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Spatiotemporal Analyses of Osteogenesis and Angiogenesis via Intravital Imaging in Cranial Bone Defect Repair

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Abstract

Osteogenesis and angiogenesis are two integrated components in bone repair and regeneration. A deeper understanding of osteogenesis and angiogenesis has been hampered by technical difficulties of analyzing bone and neovasculature simultaneously in spatiotemporal scales and in three-dimensional formats. To overcome these barriers, a cranial defect window chamber model was established that enabled high-resolution, longitudinal, and real-time tracking of angiogenesis and bone defect healing via Multiphoton Laser Scanning Microscopy (MPLSM). By simultaneously probing new bone matrix via second harmonic generation (SHG), neovascular networks via intravenous perfusion of fluorophore, and osteoblast differentiation via 2.3kb collagen type I promoter driven GFP (Col2.3GFP), we examined the morphogenetic sequence of cranial bone defect healing and further established the spatiotemporal analyses of osteogenesis and angiogenesis coupling in repair and regeneration. We demonstrated that bone defect closure was initiated in the residual bone around the edge of the defect. The expansion and migration of osteoprogenitors into the bone defect occurred during the first 3 weeks of healing, coupled with vigorous microvessel angiogenesis at the leading edge of the defect. Subsequent bone repair was marked by matrix deposition and active vascular network remodeling within new bone. Implantation of bone marrow stromal cells (BMSCs) isolated from Col2.3GFP mice further showed that donor-dependent bone formation occurred rapidly within the first 3 weeks of implantation, in concert with early angiogenesis. The subsequent bone wound closure was largely host-dependent, associated with localized modest induction of angiogenesis. The establishment of a live imaging platform via cranial window provides a unique tool to understand osteogenesis and angiogenesis in repair and regeneration, enabling further elucidation of the spatiotemporal regulatory mechanisms of osteoprogenitor cell interactions with host bone healing microenvironment.

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Keywords

osteogenesis; angiogenesis; intravital imaging; BMSC; cranial defect repair

INTRODUCTION

Bone defect healing is a dynamic progenitor cell-driven tissue morphogenetic process that requires coordinated osteogenesis and angiogenesis at the site of repair (1,2). The coordination between osteogenesis and angiogenesis is thought to be achieved by the complex interplay of auto- and paracrine factors produced by osteoblasts, endothelial cells and their precursors (3,4). While the essential role of osteogenesis and angiogenesis in bone defect repair has been established, the molecular and cellular interplay between bone forming and vessel forming cells has yet to be fully elucidated. Furthermore, due to the lack of an animal model that permits real-time, longitudinal, and high resolution analyses of osteogenesis and angiogenesis, our understanding of the spatiotemporal regulation of osteogenesis and angiogenesis during defect repair remains extremely limited. Since bone defect repair is a dynamic process controlled at multiple spatial and temporal scales, establishing the capability to analyze osteoprogenitor cell dynamics and their interaction with neovasculature is crucial for advancing our understanding of bone defect repair and for further optimizing material-based approaches to orchestrating spatiotemporal delivery of single or multiple cues capable of instructing both host and donor cells for improved bone defect reconstruction (5).

Multiphoton laser scanning microscopy (MPLSM) has emerged as a superior *in vivo* imaging modality for analyses of thick tissues in living animals (6,7). The key advantages of MPLSM include confocal-like imaging quality, reduced photo-damage, and enhanced imaging depth. Multiphoton microscopy further permits morphological and functional analyses of neovasculature with benefits of high spatiotemporal resolution, minimal invasiveness and 3D capability (8–11). In addition to imaging nonlinear fluorescence excitation, multiphoton microscopy can also be used for imaging bone matrix through second harmonic generation (SHG) (12,13). The unique capability of this technology that allows simultaneous visualization of cells, ECM, as well as the surrounding vascular networks, offers a superior imaging modality for dynamic, real-time and simultaneous analyses of osteogenesis and angiogenesis in bone tissue repair and regeneration.

The goal of our current study was to establish a MPLSM-based live imaging platform for real-time, non-destructive, and high resolution analyses of osteogenesis and angiogenesis in bone defect repair and regeneration. Utilizing a cranial defect window chamber model and an osteogenic-specific promoter-driven GFP reporter mouse model (Col2.3GFP), we demonstrated for the first time the spatiotemporal analysis of defect healing and osteogenesis and angiogenesis coupling at the site of cranial bone defect repair and regeneration. Our study highlighted the coordinated interactions between osteogenic and angiogenic compartments during repair and regeneration, further validating the use of MPLSM combined with the cranial defect window chamber model as a unique and novel tool for understanding bone defect repair and for delineating the molecular and cellular

interactions of the osteogenesis and angiogenesis coupling in bone defect repair and reconstruction.

MATERIALS AND METHODS

Animals and reagents

Col2.3GFP transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). NestinGFP mice were kindly provided by Dr. Grigori N. Enikolopov at Cold Spring Harbor Laboratories (14,15). Immunocompromised mice (bg-nu/nu-xid) were purchased from Harlan Sprague Dawley Inc. All surgical interventions were approved by the Institutional Animal Care and Use Committee at the University of Rochester.

Cranial defect window chamber model

The cranial window chamber model in mice has been previously reported for analyses of brain cell function and tumor-associated neovascularization (16,17). The model was further modified to meet the need for long-term tracking of defect healing via intravital imaging. Briefly, the surgical mouse was anesthetized with a mixture of Ketamine and Xylazine and placed on a stereotaxic frame (Stoelting Co. Wood Dale, IL) for microsurgery. To create a window chamber, a custom-made 0.5 mm-thick spacer made of poly (aryl-ether-ether-ketone) (PEEK) was glued onto skull using cyanoacrylate glue (Loctite, Cat #45404). The defect region was carefully exposed through a circular 6 mm (diameter) opening at the center of the spacer. A full thickness defect was created in the parietal bone of mouse calvarium using a tungsten vanadium inverted cone bur (Armstrong Tool & Supply Company, Livonia, Michigan) attached to a high-speed micro-drill (Stoelting Co. Wood Dale, IL). A circular cover-glass slip was gently lowered to cover the open-skull region and glued onto the top of the spacer. The wound was sealed to the edge of the optical window with a thin layer of dental cement (Stryker, Cat#0700-6-437, surgical grade). The custom-made spacer was used to position the animal head onto a platform for subsequent MPLSM imaging. A schematic illustrating the cranial defect window chamber model in mice is shown in Supplemental Fig. S1A&B. Once established, the mouse with a window chamber can survive over a 6-month period with no visible signs of distress.

