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The Control of Fracture Healing and Its Therapeutic Targeting: Improving Upon Nature

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ABSTRACT

Fracture repair is a complex process involving timed cellular recruitment, gene expression, and synthesis of compounds that regenerate native tissue to restore the mechanical integrity, and thus function of injured bone. While the majority of fractures heal without complication, this takes time and a subset of patients (~10%) experience healing delays, extending their morbidity and treatment costs. Consequently, there is a need for efficacious therapeutics for the intervention of fracture healing. Recent studies into the molecular control of fracture repair and advances in the understanding of the skeleton as a whole have resulted in the identification of numerous novel targets and compounds for such intervention. These include traditional agents such bone morphogenetic proteins and other growth factors, but also relatively newer compounds such as parathyroid hormone and modulators of the Wnt signaling pathway. These agents, along with others, are discussed in the current article in terms of their investigative status and potential for clinical implementation. Hopefully, these agents, as well as others yet to be discovered, will demonstrate sufficient clinical utility for successful intervention of fracture healing. This may have significant implications for the duration of morbidity and costs associated with traumatic bone fractures. J. Cell. Biochem. 109: 302–311, 2010.

KEY WORDS: FRACTURE HEALING; HIF; Wnt; BMP

There are over 15 million fractures treated in United States annually and many more worldwide [Andersson, 2008]. While the vast majority of these fractures heal with appropriate orthopedic management, 10–15% of patients suffer complications that result in delayed- or non-union [Einhorn, 1998]. Many factors have been associated with failure of normal fracture healing, including anatomic criteria (e.g., fracture location, extent of soft tissue damage and interposition, degree of bone loss), factors exacerbated by treatment (e.g., infection, inadequate reduction, poor stabilization/fixation), patient characteristics and comorbidities (e.g., age, sex, diabetes, osteoporosis, estrogen deficiency), and drug use (e.g., nicotine, alcohol, chemotherapeutics, and non-steroidal anti-inflammatory drugs) [Einhorn, 1998]. Among these factors, osteoporosis stands out as most significant because it is not only associated with delayed/non-union, but also with increased fracture risk. In 2004 the number of fractures in patients with osteoporosis totaled 5.7 million and directly resulted in more than 700,000 hospital and 118,000 nursing home admissions in the US [Andersson, 2008]. With the aging of the population, the incidence and burden of osteoporotic fractures is predicted to substantially increase in the next few decades with upwards of 61 million people within the US at risk for osteoporotic fractures by 2020 and cumulative costs estimated at $474 billion [Andersson, 2008]. Dollar costs are one way to assess the burden of fractures on the population, but disability is another key metric. When assessed by limitations in the ability to perform normal life activities, fractures or other bone and joint injuries result in disability in 4% of the overall US population and 11% of the aged population [Andersson, 2008]. In summary, the large incidence of fractures and associated financial costs, as well as concomitant patient disability, results in a significant ongoing and steadily increasing public health encumbrance. Understanding how the process of fracture repair is controlled, under physiological and pathophysiological conditions, is a necessary step in the development of therapeutics to enhance this process and reduce the public health burdens caused by fractures.

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CONTROL OF FRACTURE HEALING

EARLY INSIGHTS INTO THE CONTROL OF FRACTURE HEALING

In the past 100 years, our understanding of the control of fracture repair has advanced in tandem with the development of new methods for studying this process. Gross observation of limbs gave way to two-dimensional radiographic characterization of callus development, cortical bridging, and remodeling, which is now being supplanted by quantitative three-dimensional peripheral and micro-computed tomographic analyses of not only mineralized tissues, but vasculature as well. Rough determination of strength has yielded to precise determination of a multitude of whole bone and intrinsic material properties, with nanoindentation enabling measurement of mechanical properties at the microscopic level and developments in finite element modeling facilitating prediction of mechanical properties without the need for destructive testing. On the cellular level, early histological analyses of the cells responsible for fracture healing have lead to in vitro and in vivo characterization of these cells including their lineage, proliferation, differentiation, and ultimate fate. The relatively new field of molecular biology has allowed precise identification of transcriptional and translational profiles of the genes and proteins involved that ultimately control the process of fracture repair. Finally, the subsequent development of transgenic animals has enabled us to perturb these genes in a temporal and tissue-specific manner in order to definitively ascertain their role in the control of fracture repair.

Despite the aforementioned advances in scientific methodologies, the basic biologic processes that control fracture repair were understood over 50 years ago. Phemister [1951] summarized the biological principles of fracture healing as initiating with a local inflammatory reaction that is followed by callus development, bridging, and mineralization via both endochondral and intramembranous ossification, and eventual remodeling. In addition, this seminal paper discussed the importance of maintaining the biologic activity of bone grafts by limiting processing and highlighted the importance of the restoration of vasculature for successful fracture repair. It closed by stressing that equal attention should be paid to the mechanical and biological principles of fracture repair for their successful management and suggested that the biologic activity of bone grafts can be harnessed to augment fracture repair. Amazingly, more than 50 years later, bone grafts remain one of the only biologic products approved for use in augmenting fracture repair.

