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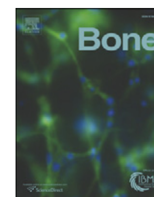

Full Length Article

# Teriparatide attenuates scarring around murine cranial bone allograft via modulation of angiogenesis

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ABSTRACT

Nearly all bone fractures in humans can deteriorate into a non-union fracture, often due to formation of fibrotic tissue. Cranial allogeneic bone grafts present a striking example: although seemingly attractive for craniofacial reconstructions, they often fail due to fibrosis at the host-graft junction, which physically prevents the desired bridging of bone between the host and graft and revitalization of the latter. In the present study we show that intermittent treatment with recombinant parathyroid hormone-analogue (teriparatide) modulates neovascularization feeding in the graft surroundings, consequently reducing fibrosis and scar tissue formation and facilitates osteogenesis. Longitudinal inspection of the vascular tree feeding the allograft has revealed that teriparatide induces formation of small-diameter vessels in the 1st week after surgery; by the 2nd week, abundant formation of small-diameter blood vessels was detected in untreated control animals, but far less in teriparatide-treated mice, although in total, more blood capillaries were detected in the animals that were given teriparatide. By that time point we observed expression of the profibrogenic mediator TGF-β in untreated animals, but negligible expression in the teriparatide-treated mice. To evaluate the formation of scar tissue, we utilized a magnetization transfer contrast MRI protocol to differentiate osteoid tissue from scar tissue, based on the characterization of collagen fibers. Using this method we found that significantly more bone matrix was formed in animals given teriparatide than in control animals. Altogether, our findings show how teriparatide diminishes scarring, ultimately leading to superior bone graft integration.

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## 1. Introduction

Human bones have a remarkable capacity for self-regeneration when fractured [1], yet their healing may be hindered due to formation of fibrosis [2,3]. Healing of cranial non-union fractures is particularly challenging, because the cranial bone has few resident stem cells and a low capacity for self-regeneration [4]. Fractured long bones efficiently recapitulate their developmental pathway of endochondral ossification, which involves a cartilaginous mold [1,5]. Cranial bone originates from intramembranous ossification, which does not include a cartilage phase [6], and little is known as to what extent it can mimic this reparative mechanism. Cranial bone reconstruction has important public health implications. Forty craniotomies are performed per million people worldwide to treat trauma and/or relieve elevated intracranial pressure [7]; this is considered a high-risk procedures [8]. One of the main concerns is the re-implantation of the dissected bone fragment following several months it is kept frozen. The re-implantation often induces fibrosis and adhesions involving parenchymal tissue [9–11]. Another major clinical challenge is oral cancer, the leading cause of mandibulectomy and maxillectomy: approximately 640,000 people worldwide are diagnosed each year [12]. Implantation of autologous bone is considered the gold standard therapy. Autologous bone contains living stem cells and growth factors needed for bone regeneration [13]. Unfortunately, the volume of harvested bone is limited, shaping the

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desired structure is not always easy, and bone harvesting entails morbidity at the donor site [14,15] as well as bone resorption [16–18]. Growth factors such as Bone Morphogenetic Proteins (BMP) may be used, although they are expensive [19] and may produce complications such as neurological impairment [20,21] and possibly carcinogenesis [22,23]. Bone allografts have been extensively used to repair long bones [24,25], as they are available in various shapes and provide structural integrity; however, bone allografts are not osteoinductive and are often encapsulated with fibrotic tissue, thereby failing to integrate with the host bone [26]. Nevertheless, allografts present a promising option for cranial regeneration when they integrate well. Teriparatide, a pharmaceutical derivative of Parathyroid hormone (PTH), is the only FDA-approved bone anabolic therapy for patients with osteoporosis [27], has been found to induce allograft integration in fractures of long bones [28,29]. Earlier we used a mouse model, which is ideal for our scientific objectives as it is well suited for quantifiable micro-imaging, to show that intermittent administration of teriparatide promotes the osteo-integration of calvarial grafts by induction of stem cell recruitment and differentiation [30], but the underlying mechanisms of the teriparatide effect on cranial allograft healing were unclear.

Interestingly, we noted that systemic teriparatide treatment delayed the mobilization of mast cells, key effector cells of fibrosis—a phenomenon that has been documented in the long bone model as well [26]. In order to study the fibrosis that forms and engulfs the cranial allograft, one has to implement a proper imaging method. For this purpose, we have refined a MRI approach based on the rate of magnetization transfer between free water molecules to collagen-bound water molecules, which will be presented in details in Section 3.

Here we report that teriparatide therapy induces formation of narrow blood vessels in the proximity of the cranial bone allograft (which may explain the previously reported delay in mast cell infiltration [30]) and eventually diminishes fibrotic scar tissue formation. To measure the extent of fibrosis we developed a unique micro-MRI (μMRI) protocol that can be used to distinguish fibrotic tissue from newly formed bone, based on the characterization of collagen fibers.