The size of the defect was controlled using a 0.9 mm or 1.8 mm size Busch inverted cone bur (Armstrong Tool & Supply Company, Livonia, MI) which generates a 1 mm or 2 mm full thickness defect, respectively, in the parietal bone. The healing dynamics of 1 mm or 2 mm-sized defects was examined by MPLSM and microCT over a 3 month period. Although both defects failed to completely heal in the window chamber over a 3 month period (supplemental Fig. S2), 1 mm defects induced a more robust healing response at the leading edge of the defect, and therefore were used in the MPLSM analyses for osteogenesis and angiogenesis.

Multiphoton-Laser-Scanning Microscopy (MPLSM)

An Olympus FV1000-AOM multiphoton imaging system (Olympus), equipped with a Titanium:Sapphire laser (Spectra-Physics), a C-Apochromat 10X/0.45 (Zeiss), and a 25X/1.05 (Olympus) water immersion objective, was used for live imaging of the cranial defect

healing. Images were acquired at 512×512 pixels, 0.2 μs pixel dwell with the laser tuned to 780nm. The fluorescence of GFP and second harmonic signals (SHG) was collected with a 517/23-nm and a 390/20-nm bandpass filters (Semrock, Rochester, NY), respectively. To visualize the blood vessel network, Q-tracker 655 nanocrystals (Invitrogen, Grand Island, NY) were injected intravenously via a femoral vein into mouse circulation according to the instruction from the manufacturer (Invitrogen). Far red fluorescence from nanocrystals was detected using a far red bandpass emission filter (Semrock), 655/40 nm). Using the 10x water immersion objective lens (Zeiss), a 1.3×1.3 mm multichannel z-series stack with 5 μm steps was obtained. The z-series stack allowed 3D reconstruction of the defect up to a depth of 300 μm (supplemental Video 1&2). The defects were imaged at the indicated time points post-surgery until euthanized. The multichannel 2D slice viewing and 3D reconstruction of defect were conducted using Amira software developed by Visualization Sciences Group (VSG, Burlington MA).

Quantification of SHG propagation and Col2.3GFP cell dynamics in cranial defect repair

To characterize collagen matrix propagation and Col2.3GFP (+) cell dynamics, the defect was imaged over a period of 9 weeks by MPLSM. To ensure accuracy of the analyses, all imaging parameters, including laser power, PMT voltages and compensation were maintained constant throughout the entire experiment. Based on the SHG microscopy, which provided the contour of the defect region, a region of interest (ROI) was established in the time-series images, referencing back to the circular defect region at the day 1 following surgery. To quantify SHG propagation within an ROI, a global thresholding method was used to quantify the total number of SHG (+) voxels above an appropriate threshold in time-series images. The percentage volume of SHG occupying the total volume of the defect was used to depict the propagation of collagen bone matrix within the defect. Similarly, the volume of GFP (+) cells occupying the defect regions within defined ROI was obtained by quantifying the total number of green fluorescent voxels above an appropriate threshold across the time-series images. SHG signal from bone matrix could be easily distinguished from soft tissue by its distinct morphology and its association with Col2.3GFP cells during healing. A schematic illustrating the measurements of SHG and GFP (+) cells can be found in Supplemental Fig. S3. All measurements were performed using a combination of Image J and Amira.

To examine the extent of bone defect healing, the area of the defect was measured in ImageJ by tracing the circular defect region in the 2D images obtained from MPLSM. Since we are using a Busch inverted cone bur to create the defect, the shape of the defect is circular or nearly circular. Based on mathematical calculations, the radius of a circle can be

approximated using the area of the circular region, i.e., $r = \sqrt{\text{Area}/\pi}$. The mean of the cell advancing distance from the edges of the bone was determined by calculating the difference of the outer radius (corresponding to the original edge of the defect) and the inner radius (corresponding to the leading edge of the expanding cells). The original radius of the defect at day 1 was calculated as $r_0 = \sqrt{A_0/\pi}$, where A_0 is the area of the defect at day 1. The area of the defect region that was not occupied by the cells at each indicated time point was

used to approximately calculate the radius of the inner circle as: $r_{\text{inner}} = \sqrt{(A_{\text{inner}}/\pi)}$, where A_{inner} is the area of the void in the defect. The advancing distance of osteoblasts at the indicated time points is then given by $r_0 - r_{\text{inner}}$. The difference was further normalized by the radius of the original defect and plotted as a function of time.

Quantitative and histomorphometric analyses of neovascularization at the site of cranial bone defect repair

A method for quantitative analyses of blood vessels was illustrated in supplemental Fig. S4 and supplemental video 3. Briefly, blood vessels (red channel) and SHG (white channel) were imaged simultaneously. The entire defect as illustrated by SHG and the surrounding vascular network (supplemental Fig. S4A) was reconstructed in 3D format using a multichannel z-series stack. To analyze the vascular network (supplemental Fig. S4B), a region of interest (ROI) was created to include all the vessels associated with the SHG signals produced by bone matrix. This vascular network was then isolated using Amira Segmentation Editor to obtain the final segmented vascular image (Fig. S4C), which was subjected to a series of morphometric analyses: vascular volume (Vasc. Vol.), total volume (T. Vol.), and vascular volume fraction (Vol. Fract.) (i.e., the ratio of Vasc. Vol. over T. Vol.). Using Amira's AutoSkeleton module, which implements a distance-ordered homotopic thinning operation, the segmented 3D vascular network was further skeletonized to generate a line-based network that was topologically equivalent to the original network (supplemental Fig. S4D) (18,19). The skeleton was superimposed on the original image to assess the relative accuracy of this method (supplemental Fig. S2E). The final skeletonized vessel network was obtained by manually retracing of the skeletons using Amira's Filamental Editor to remove false segments. Based on the skeletonized network, the number of vascular segments (NV), total vessel length (T. Length), and vessel length fraction (L.Fract.) (i.e., ratio of vessel length to total volume) were read from the Amira software. The average vessel thickness (Avg. Vess. Th.) and the associated vessel thickness distribution (Fig. S4F&G) were further calculated using an ImageJ plugin developed by Robert Dougherty (20). The complete process for quantification of vascularity was illustrated in Supplemental Video 3.

