⁵⁴	N/A
Mouse: <i>Cdh5-CreER</i>	Liu et al. ⁵⁵	N/A
Mouse: <i>R26-tdT</i>	Madisen et al. ⁵⁶	N/A
Mouse: <i>R26-RL-tdT</i>	Madisen et al. ⁴⁰	N/A
Mouse: <i>R26-Pik3ca-tdT</i>	This paper	Shanghai Biomodel Organism Co., Ltd

Oligonucleotides

Primers for genotyping	This paper	N/A
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Software and algorithms

ImageJ	NIH	https://ImageJ.net/software/fiji/
GraphPad Prism 8 software	GraphPad Software, Inc.	https://www.graphpad.com
PhotoLine 23.02	N/A	https://www.pl32.com/
Imaris	Bitplane	http://www.bitplane.com/Imaris/Imaris
AnnData	Virshup et al. ⁵⁷	https://anndata.readthedocs.io/en/latest/index.html
CellRanger	Illumina	https://www.10xgenomics.com/support/software/cell-ranger/latest
Leiden Clustering	Traag et al. ⁵⁸	https://github.com/vtraag/leidenalg
Matplotlib	Hunter et al. ⁵⁹	https://matplotlib.org/
Scanpy	Wolf et al. ⁶⁰	https://scanpy.readthedocs.io/en/stable/
Scrublet	Wolock et al. ⁶¹	https://github.com/swolock/scrublet
Scran	Lun et al. ⁶²	https://bioconductor.org/packages/release/bioc/html/scrman.html
STAR version 2.7.10a	Dobin et al. ⁶³	https://github.com/alexdobin/STAR

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

All mouse experiments used in this paper were performed strictly following the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences. *Prox1-2A-CreER*, *Prox1-RSR-CreER*, *Cdh5-Dre*, *Cdh5-CreER*, *R26-tdT*, and *R26-RL-tdT* mice lines were described previously.^{34,40,54–56} All adult experiments were performed on 8–20 weeks. All mice were kept in a C57BL6/ICR mixed background. Both male and female mice were

randomly selected for experiments. The *R26-Pik3ca-tdT* mouse was generated by targeted knock-in of CAG-loxP-Stop-loxP-Pik3ca^{H1047R}-IRES-Luciferase-P2A-tdT-WPRE-bGHpolyA cassette into the Rosa26 locus by homologous recombination using CRISPR/Cas9. All mice were fed the normal diet at the Specific Pathogen Free (SPF) animal laboratory of the Center for Excellence in Molecular Cell Science. The *Prox1-2A-CreER;R26-tdT* and *LEC-iCre;R26-RL-tdT* mice received Tam (Sigma, T5648 or ABCONE, T56488) administered by oral gavage at 0.2 mg/g body weight daily for 5 consecutive days. The *Cdh5-DreER;Prox1-RSR-CreER;R26-Pik3ca-tdT* and *Cdh5-CreER;R26-tdT* mice were treated with the same dosage of Tam (0.2 mg/g) for 3 consecutive days. The number of mice used for each experimental strategy is indicated in the figures. Genotyping PCR primer designs, their sequences, and genotyping conditions were provided in [Figure S5](#). We thank Shanghai Model Organisms Center for the generation of transgenic mouse lines.

METHOD DETAILS

Genomic PCR

Genomic DNA was extracted from the toes of transgenic mice. The toes were digested in lysis buffer (100 mM Tris HCl, pH 7.8, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 mg/mL proteinase K) for at least 5 h at 55°C. The samples were then centrifuged at maximum speed (21,130 x g) for 4 minutes to collect supernatants containing genomic DNA. DNA was precipitated using isopropanol, washed with 70% ethanol, and air-dried. Finally, the DNA was dissolved in deionized water for subsequent PCR amplification.

Irradiation and bone marrow transplantation

The mice underwent lethal whole-body irradiation with two doses of 1080 rad, administered at least 2 hours apart. Simultaneously, bone marrow (BM) cells were isolated from C57BL/6J mice and prepared in sterile PBS containing 2% fetal bovine serum. A total of 1x10⁶ BM cells were injected into the tail vein of each irradiated mouse 4-6 hours post-irradiation. Following irradiation, the mice were continuously treated with enrofloxacin in their drinking water for 2 weeks, after which they were switched to standard water.

Mouse femoral bone fracture model

Adult mice (>8 weeks) were anesthetized with isoflurane. The patella was laterally dislocated to expose the femoral condyles, after which an intramedullary pin was inserted to stabilize the femur. The fracture was then induced at the midpoint of the femur using a dentist's microdrill. Subsequently, the muscles were repositioned, and the skin was sutured to complete the procedure.

Whole-mount fluorescence microscopy

Collected mouse organs were washed in PBS and placed on agar for the whole-mount bright field and fluorescence imaging using the Zeiss stereo-scope (AxioZoom V16).

