Macrophages play important roles in fracture healing, where complete depletion of macrophages leads to impaired healing (Vi, L., et al., 2015). Macrophages found at fracture sites have different anatomical origins. Previous studies showed that spleen is a reservoir of yolk sac-derived tissue resident macrophages, which can migrate to the fracture site (Yahara, Y., et al., 2020). Other studies revealed that splenectomy can lead to delayed fracture healing (Xiao, W., et al., 2018). These data suggest that spleen macrophages (SMs) could be a potential reservoir. Here we demonstrate that depletion leads to impaired healing. However, the role and function of SMs during fracture healing remain unknown. Therefore, we hypothesize that SMs migrate to the fracture site to facilitate osteogenesis during fracture healing.

METHODS
The animal works mentioned in this study are approved by the institutional IACUC. To examine the function of SMs in fracture healing, the spleen was transplanted surgically from the conditional macrophage depleted MaFIA (Macrophage Fas Induced Apoptosis) mice to control (non-transgenic) littermates. The MaFIA mice allows inducible apoptosis of macrophages using the mouse colony stimulating factor 1 receptor promoter (Csf1r) to drive expression of a mutant human death receptor, FAS. The transgene also allows EGFP labeling of Csf1r-expressing cells, which can be used to detect macrophages. Administration of AP20187 causes apoptosis of MaFIA SMs in mice after transplant (Figure 1).

The presence of GFP+ SMs was examined by Fluorescence-activated Cell Sorting (FACS) analysis in transplanted animals 3 day post fracture (dpf) and 7dpf. The fractured and contralateral limbs were examined. The recipient control mice received a transverse mid-diaphyseal tibia fracture at the date of the last AP20187 or vehicle (control) dose. On 14dpf and 21dpf, the fractured limbs and their contralateral limbs were collected to examine the phenotype with Microcomputed tomography (microCT) and histomorphometry using analysis after Safranin-O staining and TRAP staining.

RESULTS
We successfully established the spleen transplant procedure in a fractured mouse model using mice of 3-4 month old and confirmed the long-term blood perfusion of transplanted spleens by contrast ultra-sonography. Our FACS results demonstrated that around 0.5% GFP+ cells to total live cells were found at fracture sites in the WT recipient mice, at 3dpf (Figure 2).

To study the role of SMs, we used the aforementioned model to deplete SMs and examined the fractured limbs at 14dpf and 21dpf. The bone morphometry results demonstrated that depleting SMs resulted in more fibrous tissues and less bone volume on 14dpf and, bone morphology on 21dpf. In addition, TRAP staining results showed that the number TRAP+ multinucleated osteoclasts (N.Oc/T.A, Oc.S/B.S) is significantly increased with SMs depletion (Figure 3).

DISCUSSION
Here we demonstrated that SMs migrate to a fracture site and contribute to osteogenesis. Since macrophages can be osteoclast progenitors and the spleen could be a potential reservoir of such cells, we expected to see decreased osteoclast numbers. Surprisingly, SMs depletion leads to an increase in osteoclast numbers and a trend towards increases osteoclast surface area at 14dpf. These findings suggest that the contribution of SMs, results in a compensatory increase in osteoclast of hematopoietic origin.

SIGNIFICANCE
Our findings show that SMs play an important role in osteogenesis during fracture healing, suggesting an important reparative function for a novel subpopulation of macrophages cells.