CURRENT UNDERSTANDING OF THE CONTROL OF FRACTURE HEALING

Our current understanding of the process of successful fracture repair still views it as a series of overlapping processes that begins with inflammation and proceeds to intramembranous ossification, chondrogenesis, endochondral ossification, and finally, remodeling that results in the scarless repair (i.e., regeneration) of the original bone (Fig. 1). However, we have now identified and characterized many of the signaling pathways activated in this process that together form a complex and interconnected web of transcriptional events that recapitulate aspects of embryonic skeletal development combined with normal responses to acute tissue injury [Hadjiar-

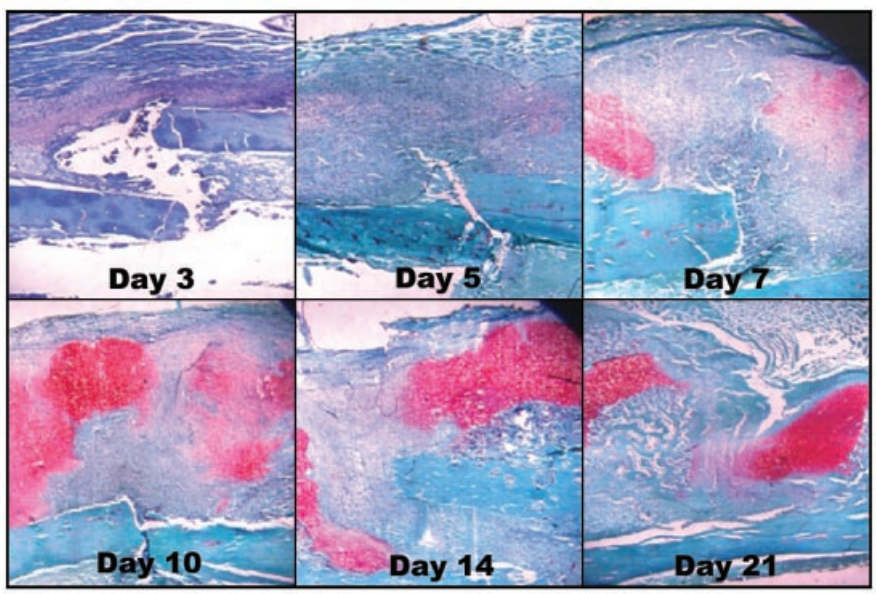

Fig. 1. Histologic progression of fracture healing. This series of photomicrographs illustrates the progression of fracture healing as seen following the generation of closed femoral fractures in rats. Longitudinal sections from decalcified fracture calluses after 3, 5, 7, 10, 14, and 21 days of healing were cut and stained with Safranin-O fast green to identify regions of cartilage (red) and bone (blue). At day 3, the initial inflammatory response is ending, although some inflamed bone marrow and periosteal swelling are visible, along with the initiation of chondrogenesis. The periosteum continues to expand and chondrogenesis increases, peaking at day 10, and then undergoing endochondral ossification. Intramembranous ossification is visible by day 7 near the fracture site and continues throughout the time course. Early signs of remodeling are present at day 21 and this process will continue until the fractured bone is restored to its original shape.

gyrou et al., 2002; Gerstenfeld et al., 2003]. The sheer number of genes involved in the control of fracture repair has been highlighted by several transcriptional profiling experiments. The first of these used a series of custom spotted cDNA microarrays and identified 588 known genes and 821 expressed sequence tags as up-regulated in the first 21 days of rat femoral fracture repair as compared to intact bone [Hadjiargyrou et al., 2002]. A later study performed using Affymetrix gene chips identified more than 2,200 genes as differentially expressed between fractured and intact bones 14 days following rat femoral fractures [Heiner et al., 2006]. More recently, a study using microarrays spotted with a murine oligo probe set reported that over 11,000 mRNAs were differentially regulated between intact and fractured murine femora in the 21 days following fracture, and suggested that more than half of the murine genome is differentially regulated during fracture repair [Bais et al., 2009].

The origins of transcriptional activation in fracture repair begin with platelets, which comprise the predominant cellular phase of the hematoma, releasing numerous cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) [Probst and Spiegel, 1997]. These trigger an inflammatory response in the local tissue which in turn leads to an accumulation of granulocytes and monocytes/macrophages, the latter of which are known to release more than 100 different bioactive molecules [Probst and Spiegel, 1997]. One of the outcomes of this signaling cascade is the initiation of osteoblast and chondroblast differentiation from mesenchymal progenitor cells residing in the periosteum, marrow, and surrounding soft tissue [Gerstenfeld et al., 2003]. The osteoblasts then initiate intramembranous ossification from periosteal sites proximal and distal to the fracture site, working toward the fracture location (giving rise to the hard callus), while the fracture site serves as a locus for chondroblasts to begin cartilage deposition (forming the soft callus). The soft callus then undergoes endochondral ossification as the chondrocytes become hypertrophic and commence apoptosis [Gerstenfeld et al., 2003]. Following these events, osteoclasts, whose activity is inhibited early in fracture repair (in part by decreases in systemic PTH levels), are activated by elevated expression of osteoclastic stimulators such as PTH and tumor necrosis factor alpha (TNFα), and begin resorbing woven bone, thereby initiating the remodeling of the callus into lamellar bone [Meller et al., 1984; Gerstenfeld et al., 2003].