Tissue collection and immunostaining

For adult bone samples, tissue preparation was performed as previously described.^{16,41} Bone were dissected and fixed in 2% paraformaldehyde (PFA) in PBS for 4-8 h at 4°C. Afterwards, the samples were washed three times in PBS, followed by incubation in 0.5M EDTA (pH 7.4) for at least 3 days for decalcification. After additional PBS washes, the samples were dehydrated in 20% sucrose and 2% polyvinylpyrrolidone (PVP360) for 48 hours. They were then embedded in 20% sucrose, 2% polyvinylpyrrolidone, and 8% gelatin, and stored at -80°C until cryosectioning. Frozen sections (25-35 μm thick) were collected on slides for further analysis. For other tissues, samples were collected in 6-well plates and fixed in 4% PFA for 1 hour at 4°C. After three PBS washes, the tissues were dehydrated overnight in 30% sucrose dissolved in PBS at 4°C. The tissues were then immersed in O.C.T. compound for 1-5 hours at 4°C before being embedded in blocks for storage at -80°C. Frozen sections, 10-12 μm thick, were collected on slides for further analysis.

For immunostaining, the protocol was followed as previously described.⁶⁴ Briefly, slides were dried at room temperature and then washed several times in PBS. The sections were then blocked for 30 min at room temperature in 5% PBSST buffer (0.1% Triton X-100 and 5% donkey serum in PBS). After blocking, the slides were incubated with primary antibodies overnight at 4°C. Primary antibodies used in this study include tdTomato (Rockland, 600-401-379, 1:1000), tdTomato (Rockland, 200-101-379, 1:500), E-cadherin (R&D system, AF748, 1:500), PDPN/T1a (DSHB, 8.1.1, 1:100), Troponin I (Abcam, ab56357, 1:200), mPeriostin (R&D system, AF2955, 1:200), CD31/PECAM (R&D system, AF3628, 1:200), LYVE1 (Abcam, ab14917, 1:100), PROX1 (Abcam, ab101851, 1:100), PROX1 (R&D, AF2727, 1:100), NeuN (Merck, MAB377X, 1:200), CD45 (eBioscience, 11-0451, 1:100), EMCN (Santa Cruz, sc-65495, 1:100), HNF4a (Cell Signalling, 3113s, 1:500). In the following day, after washing the slides with PBS to remove the primary antibodies, the sections were incubated with Alexa fluorescence secondary antibodies (Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector lab). Finally, the slides were mounted with a mounting medium after PBS washing. The secondary antibodies used in this study include Alexa donkey anti-rabbit 555 (Invitrogen, A31572), Alexa donkey anti-rabbit 488 (Invitrogen, A21206), Alexa donkey anti-rabbit 647 (Invitrogen, A31573), Alexa donkey anti-goat 488 (Invitrogen, A11055), Alexa donkey anti-goat 555 (Invitrogen, A21432), Alexa donkey anti-goat 647 (Invitrogen, A21447), Alexa donkey anti-mouse 647 (Invitrogen, A31571), Alexa donkey anti-rat 647 (Invitrogen, A21247), Alexa donkey anti-rat 488 (Invitrogen, A21208), Alexa goat anti-hamster 488 (Invitrogen, A21110). Immunostaining images were acquired by Zeiss confocal laser scanning microscope (LSM880), and Olympus confocal microscope (FV4000). Images were analyzed by ImageJ (NIH) software.

Safranin O/fast green staining of frozen sections

Frozen tissue sections were dried at room temperature and then washed several times in PBS. The slides were rinsed in PBS for 3 times following 5–10 minutes fixation in 4% PFA. Subsequently, the slides were sequentially immersed in the following solutions: hematoxylin for 5–8 minutes, running water for 5 minutes, 0.2% Fast Green solution (Sigma-Aldrich, F7252) for 3–5 minutes, 1% acetic acid (Sangon, A501931) in water for 10 seconds, and 0.5% Safranin O solution (Sigma-Aldrich, S2255) for 5 minutes. The slides were subsequently dehydrated, cleared, and mounted following the previously described protocol. Microscopic images were captured using microscope (Olympus).

H&E staining

H&E staining was performed on frozen tissue sections according to standard protocols. Briefly, sections were air-dried at room temperature and fixed in 4% paraformaldehyde (PFA) for 5–10 minutes. After fixation, slides were washed three times in phosphate-buffered saline (PBS). Nuclei were then stained by immersion in hematoxylin for 5 minutes. Following a thorough rinse in running water, sections were differentiated in 1% acid alcohol (1% HCl in 75% ethanol) for 1 minute and blued in 1% ammonia water for 1 minute, with a water wash after each step. Cytoplasm was counterstained in eosin for 10–20 seconds, followed by a brief rinse in 75% ethanol. Finally, sections were dehydrated through a graded ethanol series (95% and 100% ethanol, twice for 2 minutes each) and cleared in two changes of xylene (5 minutes each). Coverslips were applied with a neutral mounting medium for microscopic analysis.

