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Angiogenesis in Bone Regeneration

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Abstract

Angiogenesis is a key component of bone repair. New blood vessels bring oxygen and nutrients to the highly metabolically active regenerating callus and serve as a route for inflammatory cells and cartilage and bone precursor cells to reach the injury site. Angiogenesis is regulated by a variety of growth factors, notably vascular endothelial growth factor (VEGF), which are produced by inflammatory cells and stromal cells to induce blood vessel in-growth. A variety of studies with transgenic and gene-targeted mice have demonstrated the importance of angiogenesis in fracture healing, and have provided insights into regulatory processes governing fracture angiogenesis. Indeed, in animal models enhancing angiogenesis promotes bone regeneration, suggesting that modifying fracture vascularization could be a viable therapeutic approach for accelerated/improved bone regeneration clinically.

Clinical Significance of Angiogenesis in bone regeneration

An essential component of fracture healing is the appropriate development of blood vessels in the fracture callus. Immediately following injury the volume of the vascular bed of the tissue becomes increased due to vasodilation. This hyperemia contributes to edema and swelling of the injured limb, but also contributes to the formation of fracture hematoma, which is the template for the formation of the provisional, vascular callus.

Despite the immediate increase in blood flow to the injured extremity, a period of necrosis and hypoxia follows that is a normal part of healing. This necrosis results from mechanical injury to tissue in the peri-fracture region, as well as loss of nutritional support from the damage to the surrounding blood vessels. This necrotic bone must be resorbed and the region revascularized as healing progresses. When this process of normal vascularization is altered or disrupted, pathologic conditions of healing arise.

An appropriate blood supply has historically been recognized as an essential component of normal healing fracture healing [21], and defective angiogenesis at the fracture site has been a primary consideration when poor outcomes occur. Fracture non-union is a clinical diagnosis made when a patient has clinical symptoms of pain at the fracture site, pathologic motion, and radiographic findings consistent with non-union. Non-unions can be categorized into

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three types, based primarily on the amount of bone formed at the fracture site radiographically: hypertrophic, atrophic, and oligotrophic [60].

The classification scheme is also intended to guide treatment by suggesting non-union causation. Atrophic non-unions, in which there is minimal evidence of bone formation, have classically been suggested to occur secondary to poor blood supply at the fracture site [9]. These non-unions are traditionally seen as the most refractory to treatment owing to the difficulty of improving angiogenesis, and can require multiple surgeries over many years [32]. Thus, an improved understanding of vascularization in fracture union and non-union is essential for advancing orthopaedic care.

Several studies have pointed to the importance of blood flow at the fracture site. Dickson et al found a three-fold higher rate of tibial fracture non-union in patients with open fractures and abnormal distal arteriograms[21]. Open fractures in general are considered to be at greater risk of non-union, especially in long bones, such as the tibia. This increased risk is thought to result from injury to the soft-tissue envelope that deprives the fracture of normal blood supply, leading not only to diminished vascular in-growth to the callus, but also to more bone necrosis, and less resistance to infection [13]. For fractures with severely damaged or absent soft-tissue, a free-flap is the treatment of choice. A free flap provides not only containment of the microenvironment but also a limitless supply of undamaged, well-vascularized soft tissue. Yazar et al. were able to achieve 97% union rate in Gustilo type IIIB distal-third tibia fractures with free-flap technique, a substantial improvement over conventional open fracture healing, attesting to the value of improving the vascularity of the injury region[90].

Moreover, in cases where the periosteal blood supply has been disrupted it is considered of particular importance to preserve the endosteal blood supply, which is thought to contribute to revascularization in these cases through collateral circulation. This has led several authors to compare healing rates of reamed versus un-reamed intramedullary nails in treating open tibia fractures. Ziran et al. compared un-reamed and “reamed-to-fit” technique in open tibial fractures and found no significant difference in healing rates, but they did find a greater number of second surgeries required to achieve union in the un-reamed group [93]. This may suggest the importance of endosteal blood supply in achieving union.