To detail the dynamic changes of osteogenesis and angiogenesis coupling at the site of repair and to ensure clear visualization of microvessels at the site of defect, clusters of Col2.3GFP osteoblasts were tracked in time series at a higher magnification over a period of 9 weeks. Quantitative and histomorphometric analyses of neovasculature as described above were performed simultaneously with volumetric quantification of Col2.3GFP cells and SHG. Analyses were performed in 5 regions from a group of 4 mice, covering a field of view of $0.75 \times 0.75 \times 0.15 \text{ mm}^3$ for each region.

CD31 Immunohistochemical staining of vascular network in cranial defect

Mouse was perfused with 4% paraformaldehyde at week 2 following surgery. The cranial defect region of the mouse skull was harvested. The sample was treated with 3% bovine albumin in PBS containing 0.3% Triton X-100, followed by staining with CD31 antibody conjugated with Phycoerythrin (PE) (BD Pharmingen, Cat# BD 553373) or α SMA antibody

conjugated with Cy3 (Sigma. Cat# C6198, clone 1A4). The samples were imaged via MPLSM as described above. The multichannel images obtained were analyzed using Amira and Image J. Calvarial defect samples were cryo-sectioned and stained with CD31 or α SMA antibodies. Tissue sections (5 μ m thick) were imaged and photographed under a fluorescent microscope (Zeiss Axio Imager, Zeiss) using 40x lens.

Isolation, seeding and implantation of bone marrow stromal cells (BMSCs)

Bone marrow cells were isolated from 2-month-old Col2.3GFP transgenic mice as previously described with slight modification (21). Briefly, bone marrow cells were flushed from marrow cavity by slowly injecting α -MEM at one end of the bone using a sterile 21-gauge needle. The marrow suspension was dispersed gently by pipetting several times to obtain a single cell suspension. The cell suspension was further filtered through a 70 μ m cell strainer (Falcon) to remove debris. The collected cell suspension was gently overlaid onto Hitopaque1083 (Sigma) and centrifuged at 1100g for 20mins. Mononuclear cells were collected from the interphase and plated on six-well plates at a density of $2 \times 10^6/\text{cm}^2$ and cultured in α -MEM containing 15% FBS (HyClone Laboratories) for 48 hours. The medium was removed by the end of 48 hours and the culture was washed to remove all non-adherent cells. Adherent cells were further cultured in fresh MEM with 15% FBS for additional 10 days. The adherent bone marrow stromal cells (BMSCs) were released from the culture plate at day 10 using trypsin and EDTA. The recovered BMSCs were mixed with naturalized collagen solution (3mg/ml, BioMatrix) and congealed at 37°C for 30 mins (22). A 2 mm³ gel containing about 2×10^5 cells was punched out and subsequently implanted into a 2 mm defect.

Longitudinal live microCT scanning

Mice were sedated with isoflurane and restrained in a custom-made chamber containing isoflurane. The mouse skull was scanned by a Scanco Viva CT40 system (Scanco Medical) at a voxel size of 17.5 μ m. Imaging was performed on the same groups of mice repeated over three months. From the 2D images generated, the defect was reconstructed in 3D and analyzed using Amira software combined with Image J. The rate of the defect healing was evaluated by calculating the area of the defect closure over a period of 3 months.

Statistical analyses

Data are expressed as the mean $\pm$ SEM. Comparison was made among time-series data sets obtained from a group of 4 mice. Statistical significance was determined using repeated measures one-way ANOVA and a Tukey's posthoc test in GraphPad (GraphPad Prism, San Diego, CA). A p value <0.05 was considered statistically significant. Multivariate correlation analyses were performed in Excel and GraphPad. The Pearson correlation coefficients (R) between groups were analyzed in GraphPad to obtain a p value and an F value. A p value <0.05 following Bonferroni correction was considered statistically significant.

RESULTS

Real-time imaging of bone defect repair via MPLSM in the cranial defect window chamber model

A defect was created in the parietal bone of a 2.3kb collagen type 1 promoter-driven GFP transgenic mouse (Col2.3GFP), which permitted the tracking of osteoblast differentiation at the repair site (23). The superior optical sectioning capability of MPLSM enabled simultaneous visualization of GFP (+) cells (Green channel), SHG (+) collagen matrix (white channel), and neovasculature (red channel) at 2D and 3D dimensions (Supplemental Fig. S1E–G and Supplemental videos 1&2). Reconstruction of the images from live microCT scanning (Supplemental Fig. S1D) and MPLSM/SHG microscopy (Supplemental Fig. S1E) illustrated the remarkable agreement between these two approaches, which combine the high depth penetration of microCT scanning with the molecular and cellular specificity of MPLSM.

To perform longitudinal analyses, the entire defect was surveyed and reconstructed to illustrate bone (white), vessels (red), and osteoblasts (green) separately or in combination at the indicated time points (Fig. 1). As shown, the nascent blood vessels were found at week 1, extending outward to the rim of the defect (Fig. 1A2). The newly formed vessels were often irregular and disorganized. Mature vessels were visualized after week 2, radially distributed around the bone defect (Fig. 1A3). Angiogenesis at the defect region persisted during the first 6 weeks and subsided at week 9 (Fig. 1A4–6). GFP and SHG signal at the site of repair were assessed (Fig. 1, panel B). Col2.3 GFP (+) cells were first identified in the residual bone around the edge of the defect during week 1 (Fig. 1B2, arrows). More GFP (+) osteoblasts appeared at week 2 around the rim of the defect (Fig. 1B3). These GFP (+) osteoblasts expanded in size and volume, further advancing into the defect in week 3 and onward. The propagation of SHG (+) signals within the defect was evident at week 3 and progressively increased over time (Fig. 1B4–5). Quantitative analyses showed that GFP (+) osteoblast volume increased at week 2, peaked between 3–6 weeks and decreased at week 9 (Fig. 1C, n=4). The SHG signal within the defect continued to increase over a 9-week period, with significant propagation detected between week 3 and 9 (Fig. 1D). A greater percentage of defect closure at the center region was observed in the first 3–4 weeks of healing as indicated by the percent rate of defect closure (Fig. 1E). Measurement of the osteoblast advancing distance towards the center of the defect indicated that osteoblast progenitors expanded into the defect at a faster pace during the first 3 weeks of healing (Fig. 1F). The average advancing distance of GFP (+) cells at the leading edge of the defect was calculated as ~6µm/day during the first three weeks and ~2µm/day during the last 4–9 weeks (n=4). A similar faster rate of defect closure within the first 4 weeks of healing was further confirmed by live microCT scans (supplemental Fig. S2A). Collectively, these data indicate that effective bone wound closure depends on osteoblastic activity initiated at the leading edge of the bone defect during early stages of healing.