Bone fixation, decalcification, immunostaining, and clearing for light sheet imaging

The bone sample preparation and whole-bone clearing protocol were performed as previously described.¹⁶ In brief, bones were scraped to remove the attached adjoining muscles and fat, then fixed for 2.5 hours in an ice-cold, freshly prepared fixative solution comprising 4% (w/v) PFA and 0.05% (v/v) glutaraldehyde. Afterward fixation, the bones were washed three times with PBS at room temperature and decalcified in 0.5 M EDTA (pH = 7.4) for 24 h under constant rotation, followed by three additional PBS washes. After decalcification, the bones were dehydrated in an ice-cold ethanol gradient (50%, 80%, and 100%) for 30 minutes each. Subsequently, the bones were immersed in 5% (v/v) H₂O₂ for 2 hours for bleaching followed by rehydration with a decreasing ethanol gradient and three PBS washes (20 minutes each). The bones were then immersed in an ice-cold antigen retrieval and permeabilization solution containing 25% (w/w) urea, 15% (w/w) glycerol, 15% (w/w) Triton X-100, and 45% (w/v) double distilled water, and incubated for 5 hours at 4 °C. Following this, the bones were placed in 0.2% (w/v) collagenase in PBS for enzyme-based matrix digestion at 37 °C for 30 min on constant shaking. Finally, the samples were washed twice in PBS containing 2% fetal bovine serum for 5 minutes each.

For immunostaining and clearing of whole bone, samples were washed with a wash buffer containing 10% (v/v) donkey serum (Abcam, ab7475), 10% (v/v) DMSO (Sigma-Aldrich, D5879), and 0.5% (v/v) Triton X-100 at 37 °C for 30 minutes. They were then incubated with primary antibodies in an antibody dilution buffer containing 2% (v/v) donkey serum, 10% (v/v) DMSO, and 0.5% (v/v) Triton X-100 in PBS for 20 hours at 37 °C on a rocker platform shaking at 70 rpm. After primary antibody incubation, the samples were washed with a wash buffer containing 2% (v/v) donkey serum and 0.5% (v/v) Triton X-100 in PBS for 3 hours at 37 °C on a rocker platform shaking at 70 rpm. The wash buffer was changed every 15 minutes for the first hour, then every 30 minutes thereafter. Following the wash step, the samples were incubated with secondary antibodies for 6–8 hours at 37 °C on a rocker platform shaking at 70 rpm, followed by the same washing procedure as with the primary antibodies.

For clearing, the samples were dehydrated in an increasing ethanol gradient (30%, 50%, and 80%) for 30 minutes each under gentle rotation at room temperature. The samples were then subjected to 100% methanol for three washes, 20 minutes each. After methanol removal, the samples were rinsed twice with ethyl cinnamate (ECi) for 5 minutes each. The bones were subsequently cleared using a solution containing 80% (v/v) ECi and 20% (v/v) polyethylene glycol (PEG) under gentle rotation at room temperature for 30–60 minutes. Finally, the bones were placed in ECi for imaging. For light sheet imaging set-up and image acquisition, the cleared samples were imaged on a Zeiss Lightsheet Z.1. microscopy or LiTon LBS Light-sheet Microscope (Light Innovation Technology Limited) with Olympus XLFLUOR4X/340 objective lens (N.A. = 0.28). 3D image data processing was performed using Imaris software (Bitplane, <http://www.bitplane.com/Imaris/Imaris>).

scRNA-seq analysis

Raw sequencing reads were downloaded from ArrayExpress (E-MTAB-11560) and mapped against the Gencode reference genome GRCh38.p13 with genome annotations v35 (filtered for protein-coding, lncRNA, IG*, and TR* genes).

STAR version 2.7.10a⁶³ was used with the parameters “--soloType Droplet --soloUMIlen 12 --soloCellFilter None --soloCBwhitelist 10xv3_whitelist.txt --outSAMtype BAM SortedByCoordinate --soloFeatures GeneFull_Ex50pAS --soloMultiMappers EM --runRNG-seed 1 --outMultimapperOrder Random”. Whitelist was obtained from Illumina Cell Ranger.

Raw counts were imported as AnnData⁵⁷ objects. We removed low-complexity barcodes with the knee plot method, and further filtered out cells with a total contribution above 20% of reads belonging to mitochondrial mRNA, over 10,000 detected genes, or more than 15,000 counts. Doublets were predicted with Scrublet.⁶¹ Finally, each sample's gene expression matrix was normalized using Scanr⁶² (1.22.1) with Leiden clustering⁵⁸ input at resolution 0.5.