Further evidence of the importance of angiogenesis in clinical fracture healing can be seen by examining the effect of cigarette smoking on fracture union. Studies have shown that smoking decreases the rate of union in fractures. Simonis et al. demonstrated that individuals with tibial non-unions have a higher rate of smoking than the general public and that smoker’s fractures are more recalcitrant to further intervention to promote union [78]. Ueng et al demonstrated that cigarette smoking diminishes vascularization at bone healing sites through the action of nicotine, which delays mineralization as well as torsional and bending strength of regenerating bone [85]. Additionally, the Lower-Extemity Assessment Project (LEAP) study found that smokers have a 24% chance of open tibia fracture non-union at the 24-month time point compared with a 9% chance in non-smokers[15]. In many of these cases dysfunctional angiogenesis is hypothesized, but rarely proven.

Vascularization in bone repair

New blood vessels in the adult form through two processes. Angiogenesis, in which new vessels out-grow from existing vessels, is the more common. Alternatively, new blood vessels form through a process termed vasculogenesis in which new blood vessels form without a pre-exisiting vascular component. Vasculogenesis occurs through either local differentiation of endothelial progenitor cells, or more likely, circulating endothelial progenitor cells (EPC) contribute to the process [84]. In particular in ischemic skin wounds

EPC traffic to the site of injury and contribute to not only the formation of new vessels (vasculogenesis) but also contribute to the process of angiogenesis. The relative contribution of angiogenesis and vasculogenesis to fracture has not been well-studied, but Lee et al 2008, show that during both fracture healing in the mouse and distraction osteogenesis in the rat there is an increase in mobilization of EPC to the site of bone injury [53].

New blood vessels form secondary to signaling by angiogenic growth factors, in particular, vascular endothelia growth factor (VEGF). VEGF not only increases endothelial cell differentiation and proliferation, but increases tube formation and the mobilization and recruitment of endothelial progenitor cells [3]. VEGF has three isoforms, A, B, and C that form homo and heterodimers and bind to a dimeric receptor complex of 2 receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1) [26].

In fracture healing pro-angiogenic factors such as VEGF are highly elevated early post-fracture and are shown to be increased with distraction osteogenesis [14]. Indeed, blocking VEGF signaling with antibodies against VEGF receptors 1 and 2 demonstrates that intramembranous bone formation during distraction osteogenesis is dependent upon VEGF signaling [45]. VEGF in this context is likely produced by inflammatory cells as well as mesenchymal progenitors that are recruited to the site of bone injury [10,34,42]. VEGF expression can be driven secondarily to hypoxia as VEGF represents a canonical target gene of hypoxia-inducible factor [20]. Mice with enhanced HIF1-alpha transcriptional activity show profoundly increased bone mass and in a distraction model of bone regeneration show accelerated intramembranous bone formation [88].

New vessels are essential in healing bone for the obvious reason of providing nutrition and gas exchange as well as an egress for break-down (waste) products. Vascularization is also required for both intramembranous and endochondoral bone formation. During endochondral bone formation, the avascular environment of cartilage is invaded by blood vessels fronted by chondroclastic cells with osteoblast progenitors which will deposit new bone on the surface of cartilage islands. Intramembranous bone formation, while different from endochondral bone formation, also requires vascularization, presumably to provide ingress for osteoblast progenitors. Blood vessels also provide systemically circulating factors that may modify fracture healing such as PTH and Vitamin D.

Regulation of angiogenesis in bone repair

A variety of surgical, pharmacological and genetic models of fracture healing have been developed that have led to a greater understanding of the molecular mechanisms controlling callus vascularization and the essential role that vascularization plays in the process of healing. Models developed that show alterations in fracture healing in conjunction with defects in angiogenesis will be discussed in the following section.

Surgical models of disrupted vascularization have been developed and described by a number of groups. Recently Oetgen et al. developed a reproducible non-union model in mouse femur, which reliably yields fibrous non-union as far out as 35 days with no signs of bony callus formation. This model is based on devascularization of the lateral femoral periosteum with electrocautery, 2mm proximal and distal to the fracture site. Furthermore, the fractures are stabilized with intramedullary fixation and are biologically clean, pointing to a lack of angiogenesis as a primary culprit [69]. An ischemic model developed by Lu et al, uses femoral ligation to limit limb vascular perfusion. In this model there is little evidence of early callus vascularization, but the proportion of cartilage-to-bone formation is similar to vascularized fractures [59]. Instead, total callus size is diminished, cell proliferation decreased, apoptosis increased, and fibrous and adipose tissue are more likely to form than cartilage and bone [59]. These alterations ultimately led to delayed or non-union fractures [59].

