INTRODUCTION
Bone defect healing is a progenitor cell-driven tissue morphogenetic process characterized by coordinated osteogenesis and angiogenesis at the site of repair. The progenitor cells dynamics and spatiotemporal regulation of bone defect repair remain poorly understood, largely due to the lack of an animal model that permits high resolution and non-destructive analyses of progenitors and the surrounding microenvironment at the site of repair. A deeper understanding of the spatiotemporal regulation of progenitor cell interaction with the bone healing microenvironment is critically important for developing novel material-based approaches aimed at modulating progenitor cell behavior for enhanced bone defect repair.

Multiphoton laser scanning microscopy (MPLSM) has emerged as a superior in vivo imaging modality for analyses of thick tissue in living animals. The key advantages of MPLSM include confocal-like imaging quality, reduced photo damage, enhanced imaging depths, and the convenient access to the entire emission spectrum. Multiphoton microscopy further permits morphological and functional analyses of neovascularization with unprecedented advantages of high spatio-temporal resolution, minimal invasiveness and 3D capability. Compared with other existing modalities (e.g. electrodes, MRI, Doppler), multiphoton has the resolution to image non-invasively and 3D capability. Compared with other existing modalities (e.g. electrodes, MRI, Doppler), multiphoton microscopy can be used for imaging collagen matrix through second harmonic generation (SHG).

We recently established a chronic cranial defect window chamber model in mice that permits high-resolution, dynamic tracking of progenitors and their surrounding neovascularization via multiphoton microscopy. Using a 2.3kb Collagen type I promoter driven GFP reporter transgenic mice, here we describe the spatial and temporal regulation of osteogenesis and angiogenesis during cranial defect repair and regeneration.

MATERIALS AND METHODS
Animal models: Col2.3GFP transgenic mice were purchased from the Jackson Laboratory. A 1.5 mm defect was created in the parietal bone of mouse calvarium using a stainless steel drill bit with defined sized and circular shape. To create a window chamber, a custom-made 0.5 mm-thick spacer made of titanium was attached onto skull. The defect region was carefully exposed through the subcapular region and glued onto the top of the spacer. A glass window was mounted on top of the wound for imaging. The wound was sealed to the edge of the open window with a thin layer of dental acrylic. The custom-made spacer was used for stabilization of the animal head for subsequent MPLSM. Multiphoton-Laser-Scanning Microscopy (MPLSM). An Olympus FV1000-AOM multiphoton imaging system equipped with a Titanium:Sapphire laser was used for live imaging of the cranial defect healing. SHG-based collagen matrix and GFP+ cells were visualized simultaneously using the excitation wavelength 780 nm to generate multiphoton excitation signals from GFP, and SHG signals from collagen at 390 nm. To visualize the blood vessel network, we performed intravenous injection of a purified anionic dextran and SHG signals from collagen at 390 nm. To visualize the blood vessel network, we performed intravenous injection of a purified anionic dextran conjugated to a Texas Red (Invitrogen) at a dose of 40 mg/kg. Red fluorescence from Texas Red can be detected using a red bandpass emission filter (Olympus, 607±36 nm). Using a 10x water immersion objective lens (Olympus), a 1.3 x1.3 mm multichannel z-series stack at 5μm step was obtained. The z-series stack allowed 3D reconstruction of the defect at a depth of 300μm (supplemental video).

Imaging analyses: Three-dimensional reconstruction and analyses of multichannel z-series image stack were performed using Amira image analysis software (Visage Imaging). A combination of image filters was used to improve the signal/noise ratio of the acquired images for analyses. The average vessel thickness and the associated vessel thickness distribution were calculated using an ImageJ plugin.

RESULTS AND DISCUSSION
To characterize dynamic skeletal healing processes, we performed time series analyses on bone defect healing in Col2.3 GFP reporter mouse model. A marked induction of GFP+ osteoblasts coupled with propagation of SHG+ collagen matrix was recorded over a period of 9 weeks. To examine the dynamic interactions between osteoblasts and microvascularity, we tracked Col2.3GFP+ osteoblasts simultaneously with neovascularization. A representative time series of GFP+ osteoblasts, SHG and neovascularization is shown in Fig. 1 to depict the progressive cranial defect healing Quantitative and histomorphometric analyses of neovascularization, simultaneously with volumetric quantification of Col2.3GFP osteoblasts and SHG at the site of defect repair were performed using images obtained at a higher magnification. As shown, at all-time points analyzed, osteoblasts at the healing front were closely associated with micro-capillary vessels that had an average diameter of 7.3±3.2 μm (n=50, Fig. 2E-H and 4I-L, circled region). These microvessels intertwined between layers of osteoblasts at the cellular invasion front, indicating a critical role of these microvessels in supporting the migration, proliferation and differentiation of osteoblasts in vivo. Quantitative analyses of SHG, osteoblasts and neovascularization (Fig. 4M-T) further showed the induction of osteoblast volume at week 2, accompanied by enhanced SHG propagation. Analyses of neovascularization showed angiogenesis induced at week 1, peaked at week 3 and reduced at week 6, preceding and coupling with organized osteogenesis. Remarkably, we found that vessel length and length fraction (density) are the most sensitive measurements for angiogenesis (Fig. 4O and P). A decrease in vessel length and length density preceded the reduction of osteoblast volume. The changes in vessel length could be attributed to the dynamic changes of micro-capillary vessels associated with osteoblasts at the site of repair (Fig. 4I-L, circled region).

In summary, we have successfully established a cranial defect window chamber model that permits dynamic, high-resolution, quantitative, and 3D analyses of neovascularization and bone matrix propagation at the site of the defect healing via MPLSM. Using osteogenem mouse-driven GFP reporter mice, we demonstrated for the first time a feasible approach to track the migration, proliferation and differentiation of osteoblast precursor cells in association with neovascularization in vivo. This study opened up ample opportunities to use available transgenic mouse models to understand healing and to delineate the regulatory mechanisms of progenitor cell interaction with the bone healing microenvironment. The establishment of a chronic defect window chamber model further permits the development of an in vivo imaging platform to evaluate novel material-based approaches aimed at enhancing bone defect repair and reconstruction.

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