At this stage, samples were merged. For 2D embedding, the expression matrix was subset to the 3,000 most highly variable genes using Scanpy⁶⁰ (1.9.1, <<https://doi.org/10.1186/s13059-017-1382-0>>) (sc.pp.highly_variable_genes, flavor “seurat”). The top 100 principal components (PCs) were calculated and served as basis for k-nearest neighbor calculation (sc.pp.neighbors, n_neighbors=30), which were used as input for UMAP (<https://doi.org/10.48550/arXiv.1802.03426>) layout (sc.tl.umap, min_dist=0.3).

Leiden clustering was performed at resolution 0.2, and clusters were annotated based on known major cell type markers as described in the main text. Finally, expression of select marker genes was plotted using Matplotlib⁶⁹ (3.8.4) “imshow”, and clusters were annotated accordingly.

Statistical analysis

All the data were obtained from multiple individual biological mouse samples, as indicated in each Figure legend, and are presented as the means $\pm$ SD. Statistical analysis was performed using the Shapiro–Wilk test for normality. For two-group comparisons, normally distributed data were analyzed using unpaired two-tailed Student’s *t*-tests (with equal variance assumed) or Welch’s *t*-tests (with unequal variance assumed), while non-normally distributed data were analyzed using Mann-Whitney U tests. To control for multiple comparisons across multiple pairwise tests, the Bonferroni correction was applied. *P* < 0.05 was considered to be statistically significant. Each image in the Figures is representative of at least 5 individual mice. Data statistics were performed through ImageJ software.

Supplemental information

**Lymphatic vessels invade bone in disease
but are absent in health and regeneration**

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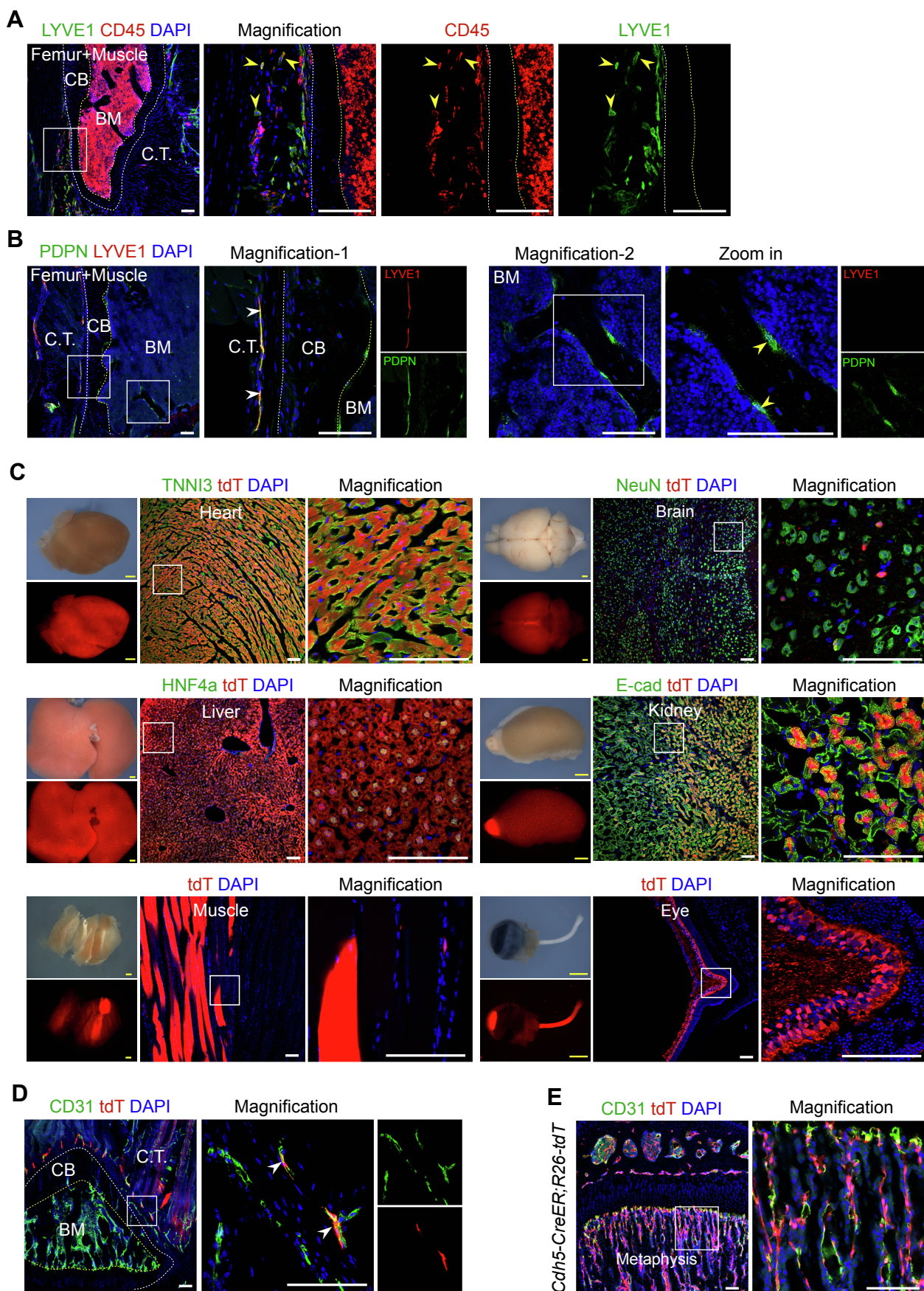


Figure S1. *Prox1-2A-CreER* labels non-LEC lineages, related to Figure 1

(A) Immunostaining for LYVE1 and CD45 on femur sections (with connective tissues). Arrowheads, LYVE1+CD45+ immune cells.