Real-time tracking of the clusters of Col2.3GFP (+) osteoblasts and the coupled angiogenesis at the site of defect repair

To examine the dynamics and coupling of osteogenesis and angiogenesis during repair, clusters of Col2.3GFP (+) cells identified at the edge of the bone defect were tracked at a higher magnification over a period of 9 weeks (Fig. 2, panel A to E). As shown, at week 1, GFP (+) clusters were found in the residual bone at the edge of the bony defect (Fig. 2, panel A, and Fig. 2F1 as indicated by arrows). These clusters of GFP (+) cells were found to be in close proximity of the invading blood vessels (Fig. 2F2, arrows). At week 2, the same clusters of Col2.3 GFP (+) cells expanded into the defect site, surrounded by numerous newly formed microvessels at the leading edge of the bone defect (Fig. 2, panel B). Weak SHG signals were detected among these cells (Fig. 2B3), indicating that they were less mature osteoblasts. At a higher magnification, micro-capillary vessels were found intertwined within these expanded Col2.3 GFP (+) cell clusters (Fig. 2G1&2 yellow arrows). A few GFP (+) cells were found extending along blood vessels in bone matrix (Fig. 2G1&2, blue arrow). At week 3, the same groups of cells expanded into the wound and increased in size and volume (Fig. 2, panel C). These cells produced a significant amount of bone matrix as indicated by the induction of high contrast SHG signals (Fig. 2C3). At a deeper level, microvessels were found embedded within new bone matrix (Fig. 2C4–6). The larger vessels inside bone matrix were surrounded by Col2.3 GFP (+) cells (high magnification Fig. 2H1&2, blue arrows). Careful examination showed that GFP (+) osteoblasts were situated along the edges of the bony channels, suggesting the active modeling of the bony channel by osteoblasts to support vascular ingrowth in bone tissue. By week 6, angiogenesis subsided as evidenced by the reduction of microvessels at the site of the defect repair (Fig. 2, panel D). Within the new bone matrix, enlarged sinusoidal blood vessels were formed (Fig. 2D4–6 and Fig. 2I1&2, yellow arrows). Continued tracking of the defect at week 9 showed a significant reduction of Col2.3GFP (+) cells at the site of healing, along with regression of vessels within and around bone matrix (Fig. 2, panel E). More mature bone matrix was built along the edge of bony defect as indicated by high contrast SHG signals. Remodeled blood vessels could be found intertwined within bone marrow spaces (Fig. 2J1&2, arrows).

Another time-series of images obtained at a different repair site further highlighted the key changes in neovasculature during repair (Fig. 3, panel A to E). Notably, the micro-capillary vessels were found intertwined within the expanding osteoblast clusters at the leading edge of the bone defect throughout weeks 2 and 3 (Fig. 3A1–3 and B1–3, circled region). Measurements of these vessels showed an average diameter of $7.35 \pm 3.2 \mu\text{m}$ ($n=50$). The quantity of these microvessels at the leading edge of the defect dwindled as the osteoblastogenesis subsided at a later stage of healing (Fig. 3C3–E3, circled regions), indicating a critical role of these microvessels for expansion and migration of the osteoblasts into the center of the defect wound. In addition to the changes in microvessels, active modeling and remodeling of blood vessel network within new bone matrix were noted (Fig. 3C3&D3&E3, arrows). The appearance of the large and irregular sinusoidal vessels in the bony tissue was evident at a later stage of healing and coincided with active remodeling of bone matrix and formation of bone marrow space.

To characterize the dynamic changes of osteogenesis and angiogenesis coupling during defect healing, quantitative histomorphometric analyses of GFP (+) cells, SHG and neovascularization were performed at the active bone repair sites using time-series images obtained at a higher magnification. Osteoblast volume increased at week 2, peaked at week 3 and decreased at week 9 (Fig. 4A). SHG progressively increased over a period of 9 weeks indicating the propagation and deposition of new bone matrix (Fig. 4B). Localized angiogenesis was characterized and quantified using a series of measurements. *T. Length* and *L. Fract.* were increased at week 1, preceding the induction of osteoblastogenesis. The two parameters peaked during weeks 2 and 3, coinciding with the peak of osteoblastogenesis, and further reduced at week 6, preceding the decline of osteogenesis at week 9 (Fig. 4C and D, n=4). Compared to *T.Length* and *L.Fract.*, other measurements namely *Vasc. Vol.* and *Avg. Vess. Th.* showed a similar trend of induction but exhibited a significant variability among samples (Fig. 4E, F and G). In the vessel thickness distribution analyses, similar trend of changes were observed, with vessels less than 10µm exhibiting a better correlation with osteoblast volume during healing (Fig. 4H).