The temporal importance of angiogenesis in contributing to non-union development is considered by Brownlow et al., which demonstrated by immunocytochemistry that at 8 and 16-weeks post-fracture, established atrophic non-unions were well-vascularized in a rabbit tibia non-union model. Earlier time points, however, did show a discrepancy in vessel concentration between union and non-union groups, particularly at 1 week [9]. This observation may suggest that angiogenesis could be most important in the early period of fracture healing. The fibrous tissue of non-unions may become revascularized eventually, but a critical window for fracture union has been missed, and some other manipulation of the fracture biology may be necessary to promote union. This has important clinical importance because it suggests that angiogenic therapy to promote healing may be required early rather than late.

In addition to surgical models demonstrating the importance of vascularization, age has been shown to decrease angiogenic potential during murine fracture healing [58] and in turn fracture healing is delayed in both human geriatric patients and aged animal models. However , aging is physiologically complex and whether alterations in fracture healing with age are associated with defeciencies in vascularization have not been established.

The importance of VEGF signaling in angiogenesis and vasculogenesis is well established, and similarly its importance in regulating endochondral bone formation was established in a model were VEGF activity was inhibited by exogenous application of Flt-IgG, a VEGF receptor. VEGF expression in hypertrophic chondrocytes is required to stimulate vascular invasion and cartilage resorption during endochondral bone formation [30]. Similarly, use of a conditional chondrocyte (Col2a1 promoter) VEGF knockout further demonstrated that VEGF activation is required for proper hypertrophic chondrocyte resorption, vascular invasion, and new bone formation during embryological endochondral ossification [71]. In fracture VEGF is also required for direct callus vascularization during fracture healing [80] and distraction osteogenesis [45].

While the previous studies demonstrate the requirement for VEGF signaling in regulating fracture vascularization, the regulation of VEGF expression in fracture tissue is also of importance. The hypoxia-inducible factor (HIF) pathway is activated in areas of low oxygen tension, such as the avascular portion of the cartilaginous callus [51]. HIF is a transcription factor that directly increases VEGF gene expression. HIF gain and loss of function models have been developed using floxed pVHL, a negative regulator of HIF-1α (OC-Cre;pVHL$^{f/f}$), and floxed HIF-1α (OC-Cre;HIF-1α$^{f/f}$) on the osteocalcin reporter, respectively. These conditional models demonstrate that the pro-angiogenic effects of HIF-1α during fracture healing are primarily mediated through its downstream activation of VEGF [88], and that enhanced callus vascularization improves bone regeneration [88]. Similarly, Komatsu et al demonstrated delayed bone regeneration in HIF-1α heterozygous mice [50].

While hypoxia is likely a driving force behind VEGF production, VEGF can also be matrix bound and its release from the matrix by matrix metalloproteinases (MMPs) may represent another regulatory pathway in the regulation of VEGF activity in healing bone. MMP play an essential role during enzymatic degradation and remodeling of extracellular matrices. MMP9 and MMP13 knockout models have been used to study effects of MMPs on coupled mechanism of cartilage resorption and callus vascularization. MMP9 is expressed in chondroclasts and osteoclasts during fracture healing. MMP9$^{-/-}$ mice fail to recruit these cells during fracture healing, which delays vascularization and results in persistence of cartilage [19]. This hypertrophic non-union phenotype is transient, suggesting compensatory mechanisms involving other pro-angiogenic factors [19]. Interestingly, while MMP9$^{-/-}$ mice did not show decreased VEGF expression in hypertrophic chondrocytes, application of exogenous rVEGF rescued the MMP9$^{-/-}$ phenotype, suggesting that MMP9-mediated

cartilage resorption indirectly activates callus vascularization by releasing VEGF bound to hypertrophic cartilage matrix [19].