(B) Immunostaining for LYVE1 and PDPN on femur sections (with connective tissues). White arrowheads, LYVE1+PDPN+ LECs; yellow arrowheads, LYVE1PDPN+ non-LECs.

(C) Immunostaining for tdT and cell lineage markers TNNI3, NeuN, HNF4a, and E-cad on tissue sections from *Prox1-2A-CreER;R26-tdT* mice treated with Tam.

(D) Immunostaining for tdT and CD31 on tibia sections (with connective tissues) from *Prox1-2A-CreER;R26-tdT* mice treated with Tam. Arrowheads, tdT+ LECs.

(E) Immunostaining for tdT and CD31 on tibia sections from *Cdh5-CreER;R26-tdT* mice treated with Tam.

Yellow bars, 1 mm; white bars, 100 μ m. CB, cortical bone. BM, bone marrow. C.T., connective tissues.

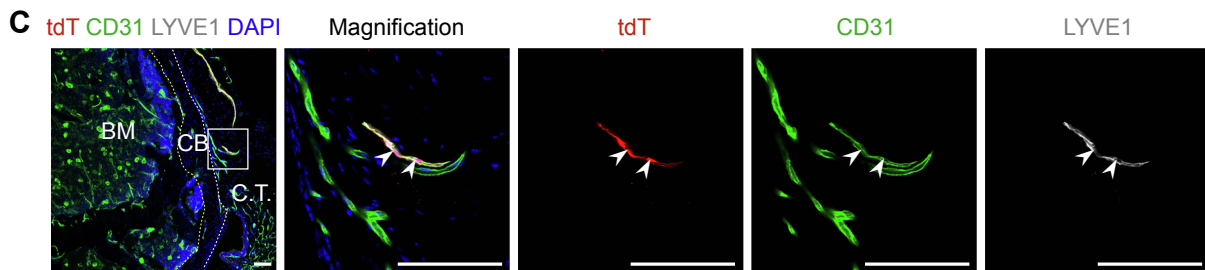
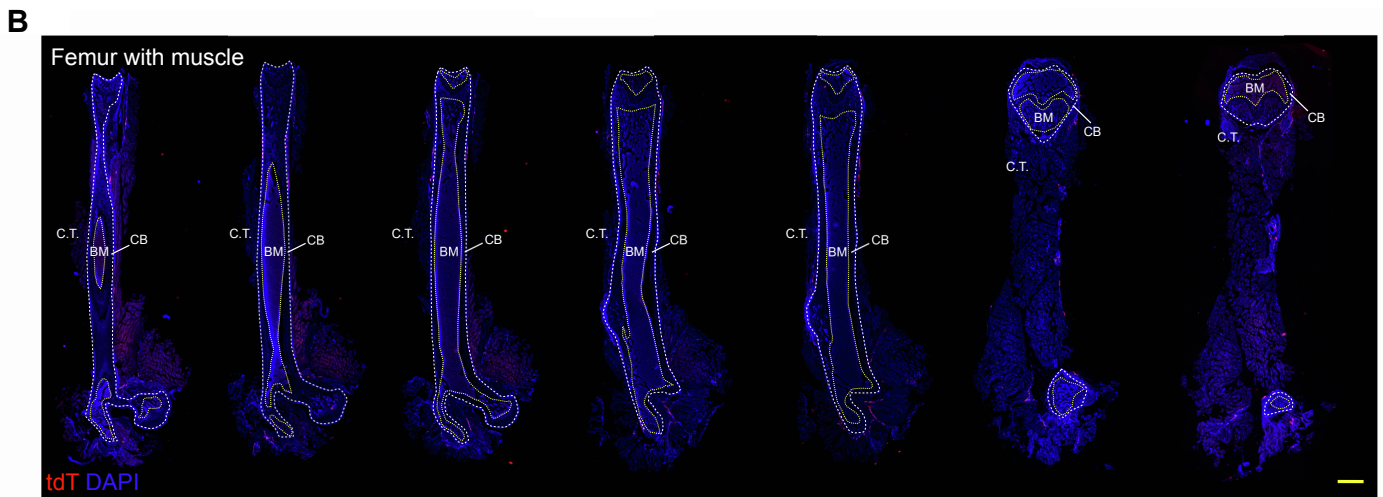
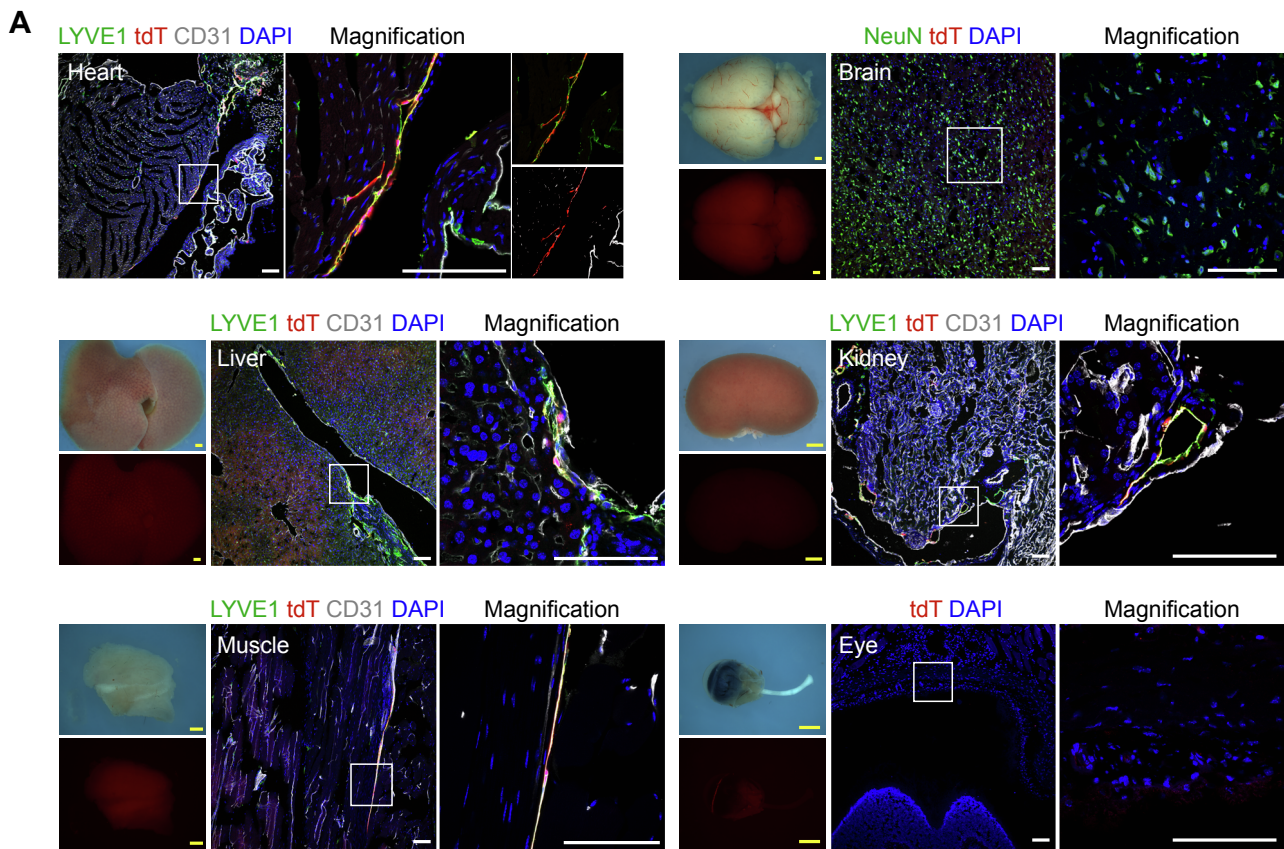
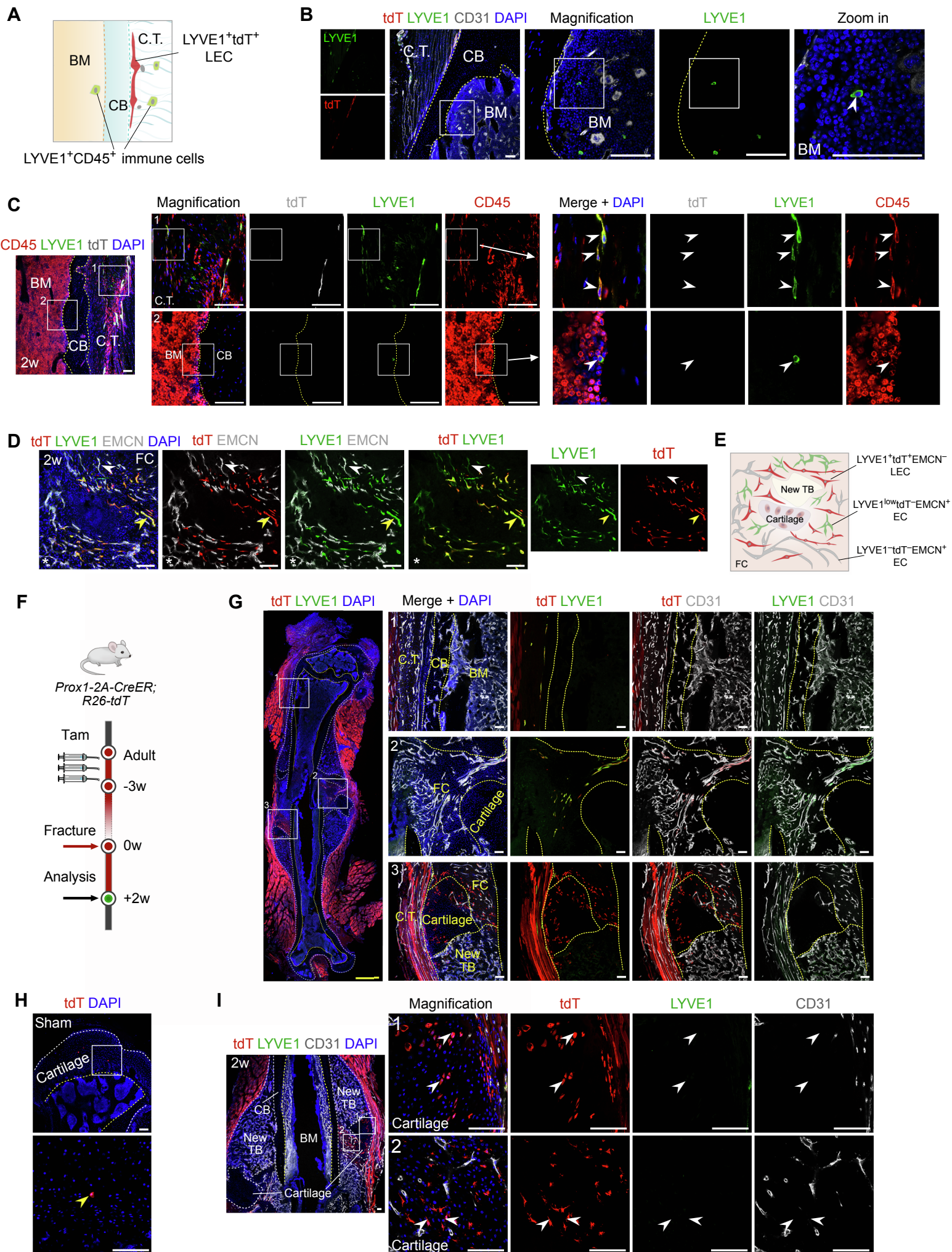


