

CXCL12+ cells in periosteum are the main origin of osteoblasts critical for fracture healing with Masquelet technique

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INTRODUCTION: Treatment of segmental critical bone defects still represents a major clinical challenge. The Masquelet technique (MT) is a two-staged approach that has been considered as a promising strategy for reconstructing large bone defects. It is broadly agreed on that an induced membrane around the PMMA spacer, which is generated after the first stage surgery, promotes bone healing, and that this membrane is indispensable after the second stage surgery. However, the cellular mechanism of this technique is not well understood. We developed a mouse Masquelet model and demonstrated that periosteal CXCL12+ cells are the origin of osteoblasts that are critical for the success of this technique.

METHODS: All experiments were approved by local IACUC.

Masquelet model: A lateral approach was used to expose the femoral midshaft. A 3-mm osteotomy on the midshaft was made with a high-speed diamond saw to induce a critical size defect. To mimic the first-stage surgery of the Masquelet technique, a 3-mm long cylindrical Polymethylmethacrylate (PMMA) spacer was put into the defect gap. A rod made from a 24-gauge needle was inserted through the center of the spacer and into the medullary canals of each side of the fractured femur. The rod not only kept the spacer in place but also served as an internal fixator of the fractured femur. The muscle and skin were then closed in layers. To mimic the second-stage surgery of the Masquelet technique, the spacer was removed with the same lateral approach 3 weeks after the first-stage surgery. The membrane pocket around the spacer was carefully protected when it was opened for the spacer removal. A cancellous iliac bone graft of donor mice was inserted into the membrane cavity left after spacer removal. The same rod used in the first-stage surgery was used to internally fixate the fracture. Mice were euthanized 6 weeks after the second-stage surgery.

The role of periosteum: CXCL12-CreERT2;TdTomato;CXCL12-GFP mice, in which all the cells that had ever expressed CXCL12 were marked with GFP, and CXCL12-expressing cells, which were marked with TdTomato reporter upon tamoxifen (2mg, I.P.) injection at 7 and 3 days prior surgeries, on the day of surgery, and 3 and 7 days after surgeries, underwent the first-stage surgery on the right femur. Periosteum on the remained shaft of the fractured femur was removed by surgical scalpel or preserved without disruption (n=12/group) before the skin and muscle closure. At 3 weeks after the first-stage surgery, 6 mice in each group were sacrificed for histology and biofluorescence analysis while the other 6 mice underwent identical second-stage surgeries.

The role of CXCL12-expressing cells: CXCL12-CreERT2;TdTomato;iDTA mice were used to ablate CXCL12-expressing cells. The ablation was induced by tamoxifen given in the same intervals as described above. CXCL12-Cre; Ai14 mice were used as controls (n=8/group). Mice in both groups underwent the first-stage surgery on both femurs without periosteum disruption. In each group, 4 mice were sacrificed at 3 weeks after the first-stage surgery and the other 4 mice underwent the second-stage surgery.

Faxitron radiograph imaging was used to monitor the progress of fracture healing. All mice were euthanized 4 or 6 weeks after the second surgery. MicroCT analysis was used to measure the bone volume fraction at the fracture gap. Histology and immunofluorescence were used to analyze fracture healing status and cellular components at the fracture site.

Statistical analysis: Student's T-test was used. A P<0.05 was considered as significant.

RESULTS: Serial radiographs and histology analysis showed that a membrane pocket formed and partially converted to new bone around the spacer over the 3 weeks after the first-stage surgery (**Figure 1A and B**) in periosteum preserved mice. CXCL12+ cells were abundant in this membrane (**Figure 1C**). Conversely, in the mice after periosteum removal, no callus bridge was formed and the soft tissue around the spacer had minimal CXCL12+ cells (**Figure 1D, E and F**). Furthermore, the periosteum removal at first-stage surgery caused the non-union of 6 out of 6 mice at 6 weeks after the second-stage surgery while only 1 out of 6 mice with periosteum preserved had non-union (**Figure 2A, B, and C**). The bone volume fraction (BV/TV) measured by microCT (**Figure 2D**) at the fracture site was significantly lower in the periosteum removal group. Similarly, CXCL12 cell ablation caused non-union in 4 out of 8 mice while only 2 out of 8 mice in the controls showed non-union at 4 weeks after second surgery. The ablation also decreased the BV/TV at the fracture gap.

DISCUSSION: We have established a reproducible mouse Masquelet model. CXCL12+ cells in the periosteum are the origin of osteoblasts in the membrane induced around the PMMA spacer after the first-stage surgery, which are critical for fracture healing after the second-stage surgery.

SIGNIFICANCE/CLINICAL RELEVANCE: This mouse model can be used to further study the cellular and molecular mechanisms of fracture healing by taking the advantage of numerous genetically engineered mouse lines. CXCL12+ cells can be a potential target to enhance bone formation after a critical size bone defect.

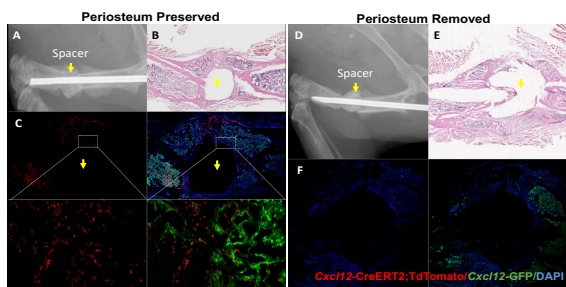


Figure 1. Radiograph (A, D), histological images (H&E, B, E), and fluorescence images 21 days after the first-stage surgery of the Masquelet model, showing the bone callus and membrane around PMMA spacer and abundant CXCL12+ cells in membrane in the periosteum preserved mice and the lack of them in the periosteum removed mice.

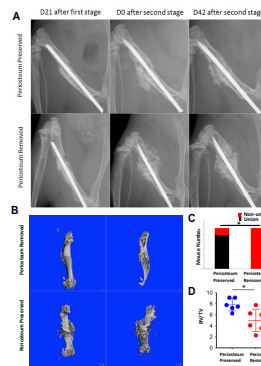


Figure 2. Faxitron (A) and micro-CT (B) images showing the union or non-union in mice with periosteum preserved or removed 6 weeks after the second-stage surgery. Periosteum removal led to 6/6 non-union compared with the 1/6 in mice with periosteum preserved (C). Bone volume fraction (BV/TV) was significantly higher in the mice with periosteum preserved (D).

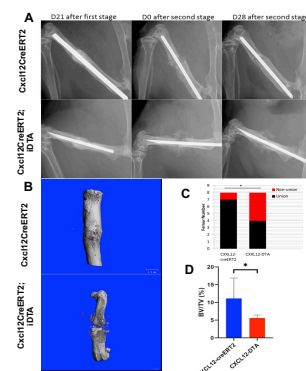


Figure 3. Faxitron (A) and micro-CT (B) images showing the union or non-union in Cxcl12-CreERT2 mice or Cxcl12-CreERT2;iDTA mice 28 days after second stage surgery. Ablation of CXCL12+ cells led to 4/8 non-union compared with the 2/8 in the controls (C). Bone volume fraction (BV/TV) was significantly lower in mice with CXCL12 cell ablation (D).