Another MMP expressed during fracture healing is MMP13. MMP13 is expressed in hypertrophic chondrocytes and osteoblasts. While fracture healing in MMP13$^{-/-}$ mice results in delayed cartilage resorption and altered callus vascular invasion similar to the MMP9$^{-/-}$ phenotype, the molecular mechanisms controlling these processes differ [6,52]. In contrast to MMP9$^{-/-}$ healing, the MMP13$^{-/-}$ fracture callus does not show reduced angiogenic potential or osteoclast number. Instead, MMP13$^{-/-}$ mice exhibit decreased proteoglycan degradation within the cartilage matrix, preventing recruited blood vessels and resorbing cells from penetrating the cartilage. This intrinsic role for MMP13-mediated cartilage ECM degradation in chondrocytes is paralleled in osteoblasts during bone remodeling as well [6]. Therefore, while impaired cartilage resorption delayed vascular invasion of the MMP13$^{-/-}$ callus, the amount of new immature bone present in the callus at later time points was not reduced and in fact persisted because of retained woven bone [6]. The MMP13-mediated degradation mechanism in chondrocytes and osteoblasts appears to occur prior to and independent of MMP9-mediated degradation in chondroclasts and osteoclasts [6].

Vascularization activity is also likely regulated by the inflammatory environment of early callus. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that is activated in fracture. By knocking out its receptors (p55$^{-/-}$p75$^{-/-}$), TNF-α was shown to be required for timely cartilage formation and resorption [31], acting upstream of MMPs 2, 9, 13, and 14 [54]. While TNF-α did not directly regulate VEGF signaling, TNF-α did stimulate other angiogenic factors such as angiopoietin 2 and PEDF [54].

Prostaglandins are additional key inflammatory mediators present during early fracture healing. The activity of COX2, a cycloxyganse that is required for the synthesis of pro-inflammatory prostaglandins, is a rate-limiting step in prostaglandin production. Pharmacologic inhibition of COX2 via clinically available NSAIDs, or using genetic knockout models (COX2$^{-/-}$) has shown a severe vascular reduction during fracture healing that is in part mediated through MMP9 inhibition [77,89,91]; however, similar to MMP9 and TNF-a, COX2 did not directly regulate VEGF expression. In keeping with this hypothesis, Murnaghan et al in 2006 displayed a decrease in laser Doppler blood flowmetry through mouse fracture sites in mice treated with rofecoxib, a selective COX-2 inhibitor [64]. Of note, long-acting COX-2 inhibitors were shown by O'Connor et al. to have a more deleterious effect on fracture histological and mechanical healing in rabbit tibial fractures than short acting NSAIDs, although they did not identify deficient angiogenesis as the primary cause.[68]

In addition to signaling molecules playing a role in regulating callus vascularization, the extracellular matrix (ECM) also regulates angiogenesis. TSP2 is a matricellular protein that modulates cell-matrix interactions [8]. TSP2$^{-/-}$ fractures show increased callus vascularity coupled with a shift in early mesenchymal differentiation from the chondrogenic to the osteoblastic lineage [83]. Cartilage is inherently hypoxic. This model of enhanced vascularization demonstrated that a hyperoxemic environment encourages osteoblastogenesis at the expense of chondrogenesis in order to advance the endochondral transition from cartilage to bone [83]. Genetic knockouts of osteopontin, another matricellular protein, during fracture healing exhibited a transient delay in callus neovascularization that was ultimately recovered [23].

Therapeutic angiogenesis for enhanced bone repair

Therapeutic angiogenesis has been suggested for management of fracture healing in acute injuries, non-unions, and distraction osteogenesis. Various growth factors in the angiogenic

cascade, including VEGF, FGF, and PDGF, are potential targets for upregulation and direct administration. Additionally, other suggested therapies to improve angiogenesis include enhancement of HIF signaling, blockade of angiogenesis inhibitors, delivery of endothelial progenitor cells, ultrasound therapy, fracture stabilization, and enhanced weightbearing. The following section outlines the above therapeutic options for improved angiogenesis in animal and clinical models of bone injury.

