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# The temporal and spatial development of vascularity in a healing displaced fracture

Masato Yuasa [a,f,1], Nicholas A. Mignemi [a,b,1], Joey V. Barnett [c], Justin M.M. Cates [b], Jeffry S. Nyman [a,d], Atsushi Okawa [f], Toshitaka Yoshii [f], Herbert S. Schwartz [a,b], Christopher M. Stutz [a], Jonathan G. Schoenecker [a,b,c,d,e,*]

[a] Vanderbilt University Medical Center, Department of Orthopaedics, 2200 Children's Way, Nashville, TN 37232-9565, USA
[b] Vanderbilt University Medical Center, Department of Pathology, C-2314 Medical Center North, Nashville, TN 37232-2561, USA
[c] Vanderbilt University Medical Center, Department of Pharmacology, 438 Robinson Research Building, Nashville, TN 37232-6600, USA
[d] Vanderbilt University Medical Center, Center for Bone Biology, 1255 MRB IV, Nashville, TN 37232-9565, USA
[e] Vanderbilt University Medical Center, Department of Pediatrics, 2200 Children's Way, Nashville, TN 37232-9565, USA
[f] Tokyo Medical and Dental University, Department of Orthopaedic and Spinal Surgery, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

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ABSTRACT

Underlying vascular disease is an important pathophysiologic factor shared among many co-morbid conditions associated with poor fracture healing, such as diabetes, obesity, and age. Determining the temporal and spatial patterns of revascularization following a fracture is essential for devising therapeutic strategies to augment this critical reparative process. Seminal studies conducted in the last century have investigated the pattern of vascularity in bone following a fracture. The consensus model culminating from these classical studies depicts a combination of angiogenesis emanating from both the intact intramedullary and periosteal vasculature. Subsequent to the plethora of experimental fracture angiography in the early to mid-20th century there has been a paucity of reports describing the pattern of revascularization of a healing fracture. Consequently the classical model of revascularization of a displaced fracture has remained largely unchanged. Here, we have overcome the limitations of animal fracture models performed in the above described classical studies by combining novel techniques of bone angiography and a reproducible murine femur fracture model to demonstrate for the first time the complete temporal and spatial pattern of revascularization in a displaced/stabilized fracture. These studies were designed specifically to i) validate the classical model of fracture revascularization of a displaced/stabilized fracture, ii) assess the association between intramedullary and periosteal angiogenesis and iii) elucidate the expression of VEGF/VEGF-R in relation to the classical model. From the studies, in conjunction with classic studies of angiogenesis during fracture repair, we propose a novel model (see abstract graphic) that defines the process of bone revascularization subsequent to injury to guide future approaches to enhance fracture healing. This new model validates and advances the classical model by providing evidence that during the process of revascularization of a displaced fracture 1) periosteal angiogenesis occurs in direct communication with the remaining intact intramedullary vasculature as a result of a vascular shunt and 2) vascular union occurs through an intricate interplay between intramembranous and endochondral VEGF/VEGF-R mediated angiogenesis.


## Introduction

Fracture is arguably the most common medical condition treated by orthopedic surgeons, with over 5 million fractures treated annually in the United States alone [1]. Between 2.5 and 10% of these fracture cases are complicated by delayed union or non-union [2–8]. The increasing incidence of comorbid conditions commonly associated with non-union, such as obesity, diabetes, and age suggests that the rate of non-union will only continue to rise [9–13]. In addition to substantial morbidity for patients, these complications impose a significant cost burden on the health care system. Thus, there are considerable ongoing efforts to develop novel methods of fracture fixation and/or application of bone-inducing biological agents to stimulate fracture healing. Given that angiogenesis is a requisite for fracture healing [14–19] and that vascular dysfunction is common to many comorbid conditions associated with poor fracture healing [20–24], it has been hypothesized that a primary cause of delayed union or non-union is

* Corresponding author at: Vanderbilt University Medical Center, Department of Orthopaedics, 2200 Children's Way, Nashville, TN 37232-9565, USA.
*E-mail address:* Jon.Schoenecker@vanderbilt.edu (J.G. Schoenecker).
[1] These authors contributed equally on this publication.

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impaired angiogenesis. A necessary step in developing approaches to augment angiogenesis during fracture repair is the identification of the temporal and spatial patterns of vascularization following an injury.

Seminal studies conducted in the last century have investigated the pattern of vascularity in bone following a fracture. Investigations by Kolodny [25], Teneff [26] and Göthman [27] suggest a model in which the dominant source of fracture revascularization is the periosteum and other extra-osseous vascular sources such as adjacent skeletal muscle. Conversely, Ladanyi and Trueta discovered patterns of revascularization that apparently contradicted this model by demonstrating the essential nature of the intramedullary vasculature in revascularization of a fracture [28,29]. In a series of papers published in the 1960s, this controversy was revisited by Rhinelander who showed that the mechanism of revascularization was dependent on the type of experimental fracture model employed [30,31]. He made a key observation that in non-displaced fractures, the intramedullary vascularity reunited relatively early, without development of a significant periosteal component (so-called primary bone healing through "trans-medullary revascularization"). However, a different pattern of revascularization was observed in fractures with significant displacement, even with subsequent reduction and stabilization. In the displaced fracture model, initial formation of new periosteal vasculature was followed by intramedullary revascularization (so-called secondary bone healing or "trans-periosteal revascularization"). Thus, Rhinelander's work resolved the apparent discrepancy by showing that different mechanisms of revascularization are employed in different experimental models (displaced vs. non-displaced fractures). The consensus model of revascularization of a displaced fracture culminating from these classical studies depicts a combination of angiogenesis emanating from both the intact intramedullary and periosteal vasculature.