Figure S2. *LEC-iCre* specifically labels lymphatic vessels in tissues outside the bone, related to Figure 2

(A) Immunostaining for tdT, CD31, LYVE1, NeuN on tibia serial sections (with muscle) from *LEC-iCre;R26-RL-tdT* mice treated with Tam.

(B) Immunostaining for tdT on femur serial sections (with muscle) from *LEC-iCre;R26-RL-tdT* mice treated with Tam.

(C) Immunostaining for tdT, CD31, and LYVE1 on bone sections from *LEC-iCre;R26-RL-tdT* mice treated with Tam. Arrowheads, tdT+ LECs. Yellow bars, 1 mm; white bars, 100 μ m. CB, cortical bone. BM, bone marrow. C.T., connective tissues.



(legend on next page)

Figure S4. Lymphatic vessels do not invade the bone following fracture healing, related to Figure 5

(A) A cartoon image showing LYVE1+CD45+tdT immune cells in the connective tissue and bone marrow after fracture.

(B) Immunostaining for tdT, LYVE1, and CD31 on femur sections from 2w post-fracture *LEC-iCre;R26-RL-tdT* mice (with connective tissues). Arrowheads, LYVE1+CD31 non-LECs.

(C) Immunostaining for tdT, LYVE1, and CD45 on femur sections from 2w post-fracture *LEC-iCre;R26-RL-tdT* mice (with connective tissues). Arrowheads, LYVE1+CD45+ immune cells.

(D) Immunostaining for tdT, LYVE1, and EMCN on femur sections from 2w post-fracture *LEC-iCre;R26-RL-tdT* mice (with connective tissues). White arrowheads, tdTLYVE1lowEMCN+ ECs; yellow arrowheads, tdT+LYVE1+EMCN LECs.

(E) A cartoon image showing tdT+LYVE+EMCN LECs, tdT+LYVE1lowEMCN+ capillary vascular ECs that upregulate the LYVE1 expression after fracture, and the tdTLYVE1EMCN+ capillary ECs in fibrous callus.

(F) A schematic diagram illustrating the experimental design.

(G) Immunostaining for tdT, LYVE1, and CD31 on femur sections from 2w post-fracture *Prox1-2A-CreER;R26-tdT* mice (with connective tissues).

(H) Immunostaining for tdT on femur sections from *Prox1-2A-CreER;R26-tdT* mice (sham).