The United States Food and Drug Administration has granted approval for use of osteogenic growth factors, BMP-2 and BMP-7, for fracture and non-union management. Similarly, exogenous administration of angiogenic growth factors, including VEGF, FGF, and PDGF, have been investigated in animal models, with therapeutic goals of combined angiogenic and osteogenic induction. As previously highlighted endogenous VEGF is important for endochondral bone formation, and is essential for blood vessel invasion of hyaline cartilage, growth plate morphogenesis, and cartilage remodeling[30]. Considering the importance of endogenous VEGF in normal bone formation and repair, treatment with exogenous VEGF would also be useful to promote angiogenesis and fracture healing. Exogenous VEGF enhances angiogenesis [57,80,82] and improves blood flow [24] in animal models. Moreover, Street et al. noted improved ossification, and callus maturation in a mouse femur fracture model, and improved bony bridging in a rabbit radial segmental gap defect [80]. Eckardt et al. showed increased failure torque, failure angle, stiffness, and cross-sectional area of callus, with similar results to autograft in a rabbit nonunion model [25]. Tarrka et al. noted faster healing and enhanced mineral density in a rat model [82].

Fibroblast growth factor-2 (FGF2) is another growth factor that increases vascularization. However, the role of FGF in fracture healing is not well understood because FGF2 not only induces angiogenesis [18,37,61], but also stimulates mitogenesis of mesenchymal progenitors and osteoblasts [33]. Several studies have demonstrated positive angiogenic and healing effects of exogenous administration of FGF following fractures [2,7,16,47,48,65,66,72]. Treatment with a single dose of FGF-2 increased the callus formation and structural stability of fibular fractures in normal and diabetic rats [47]. Similarly, incorporation of FGF2 in a gelatin hydrogel increased bone mineral density and cancellous bone area in a rabbit osteotomy model at 4 and 8 weeks post-injury[16]. However, while a single dose of FGF2 in a hydroxyapatite graft increased development of hyaline cartilage and vascularity two weeks following segmental defect in a rat; mechanical strength was transiently reduced[2]. Local administration of either FGF1 and FGF2 four days after tibial defect in a rat model did not affect the size or amount of cartilage and bone in the callus [7].

Studies in large animal models also support a positive role for exogenous administration of FGF2 in fracture healing [48,66,72]. In a canine tibial fracture model, injection of 200 μg of FGF-2 at the fracture site increased callus area, mineral content, and mechanical strength[66]. Two non-human primate studies demonstrated increased callus area, mechanical strength, and improved vascularity with local application of FGF2 in gelatin hydrogels[48,72]. Results from these various small and large animal *in vivo* studies show that the effectiveness of FGF2 in promoting fracture healing is likely a result of the time and dose of FGF. The minimum dose required for increased bone volume and mineral content in a rabbit segmental defect was shown to be 100 μg [46]. Similarly, a slow release formula allowed for lower effective doses (to 1.4 μg) in a rabbit segmental defect model, whereas a single dose of 2 μg was not sufficient to promote repair [43].

Platelet-derived growth factor (PDGF) is another key growth factor in fracture healing that appears to stimulate angiogenic as well as osteogenic pathways. PDGF is released by degranulating platelets and is chemotactic and mitogenic for osteoblasts, and is both directly angiogenic and upregulates VEGF expression [39]. Animal studies have evaluated the effects

of recombinant PDGF on fracture healing [40,67]. PDGF administration improved the mechanical strength in rabbit tibial metaphyses following osteotomy [40]. Similarly, local administration of PDGF in a collagen gel increased callus density and volume, and improved mechanical strength following proximal tibial osteotomy in a rabbit model [67]. One recent review illustrated the unpublished results of a human pilot study, which evaluated effects of combined external fixation and local PDGF following distal radius fractures in the geriatric population. These authors showed improved bone filling by computed tomography and equivalent functional outcome scores at 3 and 6 weeks following injury [40].

Thrombin-peptide 508 (TP-508) is a thrombin peptide that mimics the thrombin healing response, without promoting clotting. It stimulates cell chemotaxis and proliferation, and stimulates angiogenesis [86]. *In vivo* studies have examined the effect of exogenous application of TP-508 [56,74,75,87]. A single injection of TP-508 increased the number of blood vessels per unit area by 40% and increased the area occupied by blood vessels by 80% in a rat femur fracture model [87]. Mechanical strength was also significantly increased 3 weeks post-injury. Similarly, a controlled-release microsphere containing TP-508 improved the mechanical strength of healing radius and ulna fractures in rabbits [75]. Moreover, increased bone consolidation and blood-vessel development was noted following TP-508 administration in a rabbit distraction osteogenesis model [56]. Single injection of TP-508 in a closed rat femur fracture model increased the mechanical strength of the healing fracture at 3 weeks post-injury, and resulted in increased vessel number and callus size as compared to controls [74]. One recent review article illustrated the unpublished results of a human pilot study, which evaluated the effects of local administration of TP-508 in combination with external fixation, percutaneous fixation, or casting of geriatric distal radius fractures. Preliminary results demonstrated safety and accelerated radiographic healing [74].