Multivariate correlation analyses were performed using time-series data obtained from histomorphometric imaging analyses to determine the correlation among SHG volume, Col2.3 GFP (+) cell volume, and vascularity. SHG was found to be correlated with neither GFP volume nor vascularity (Supplemental Table 1.). In contrast, Col2.3GFP (+) cell volume demonstrated statistically significant correlations with a number of vascular measurements, including *T. Length*, *L.Fract.*, *Avg. Vess. Th.*, *Vasc. Vol.* and *Vol. Fract.* (Table 1). Among them, *T. Length Fract.* showed the strongest correlation with GFP (+) cell volume ($R=0.69$, $p=0.0016$), and *Vasc. Vol.* and *Vol. Fract.* showed the weakest ($R=0.52$, $p=0.02$). If three parameters were combined, e.g., *Vasc. Vol.*, *T. Length* and *Avg. Vess. Th.* in a multivariate regression analysis, an improved correlation coefficient (R) reached to 0.82, with a R^2 of 0.62 ($p<0.001$), demonstrating a significant correlation of vascularity with Col2.3GFP (+) cells at the regions of active repair. In vessel distribution analyses, we found that vessels less than 10 µm showed a better correlation with osteoblast volume than vessels greater than 10 µm ($R=0.60$, $p=0.02$), again suggesting that microvessel angiogenesis is critical for osteoblast-dependent repair.

To further characterize the regenerative microvasculature at the leading edge of the cranial defect, a nestin-GFP transgenic mouse model, which has been shown to mark nascent vessels in collagen-mediated angiogenesis and in skin wound healing (24,25), was utilized. In un-injured mice, nestin-GFP (+) cells could be occasionally identified in bone marrow space adjacent to a blood vessel in parietal bone. In the suture, a large number of nestin-GFP (+) cells could be found associated with blood vessels (data not shown). Following cranial defect surgery, nestin-GFP (+) cells were identified at the repair site at week 1, attaching to the irregularly shaped nascent blood vessels at the surface of the bone wound (Fig. 5A). For a duration of week 2 and onward, a large number of nestin-GFP (+) blood vessels were found at the surface of bone wound, primarily located in soft tissue (Fig. 5B). Only a very small number of nestin-GFP (+) cells were identified at the leading edge of the bone defect (Fig. 5C), indicating that the microvasculature at the bone repair site was mostly nestin-GFP negative. To establish the identity of nestin-GFP (+) cells associated with blood vessels,

immunofluorescent stainings on intact calvaria sample harvested from the defect site were performed. As shown, nestin-GFP (+) vessels co-localized with CD31 (+) vessels primarily in soft tissue on the surface of the bone wound (Fig. 5D). Microvessels at the leading edge of the bone defect were largely negative for nestin (Fig. 5E). Both endothelial and pericytic location of nestin-GFP (+) cells were observed in MPLSM imaging (Fig. 5F). Higher magnification MPLSM images depicted nestin-GFP (+) cells as typical endothelial lining cells along CD31 (+) vessels (Fig. 5G–I, arrows). Nestin-GFP (+) cells also stained positive for α SMA (Fig. 5K–M, arrow). Further immunohistochemical stainings of CD31 and α SMA using histologic sections prepared from calvaria samples confirm the co-localization of nestin-GFP (+) cells with CD31 (+) endothelial cells (Fig. 5J, arrow) and α SMA(+) pericytes (Fig. 5N, arrow), demonstrating that nestin marks both endothelial cells and pericytes on blood vessels. To further establish the expression of nestin in nascent forming vessels, we performed tubular formation assay using stromal vascular fraction of adipose tissue from nestin-GFP mice (26). Extensive nestin (+) tubular networks were identified in the culture, co-localizing with CD31 (supplemental Fig. S7).

Real-time tracking of donor BMSC-mediated bone defect repair and reconstruction

The establishment of a chronic window chamber model further enables us to track donor progenitor cell-mediated bone regeneration at the defect site. To track donor cell differentiation and bone formation in real-time and longitudinal fashion, bone marrow stromal cells (BMSCs) derived from Col2.3 GFP mice were implanted into the site of a cranial defect. Prior to implantation, all BMSCs were GFP (–). Upon differentiation, osteogenic cells including osteoblasts and early osteocytes in forming osteoid become GFP (+), facilitating tracking of osteogenic differentiation and bone forming activity *in vivo* (23,27). As illustrated, progressive new bone formation within the defect region was detected by live longitudinal microCT scans over a period of 14 weeks (Fig. 6, panel A). A similar time course of defect healing was depicted by 3D reconstruction of MPLSM images obtained from the same defect (Fig. 6, panel B). The analyses of the MPLSM time-series showed that clusters of Col2.3GFP (+) BMSCs appeared at day 10 post-implantation, surrounded by nascent blood vessels (Fig. 6B1). Col2.3 GFP (+) cells were quickly replaced with bone tissue between days 14–20 as indicated by live microCT scanning (Fig. 6A2) and MPLSM imaging (Fig. 6B2). A significant number of GFP (+) cells were found embedded in new bone matrix indicating direct donor cell-dependent new bone formation during the early stages of healing (Fig. 6C2). Continued monitoring of the defect over the next 11 weeks showed progressive bone wound closure (Fig. 6B3–B6&C3–C6). Based on the presence of the donor GFP (+) cells in the time-series images, it was evident that late stage bone formation and bone wound closure were largely dependent on the host (Fig. 6C3–6 and D3–6). Although scattered donor GFP (+) bone nodules (yellow arrows in Fig. 6C3&C4) were found within the defect, tracking of these isolated bone nodules showed that the GFP (+) donor osteoblasts slightly expanded in volume and were eventually embedded within host bone at a later stage. While host-dependent bone formation primarily arose from the edge of the defect, scattered GFP (–) bone nodules (host origin) could be identified within the center region of the defect. Examination of the neovascularization at the defect site showed that induction of overall vascularity coincided with early osteogenesis (Fig. 6. Panel D). Blood vessels remodeled and regressed as the bone matrix built up and became more

mature. Only modest induction of vascularity was shown at the localized region, corresponding to the enhanced host bone formation at weeks 9 and 14 (Fig. 6D5&D6, white boxed region).

Donor-dependent bone formation and the associated neovascularization were further illustrated at a higher magnification over time (Fig. 6, panel E&F). At day 10, nascent vessels were found invading the differentiated GFP (+) osteoblastic donor cells (Fig. 6E1). These cells quickly laid down bone matrix between days 10 and 20 as indicated by SHG (Fig. 6E2). Microvessels were found closely encircled around GFP (+) cells within new bone at days 20–30 following implantation (Fig. 6E2–3 and F2–3). These blood vessels were subsequently remodeled and regressed as more bone matrix was built, leading to the formation of vascular channels and bone marrow spaces (Fig. 6E4–6& F4–6). Interestingly, we found that some donor GFP (+) cells persisted in newly formed bone, particularly in areas where marrow cavity and sinusoidal vessels were formed at a later stage (Fig. 6F2–6). Histology of the same sample harvested at week 14 illustrated the donor-derived bone in the calvaria defect (supplemental Fig. S8).