(I) Immunostaining for tdT, LYVE1, and CD31 on femur sections from 2w post-fracture *Prox1-2A-CreER;R26-tdT* mice (with connective tissues). Arrowheads, tdT+LYVE1CD31 non-LECs.

Yellow bars, 1 mm; white bars, 100 μ m. CB, cortical bone. BM, bone marrow. C.T., connective tissues. P, periosteum. FC, fibrous callus. P&FC, periosteum and fibrous callus. New TB, new trabecular.

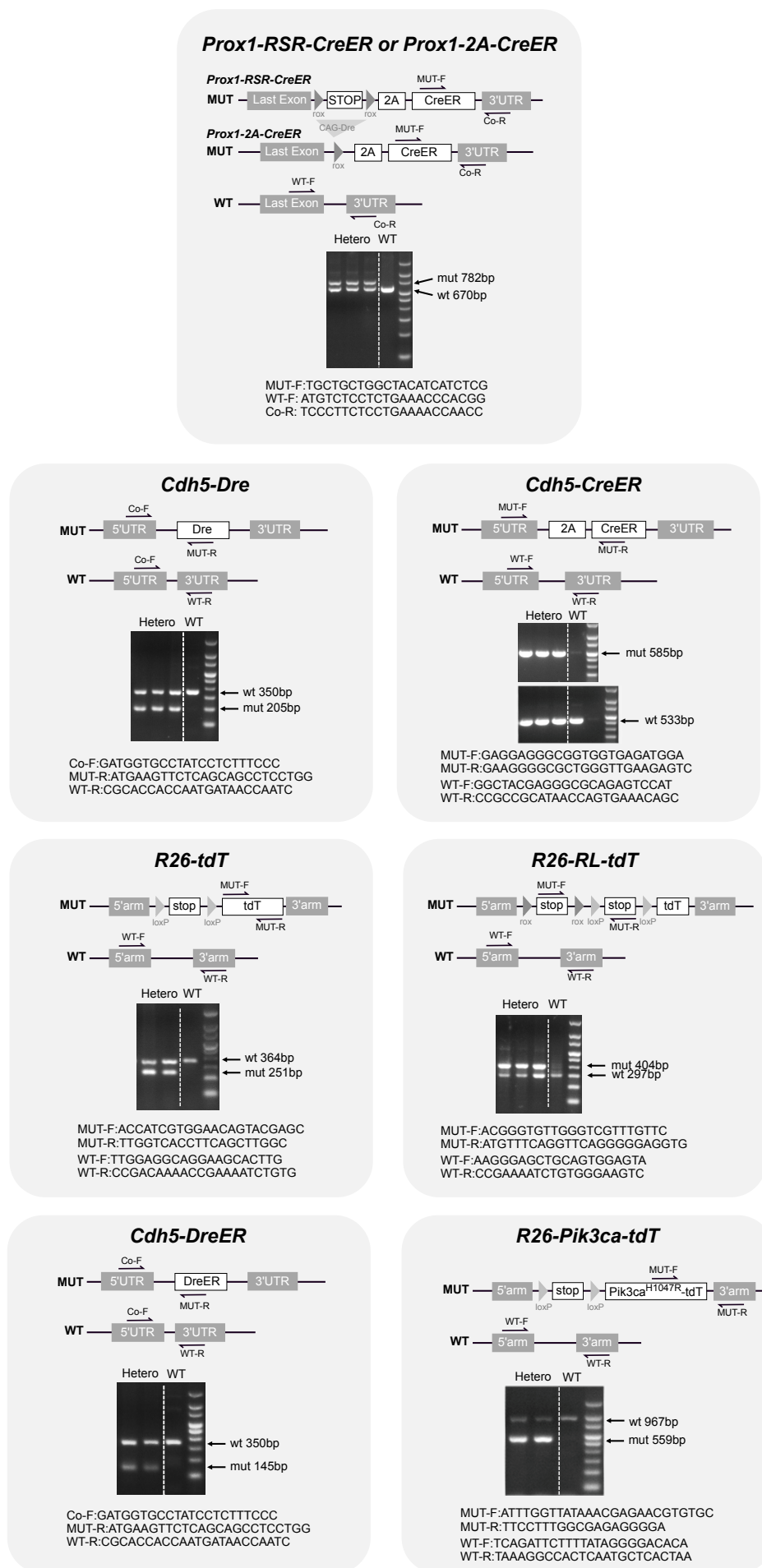


Figure S5. Genotyping information for all mouse lines used in this study, Related to the STAR Methods – Mice section

The representative results of PCR genotyping, primer design, and primer sequences for all mouse lines used in the study. Hetero, heterozygote; MUT, mutant allele; WT, wild type allele; MUT-F, forward primer for detecting mutant allele; MUT-R, reverse primer for detecting mutant allele; WT-F, forward primer for detecting wild type allele; WT-R, reverse primer for detecting wild type allele; Co-F, common forward primer; Co-R, common reverse primer.