Erythropoietin (EPO) is a cytokine that has significant homology with VEGF [36,79]. Both VEGF and EPO are similarly stimulated by hypoxia through an analogous pathway [36,79]. In addition to established effects on red blood cell proliferation, EPO promotes angiogenesis [1]. In a murine femur fracture model, EPO injected systemically induced better mechanical strength, callus formation, and bone mineral density [41].

An indirect mechanism for enhancing bone formation, presumably through enhanced expression of VEGF and increased angiogenesis, has recently been demonstrated by Gilbert et al [76]. Inhibitors of proyly hydroxylase, which result in the stabilization of HIF1-alpha, were shown to increase callus bone mass.

While there are few examples of this in the literature, another approach to promote angiogenesis would be to block the activity of an angiogenesis inhibitor. Pro-angiogenic and anti-angiogenic growth factors exist in a dynamic balance. As an example, the thrombospondin family of proteins are potently anti-angiogenic, and data from a model in which thrombospondin-2 (TSP2) is knocked out demonstrates an increase in fracture vascularization and accelerated intramembranous bone formation [83]. Blocking the CD47 binding activity using soluble inhibitors has been shown to promote revascularization of ischemic limbs in murine studies [44]. Thus, blocking TSP2 activity (or another angiogenesis inhibitor) could also be used to promote bone healing.

Cell-based therapies for bone regeneration have been extensively investigated. While these studies have mostly considered the delivery of mesenchymal progenitor cells to provide precursor cells to become osteoblasts and chondrocytes, a growing body of literature suggests that these mesenchymal progenitor cells may secrete positive acting factors that could increase angiogenesis if delivered to an injury site [12,81]. In some cases, delivered cells

may even be incorporated into the vasculature as has been demonstrated in a recent study in which EPC were delivered to a segmental defect site [4] .

Beyond modulation of growth factors and cell delivery, low-intensity pulsed ultrasound (LIPUS) has been utilized extensively to promote healing in animal and clinical models of fractures, non-unions, and distraction osteogenesis[5,11,27–29,35,38]. LIPUS is based on the theory that acoustic pressure waves are transmitted through the body by molecular vibrations and collisions [49]. Numerous clinical studies have shown that 20 minutes of LIPUS therapy per day can reduce the time to fracture healing by 17–51%, and are associated with nonunion healing rates of approximately 85%, which are equivalent to surgical intervention rates[11,27,28,35,38]. Although the exact mechanism of LIPUS on fracture healing is not well understood, *in vitro* and *in vivo* studies have suggested that it upregulates VEGF expression in osteoblast and periosteal cells [22,55], as well as increasing aggrecan and proteoglycan synthesis in chondrocytes [63,70,92]. To that end, doppler sonography of canine ulnar shaft fractures showed a 3-fold increase in blood flow 1 week after initiation of LIPUS therapy [73]. Together, these studies suggest that LIPUS may affect angiogenesis and chondrogenesis after fractures.

Lastly, various mechanical factors, including fracture fixation techniques and weight-bearing strategies, influence angiogenesis at fracture sites [17,62]. It is well-accepted that gross mechanical instability in the fracture healing zone leads to inhibition of vascularization and formation of excessive fibrocartilage. However, micromotion at the fracture site, through early weightbearing, may offer improved vascularization at the fracture site [62]. The balance between favorable micromotion and excessive macromotion at the fracture site needs to be better elucidated to optimize the mechanical fracture healing environment.

In summary, manipulation of angiogenesis during bone regeneration should be a promising therapeutic strategy for enhancing fracture healing. Numerous small and large animal pre-clinical studies have demonstrated the positive effects of promoting angiogenesis during bone regeneration. Future studies must identify ideal delivery methodologies and should focus on studies combining angiogenic therapy with osteoblastic inductive BMP treatment. Furthermore, appropriate clinical studies must be pursued.

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