DISCUSSION

To gain a better understanding of the spatiotemporal regulation of osteogenesis and angiogenesis in defect repair, we established a cranial defect window chamber model in mice that allows high-resolution, longitudinal, and real-time analyses of bone defect healing and osteogenesis and angiogenesis coupling via Multiphoton Laser Scanning Microscopy (MPLSM). Using this novel intravital imaging approach, we demonstrate for the first time the longitudinal and spatiotemporal analyses of osteogenesis and angiogenesis in cranial bone defect repair and regeneration. Our study provides basis for understanding molecular and cellular interactions of bone and vessel forming cells in cranial bone defect repair and regeneration, further offering a unique *in vivo* real-time imaging tool to track the fate of the progenitor cells and their interactions with host healing microenvironment.

Bone defect repair is a dynamic tissue morphogenetic process orchestrated by progenitor cells, chemokines and growth factors produced at the site of repair. Despite the fact that the cranial bone defect model is being used as the first stage of *in vivo* study for bone regeneration and bone tissue engineering, the dynamic morphogenetic process of cranial bone defect healing has been poorly described. The spatiotemporal regulation of osteogenesis and angiogenesis in defect repair remains superficially understood. By simultaneously tracking SHG, Col2.3GFP (+) osteoblasts, and neovascularization in a windowed cranial defect model via MPLSM, we established the morphogenetic sequence and spatiotemporal coordination of osteogenesis and angiogenesis in cranial bone defect healing. Our study showed that at the early stage of healing, the expansion of the Col2.3GFP (+) osteoblasts coupled with vigorous angiogenesis and characteristic formation of micro-capillary vessels at the leading edge of the defect. The significance of the coupling with micro-capillary vessels is further corroborated by multivariate correlation analyses which demonstrated a strong correlation of osteoblast volume with vascular parameters, particularly the blood vessel length and vessels less than 10µm in diameters at the site of repair. In concomitance with the early small vessel angiogenesis, the rate of the defect

healing, as measured by the advancing distance of GFP (+) osteoblasts into the defect, was 3 times faster in the first 3 weeks than the last 6 weeks. These data collectively suggest that localized small vessel angiogenesis at the early stage of repair is critical for the expansion of osteoblasts and the induction of bone formation. Hence, prolonging this early osteogenic and angiogenic phase could be beneficial for enhancing defect closure.

In contrast to the early stage, the late stage bone defect healing was characterized by slow expansion of osteoblasts at the leading edge and active vascular network remodeling within the newly formed bone matrix. Vascular remodeling and regression are integral components of angiogenesis in tissue repair (28). The proper remodeling and pruning of neovasculature are critical for establishing bone homeostasis, developing sinusoids and restoring bone marrow in repair (29–31). How this remodeling process is regulated in repair and how it is affected by bone healing microenvironment in defect repair remain poorly understood. Further detailed studies using available fluorescent transgenic reporter mouse models will facilitate mapping of the spatiotemporal cues of the “morphogenetic field” of cranial defect repair, offering new insights for development of material-based approaches to orchestrating controlled delivery of growth factors and chemokines for repair and reconstruction.

The origin and sources of osteoblast precursors remains as one of the key interests in skeletal repair and regeneration. While periosteal progenitor cells have been recognized as a major source (32,33), studies have reported the contribution of skeletal progenitors from bone marrow, dura, as well as perivascular cells in cranial repair and regeneration (34–37). A 2.3kb collagen type I promoter driven GFP transgenic mouse model (Col2.3GFP) was used in our current study. It is reported that Col2.3GFP labels mature osteoblasts and osteocytes in forming bone (23,38). However, in our experiments, we found that Col2.3GFP also labeled early osteoprogenitors that had the capacity to migrate and expand into the wound healing site. Careful tracking of these cells showed that the osteoprogenitors originated from the residual bone at the rim of the bone defect (Fig. 1 and 2). The unique location of these cells suggests a potential contribution of osteoblasts from the living cells dwelling inside the bone tissue. It is known that skeletal progenitors can be directly isolated from the crushed bone chips (39), and several studies show that osteocytes can undergo dedifferentiation and migrate out of lacunae to form osteoblasts *in vitro* (40,41). Further lineage tracing experiments in cranial windowed defects will assist in establishing the key sources of the osteoblasts in cranial defect repair.

Perivascular cells, often referred to as pericytes, have been shown as a source of skeletal stem/progenitor cells (31,42,43). The unique pericytic location of osteoblastic cells in repair tissue has been described by Maes et al. who suggest that osteoblast precursors (OSX +ve cells) enter the cartilage and bone along with blood vessels to form new trabecular bone during endochondral bone formation (44). A similar close location of osteoblasts with blood vessel was observed in our current study in both un-injured (supplemental Fig. S5C, arrows) and injured bone (Fig. 2G2&H2). Careful analyses showed that the majority of the GFP (+) osteoblasts along blood vessels were situated along the surface of bony channels, suggesting a mechanism that involves the induction of osteoblasts along vessel paths to facilitate dynamic vascular ingrowth and remodeling within the newly formed bone.

In attempt to further characterize the neovascularization in repair, we examined the distribution of nestin-GFP (+) cells in repair tissue. Nestin (+) perivascular cells have been shown to constitute hematopoietic stem cell niches in bone marrow and further participate in bone remodeling and homeostasis (14). Nestin-GFP has also been associated with nascent vessels in collagen-induced angiogenesis and in skin wounds (25). Using nestin-GFP transgenic mice in cranial defect model, we found that nestin-GFP expressions were primarily associated with blood vessels in inflammatory soft tissues at the surface of the defect. In contrast, the regenerative micro-vasculature at the leading edge of bone defect was mostly negative for nestin-GFP (Figure 5). Further immunofluorescent staining established the localization of nestin-GFP (+) cells as CD31 (+) endothelial cells and α SMA (+) perivascular cells on blood vessels. These data confirm the heterogeneity of the nestin-promoter driven GFP cells on blood vessels (42,45) and further demonstrates the preferential distribution and localization of nestin-GFP (+) vessels in repair.