## 2. Materials and methods

### 2.1. Calvarial defect surgery

All procedures described in this study were approved by The Hebrew University of Jerusalem"s institutional animal care and use committee. The calvarial defect model was previously described [30,31] and is depicted in detail in a Supplemental data section. Briefly, structural allografts (4.5 mm in diameter) were harvested from wild-type B57 mice. Any soft tissue that we encountered was scraped off, and the excised bone was washed extensively with PBS solution followed by application of 70% ethanol to decellularize the graft. The grafts were frozen at −70 °C for at least 1 week prior to transplantation. To create the calvarial defects, a total of 36 female FVB/N mice, 8-week-old young adults, were anesthetized and a 5-mm-diameter circular full-thickness defect was created at the lambda suture of the calvaria. The decellularized allografts were placed into the calvarial defect, leaving no direct contact with the host bone, after which the scalp was sutured. Mice in the allograft-implanted groups were randomly treated daily with subcutaneous administration of either teriparatide (40 μg/kg body weight, teriparatide dose was determined to meet the accepted dose in the literature [32,33]) or PBS mock treatment for 3 weeks.

### 2.2. Molecular fluorescence imaging (FLI)

Neovascularization was assessed by imaging an integrin-$a_3\beta_v$–targeted probe (IntegriSense750, PerkinElmer, Waltham, MA). A solution of 2 nmol probe dissolved in 100 μl PBS was injected into the mice via the tail vein on Day 13, one day before imaging, which took place on Day 14. Images were acquired using the IVIS Kinetic system (PerkinElmer) with an excitation light of 710 nm. Light emission was collected using a 750 nm–770 nm wavelength filter.

Mineralization of newly formed bone was quantified by imaging of Alexa Fluor 647–labeled risedronate (AF647-RIS), a fluorescent nitrogen-containing bisphosphonate analogue, was found to be bound to bone surfaces [34]. Imaging sessions were performed weekly beginning a week after allograft implantation until the fourth week, 24 h after the probe that was administrated intraperitoneally, using an excitation light of 640 nm. Light emission was collected using a 670 nm–690 nm wavelength filter.

### 2.3. Laser Doppler imaging (LDI)

Functional analysis of the vascular tree was performed using LDI. The mice were anesthetized by continuous inhalation of 1%–3% isoflurane mixed with 100% medical-grade oxygen. The scalp was surgically removed, and each mouse was scanned using an infrared laser Doppler imager (Moore Instruments, Devon, UK) with the bandwidth set at 250–150 kHz.

### 2.4. Micro-computed tomography (μCT) analysis

The μCT analysis protocol has been thoroughly described [31] and is depicted in detail in the Supplemental data section. Blood vessels were demonstrated on μCT scans obtained 1 week and 2 weeks after surgery. In brief, the mice were anesthetized by an intraperitoneal injection of ketamine/medetomidine. We dissected each mouse's thoracic cavity and flushed the vasculature via a needle inserted into the left ventricle with heparinized 0.9% normal saline (100 U/ml) followed by 10% neutral buffered formalin, and a radiopaque silicone rubber compound containing lead chromate, Microfil MV-122 (Flow Tech Inc., Carver, MA). Samples were stored at 4 °C overnight, after which the calvarial region was isolated. μCT scanning and analysis were performed using a Desktop μCT 40 (Scanco Medical AG, Bassersdorf, Switzerland). ANOVA and the Bonferroni post hoc test were used for statistical analysis of each vessel diameter [26].

Bone formation was measured by μCT using parameters detailed in the Supplemental Data section [35].

### 2.5. Intravital microscopy

Prior to this study the mice were anesthetized by means of 3% isoflurane inhalation. The animals were scalped and imaged immediately using a visible channel of a Nikon two-photon microscope with NIS-Elements image acquisition software (Nikon, Tokyo, Japan). In each animal, four fields containing the graft-host interphase were randomly chosen and imaged. Each field was imaged between four and seven times using different focus planes, which were merged into one image. The blood capillaries were manually contoured; this was followed by segmentation and generation of the thickness map using ImageJ software. For statistical analysis, ANOVA followed Bonferroni tests.

### 2.6. Histological and immunofluorescence analyses

Histological and immunofluorescence analyses are fully described in the Supplemental Data section.

### 2.7. Magnetization transfer contrast (MTC) μMRI

Each mouse was anesthetized by 3% isoflurane inhalation. Imaging was performed at 37 °C using a 300WB Bruker biospectrometer (7 T) with gradient-echo sequences (1.0173-sec TR, 4-msec TR, 30° field angle, and 11-mm FOV). High-resolution MRI was performed ex vivo. Freshly isolated samples of calvaria were embedded in Fluorinert FCC-77 (3 M electronics, Belgium NV). Imaging was performed at 293.3 K using an Avance 360 NMR spectrometer (8.4 T, Bruker Corp., Billerica,

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