Subsequent to the plethora of experimental fracture angiography in the early to mid-20th century there has been a paucity of reports describing the pattern of revascularization of a healing fracture. Consequently the classical model of revascularization of a displaced fracture has remained largely unchanged. Determination of the molecular elements responsible for and the pattern of revascularization following a fracture are essential for targeting and augmenting discrete events during fracture healing. Importantly, key elements of fracture revascularization were unclear following the development of this model. Specifically, the association of angiogenesis in the intramedullary and periosteal space was unknown. It was hypothesized, that fracture angiogenesis in these anatomically distinct compartments occurred independently and only formed an anastomosis in the later stages of fracture healing [31]. It was proposed that intramedullary angiogenesis occurred as a result of direct vascular growth from the remaining intact intramedullary vascularity and periosteal angiogenesis occurred as a result of direct vascular growth from the surrounding musculature. Additionally, the factors directing vascular union were unknown as vascular endothelial growth factor (VEGF) and its receptors (VEGF-R) had yet to be discovered [32]. Thus, the molecular patterning leading to vascular union has not been incorporated into this classical model. In addition to these unanswered questions, the principle reason for the lack of advancement in this field has been critical methodological restraints prohibiting high throughput animal studies on fracture vascularity. Specifically, the classical models were performed primarily in relatively cost-inefficient larger animal models without the benefit of axial imaging allowing for detailed evaluation of fracture related angiogenesis.

Here, we have overcome the limitations of animal fracture models performed in the above described classical studies by combining novel techniques of bone angiography and a reproducible murine femur fracture model to demonstrate for the first time the complete temporal and spatial pattern of revascularization in a displaced/stabilized fracture. These studies were designed specifically to i) validate the classical model of fracture revascularization of a displaced/stabilized fracture, ii) assess the association between intramedullary and periosteal angiogenesis and iii) elucidate the expression of VEGF/VEGF-R in relation to the classical model.

From the studies, in conjunction with classic studies of angiogenesis during fracture repair [25,26,28,30,31,33–43], we propose a novel model (see abstract graphic) that defines the process of bone revascularization subsequent to injury to guide future approaches to enhance fracture healing. This new model validates and advances the classical model by providing evidence that during the process of revascularization of a displaced fracture 1) periosteal angiogenesis occurs in direct communication with the remaining intact intramedullary vasculature as a result of a vascular shunt and 2) vascular union occurs through an intricate interplay between intramembranous and endochondral VEGF/VEGF-R mediated angiogenesis.

## Materials & methods

### *Murine fracture model and X-ray imaging*

Protocols were approved by the Vanderbilt University IACUC. Open femur osteotomies were performed and fixed as previously described using a medial approach to the mid-shaft femur of 8 week old c57/b6 mice. The fracture was fixed through the placement of a 23-G (0.6414 mm) retrograde intramedullary pin and the mice were examined from 7 to 42 days after fracture [44]. X-ray was performed as previously described [45]. Briefly, the mice were placed in the prone position and imaged for 4 s at 45 kV using a Faxitron LX 60. The mice were sacrificed at various time points (7–42 days) following a fracture.

### *Angiography*

Perfusion with Microfil (MV-122 Flow Tech Inc., Carver, MA) vascular contrast was conducted as previously described [46–49]. Briefly, the mice were euthanized, positioned supine and a thoracotomy extending into a laparotomy was performed. The left ventricle of the heart was cannulated using a 25-G butterfly needle. The inferior vena cava (IVC) was transected proximal to the liver and the entire vasculature subsequently perfused with 9 ml of warm heparinized saline (100 units/ml in 0.9% saline) through the left ventricle cannula to exsanguinate and anticoagulate thus preventing erythrocyte aggregation and thrombosis thereby promoting consistent perfusion of subsequent infusion materials. Exsanguination and anticoagulation was deemed complete upon widespread hepatic blanching with clear fluid extravasating from the IVC. The mice were then perfused with 9 ml of 10% neutral buffered formalin followed by 3 ml of Microfil (Flow Tech inc. Carver Massachusetts) vascular contrast polymer. To best verify complete filling of the vasculature gross images of the liver (Supplemental Fig. 1a), the last organ to be perfused prior to extravasation through the IVC, was examined for extravascular pooling. The mice were excluded from the study if complete hepatic blanching prior to Microfil was not achieved, if contrast was not clearly or uniformly visible in the hepatic vasculature or if extravascular pooling occurred. Inspection of hepatic perfusion revealed no differences between manual and infusion pump perfusion set at a constant rate of 0.5 ml/min (Supplemental Fig. 2a). Manual filling was conducted at a goal rate approximately equal to the pump rate.

### *Generation of angiogram images*

The mice perfused with Microfil were then stored overnight at 4 °C to allow the vascular contrast to polymerize. The femurs were dissected and fixed in 4% PFA for another 24 h. X-rays of the samples were then taken to visualize the femur and the vascular contrast (Supplemental Fig. 1b). The femur was placed in 0.5 M EDTA pH 8 for decalcification then imaged via X-ray (Supplemental Fig. 1c). The muscles surrounding the femur were removed, and the leg was photographed and X-rayed (Figs. 1d & e). Demineralized specimens were imaged by μCT (μCT40, Scanco-Medical-AG, Bassersdorf, Switzerland) with a 20 μm isotropic