While BMSCs have been used in repair and reconstruction of bone tissue in animal models and in human trials, the mechanisms by which BMSCs participate in bone defect repair are not completely understood (46–48). The establishment of an intravital bone healing platform provides a unique tool to track BMSC differentiation and further investigate the role of BMSC in repair and reconstruction. Using this novel imaging approach combined with longitudinal microCT scanning in the cranial defect window chamber model, we obtained a sequence of images illustrating BMSC-mediated bone regeneration *in vivo* (Figure 6). Our analyses on these images showed that 1) donor BMSCs underwent differentiation in concert with early angiogenesis; 2) donor BMSC-dependent regeneration occurred rapidly within the first 3 weeks of implantation, with significant numbers of Col2.3GFP (+) BMSCs incorporated into bone; 3) active vascular remodeling occurred in donor-derived bone tissue with potential participation of donor derived Col2.3GFP (+) osteoblasts; 4) late stage bone repair and defect closure occurred at a slower rate and were mostly host dependent; 5) the persistent late stage bone healing was associated with localized modest induction of angiogenesis and active remodeling of blood vessels. These observations collectively support a mechanism of coordinated and joint contribution of both the donor and host bone forming cells in bone defect repair. Since only modest and localized angiogenesis was observed during late stage bone defect healing, the persistent bone formation in these regions could be attributed to the unique capabilities of BMSCs to produce a wide array of trophic factors that stimulate host osteogenesis and modulate local inflammatory and immune responses in repair (47,49–51). The anti-inflammatory and anti-fibrotic actions of BMSCs have been extensively reported in cardiac and skin injury models (52–54).

Current analyses of osteogenesis and angiogenesis in bone defect repair largely rely on histology, which is destructive and challenging in providing a three-dimensional perspective of the repair tissue. A micro CT-based approach has been successfully used in quantitative analyses of osteogenesis and angiogenesis in bone tissue repair and regeneration (55–57). However, micro CT-based analysis does not permit longitudinal analyses of the neovasculature and has limitations when imaging small vessels less than 20 μ m in diameter (58,59). Other methodologies such as Optical Coherence Tomography (OCT) (60), Doppler (61,62) and Magnetic Resonance Imaging (MRI) can provide functional assessment of blood

vessels but lack the resolution to correlate the neovascularization with cellular differentiation during bone tissue repair and reconstruction. MPLSM, due to its high 3D spatiotemporal resolution and deep tissue penetration, is ideal for real-time tracking of clusters of progenitor cells and their complex residing vascular microenvironment *in vivo*. This unique capability offers distinctive advantages over MicroCT and histology for simultaneous and dynamic analyses of osteogenesis and angiogenesis coupling in real-time and 3D format. In conjunction with fluorescent transgenic mouse models and cell/vessel labeling technologies, MPLSM could provide a valuable tool for analyses of cell-cell, cell-vessel, and cell-matrix interactions in dynamic biological processes, such as bone defect repair and bone tissue engineering (4,12,34,63,64).

As every other available technology that has pros and cons, MPLSM also has a number of limitations when imaging bone tissue in living animals. These include: 1) the surgical challenge in establishing a clean window for repeated imaging; 2) the limitation in maximum penetration in bone tissue (250–300 μm); 3) the potential tissue response from repeated imaging; and 4) the reliance on technical innovations in fluorochrome and cell labeling system to probe biological system. However, despite these disadvantages, MPLSM remains the top choice for *in vivo* imaging of thick and complex tissue. Technology development will continue to push the boundaries of multiphoton-based imaging technology to provide a better and more suitable platform for obtaining comprehensive pictures of biological systems at high macroscopic resolutions (65).

In summary, by establishing a MPLSM-based intravital imaging modality in a cranial defect window chamber model, we examined the morphogenetic process of cranial bone defect healing and further established the spatiotemporal analyses of osteogenesis and angiogenesis coupling in repair and regeneration. Our study provides the basis for real-time tracking of progenitor cell fate and understanding of the molecular and cellular interactions of bone and vessel forming cells in bone defect repair. The establishment of an intravital imaging modality further facilitates elucidation of spatiotemporal regulatory mechanisms of osteoprogenitor cell interactions with the host bone healing microenvironment, aiding in development of novel material-based approaches aimed at modulating progenitor cell behavior and engineering bone healing microenvironment for enhanced bone defect repair. With the increasing need for design of smart biomaterials for controlled delivery of growth factors, a deeper understanding of the spatiotemporal cues of osteogenesis and angiogenesis, as well as vascular remodeling and regression will facilitate the development of novel material-based therapeutics for improving defect repair and reconstruction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Longitudinal analyses of cranial defect healing via MPLSM in Col2.3GFP mice
 A 1 mm cranial defect healing was tracked over a period of 9 weeks using MPLSM. Progressive defect healing and neovascularization are illustrated in a time-series images reconstructed by Col2.3GFP(+) osteoblasts (green), SHG (+) bone matrix (white) and blood vessels (red) in combination as indicated (panel A and B). Volumetric analyses of GFP (+) osteoblasts (C) and SHG (D) over a 9 week period within the defect region are shown. Using the area and radius of the defect on day 1 as a reference, the % area of the defect in the center region (E), and the cell advancing distance relative to the radius of the original defect (F) were further calculated. Data are expressed as the mean $\pm$ SEM (n=4). * indicates significant difference ($p < 0.05$). Scale bar = 100 μ m.

Figure 2. Real-time tracking of the clusters of Col2.3GFP (+) osteoblasts and the coupled angiogenesis at the site of defect repair

Clusters of GFP (+) osteoblasts at the edge of bone defect were tracked via MPLSM at a high magnification at week 1 (A1–6), 2 (B1–6), 3 (C1–6), 6 (D1–6) and 9 (E1–6). The region of interest was reconstructed and shown at a depth of 20–250 µm (panel 1–3) and 100–250 µm (panel 4–6). The cluster of Col2.3GFP (+) cells (indicated by yellow arrows) and the associated vessels were further zoomed-in to illustrate the spatial interaction of clusters of GFP (+) cells and neovasculature at week 1 (G1–2), 2 (H1–2), 3 (I1–2), 6 (J1–2) and 9 (K1–2). Osteoblasts were identified along microvessels or closely associated with vasculature at week 2 and 3 (G1&2, H1&2, blue arrows). Scale bar = 100 µm in B–F, 40 µm in G–K.

Figure 3. Real-time tracking of angiogenesis and vascular modeling in cranial defect repair
Dynamics of defect healing and neovascularization was shown in time-series images reconstructed by Col2.3GFP (+) osteoblasts (green), blood vessels (red) and SHG (+) bone matrix (white) at a depth of 50–250μm (A1–3, B1–3, C1–3, D1–3 and E1–3) at week 2, 3, 6, 9 and 14. The purple dash lines marks the defect at day 1 in all images. The combined images of SHG, GFP, and vessels demonstrate the progressive new bone formation at the leading edge of the defect (panel A1, B1, C1, D1 and E1). Images of GFP and blood vessels combined (panel A2, B2, C2, D2 and E2), and images of blood vessels alone at the same site (panel A3, B3, C3, D3, E3) are shown to illustrate the spatial interaction of osteoblasts and blood vessels over time. The association of osteoblasts with micro-capillary vessel is indicated by yellow circles. The formation and remodeling of vascular channels within newly formed bone are indicated by blue arrows in B3–D3. Scale bar =125μm.

Fig. 4. Quantitative and histomorphometric analyses of GFP (+) cells, SHG, and neovasculture during defect healing

Quantitative and histomorphometric analyses of SHG, GFP and vascularity were performed as described. Osteoblast volume peaked at week 3 and 6, decreased at week 9 (A) whereas SHG progressively accumulated up to 9 weeks (B). Blood vessel length (C), length fraction (D), vascular volume (E) and volume fraction (F) increased at week 1 and peaked at week 3, coupled with osteoblast expansion and differentiation. Average vessel thickness (G) and thickness distribution (H) at the site of the defect repair are shown. Data are expressed as the mean + SEM. * indicates significant difference ($p < 0.05$). $n = 4$.

Fig. 5. Distribution of nestin-GFP (+) cells at the cranial defect site

A windowed defect was created in nestin-GFP transgenic mouse. The reconstructed live MPLSM images at week 1 and 2 show nestin-GFP (+) cells associated with nascent blood vessels at week 1 (A) and 2 (B) in soft tissue on top of bone. Microvasculature at the leading edge of the defect was largely negative for nestin-GFP (C). CD31 immunofluorescent staining of intact calvaria samples demonstrated that nestin-GFP (+) cells were primarily associated with blood vessel at the surface of bone wound (D). Only a few nestin-GFP (+) cells were identified on vessels leading into the bone tissue (F). Higher magnification 3D reconstruction of MPLSM image depicts CD31 (+) nestin-GFP (+) vessels at the defect site (F). Nestin GFP (+) cells demonstrated typical morphology of endothelial lining cells (G) and were co-localized with CD31 (H: CD31 only, I: nestinGFP overlaying with CD31). Immunofluorescent staining of CD31 using histologic section confirmed the co-localization of nestin-GFP (+) cells with CD31 (+) endothelial cells (J). Immunofluorescent staining of α SMA showed co-localization of nestin-GFP (+) cells with α SMA (+) pericytes (K: nestinGFP only; L: α SMA(+) vessel only; M: nestinGFP overlaying with α SMA; arrow indicate α SMA+ perivascular cells). Immunofluorescent staining of CD31 using histologic section confirmed the co-localization of nestin-GFP (+) cells with α SMA (N).

Fig. 6. Live tracking of donor BMSCs and neovascularization during cranial defect repair and regeneration

Donor BMSCs isolated from Col2.3GFP mice were implanted into a cranial defect window chamber created in an immunocompromised mouse. The entire defect was scanned and reconstructed at the indicated time points by microCT (panel A) and MPLSM (panel B&C&D), with yellow circle indicating the defect region. The 3D time-series images of the defect (panel B) were reconstructed by Col2.3GFP(+) osteoblasts (green), SHG (+) bone matrix (white) and blood vessel (red) to show progressive defect healing over a period of 14 weeks. The same time-series 3D images reconstructed by GFP (green) and blood vessels (red) were superimposed with a 2D image of SHG to show the distribution and incorporation of donor GFP (+) cells into bone matrix (panel C). The same time series 3D images reconstructed by GFP (green) and blood vessels (red) (panel D) illustrate the spatial interaction of GFP (+) cells with neovasculature during the entire process of repair. Blue boxed region in A3 corresponds to the region undergoing donor-dependent bone formation. White boxed region in A4–6 and D5–6 corresponds to the region primarily undergoing host-dependent bone formation. Arrows in C3 and C4 indicates donor derived bone nodules.

Arrows in C5 and C6 indicate the formation of bone marrow space and remodeling of sinusoidal vessels in new bone. Blue boxed region in A3 is further zoomed-in to illustrate the dynamic progression of donor dependent bone formation (panel E) and spatial interaction of donor cells with neovasculature (panel F). Scale bar =200 μ m in panel B, C and D. Scale bar=100um in panel E and F.

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Table 1

Pearson’s Correlation Coefficient (R) for histomorphometric analyses of neovasculature

	Vasc. Vol. Fract.	AVG. Vess. Th.	T. Vess. Length Fract.	Vessel <10mm	Vessel 10–30mm	Vessel >30mm
Cell Vol.	0.52 (p=0.026) (F=6.032)	0.65 (p=0.0037) (F=11.53)	0.69 (p=0.0016) (F=14.28)	0.60 (p=0.02) (F=7.14)	0.47 (p=0.05) (F=4.785)	0.48 (p=0.05) (F=4.529)

The table lists Pearson’s Correlation Coefficient (R) for histomorphometric analyses of vascularity and osteoblastogenesis as determined by Col2.3GFP (+) cell volume. The p and F values of the indicated coefficient are listed in parenthesis. A p value <0.05 is considered statistically significant.