BONE MORPHOGENETIC PROTEINS

The bone morphogenetic proteins (BMP) belong to the TGF-β superfamily and are vital to skeletal development, growth, homeostasis, and fracture healing. BMP signaling is initiated by BMP binding to type 1 and type 2 serine/threonine kinase receptors. This results in the formation of a heterodimeric receptor complex and the phosphorylation of the type 1 receptor. Once phosphorylated, the type 1 receptor is activated and in turn phosphorylates intracellular pathway components, of which the Smads are the most prominent. Phosphorylated Smads then translocate to the nucleus and initiate transcription of BMP responsive genes [Wu et al., 2007]. Although more than 20 distinct BMP isoforms have been described, the function of a few isoforms, notably BMP-2, -4, and -7, has garnered the most attention. Immunohistochemical analysis of rat fracture calluses has shown that the expression of these three isoforms is strongly up-regulated at day 3 in the periosteum near the fracture site followed by a gradual reduction in expression [Onishi et al., 1998]. In regions of intramembranous ossification, osteoblastic expression of all three isoforms peaked at day 7, while in regions of endochondral ossification steady expression was seen in fibrocartilage, as well as in proliferating, mature, and hypertrophic chondrocytes, though BMP-7 expression dropped off rapidly in the mature and hypertrophic chondrocytes. Finally, osteoblasts invading the cartilaginous matrix showed strong BMP-2 and -4 staining with only weak BMP-7 reactivity. Studies of human fracture callus samples reveal similar spatial distribution of BMPs, with BMP-2, and -4 strongly expressed in mature and hypertrophic chondrocytes and moderately expressed in young active osteoblasts [Kloen et al., 2003]. In addition, strong BMP-7 expression was noted in young osteoblasts along with weaker and more varied expression in chondrocytes. These expression studies suggest that BMPs function to control fracture healing by inducing the proliferation and differentiation of osteoblasts and chondrocytes, thus affecting both endochondral and intramembranous ossification. This role is consistent with in vitro studies reporting that BMP-2 induces the differentiation of mesenchymal stem cells (MSC) into osteoprogenitors and that BMP-2, -4, and -7 further induce osteoprogenitor differentiation into osteoblasts [Cheng et al., 2003]. Similarly, it is consistent with the observed important role of BMP-2 signaling in chondrocyte proliferation and differentiation [Minina et al., 2001]. Definitive evidence that BMP-2 controls fracture repair was obtained from a fracture healing study conducted in mice with limb targeted deletion of BMP-2, which resulted in abrogation of healing [Tsuji et al., 2006]. Surprisingly, when these experiments were repeated with similarly targeted BMP-4 deletion, no adverse effects on fracture healing were seen [Tsuji et al., 2008], indicating that while BMP-4 is expressed in the fracture callus, its activity is not required for successful healing.

HYPOXIA-INDUCIBLE FACTOR 1 ALPHA AND ANGIOGENESIS

The hematoma formed following fracture not only provides a rich source of cytokine signaling but also isolates the injury site from perfusion, leading to low oxygen tension and regional hypoxia [Brighton and Krebs, 1972]. The chondrocyte locus lies in the center of this hypoxic zone and these cells must proliferate, differentiate, and begin matrix deposition in the complete absence of physiological perfusion. These events necessitate anaerobic metabolism for cellular energy and vascular in-growth for subsequent endochondral ossification [Schipani et al., 2001]. The process of intramembranous ossification begins on the border of the hypoxic region, requiring prompt blood vessel in-growth to deliver not only oxygen, nutrients, and mineral but also the majority of the newly differentiated osteoblasts [Einhorn, 1998]. An integral transcriptional regulator of these two vital processes, anaerobic metabolism and angiogenesis, is hypoxia-inducible factor (HIF) [Semenza, 2009].

HIF-1 is a heterodimeric transcription factor consisting of alpha (HIF-1α) and beta (HIF-1β) subunits, each with several isoforms [Semenza, 2009]. Both subunits are constitutively expressed throughout mammalian tissues and while HIF-1β is stable, the

stability and activity of HIF-1α is dependent on local oxygen tension. Under conditions of normoxia (~20% O_2), two proline residues in the oxygen-dependent degradation (ODD) domain of HIF-1α are hydroxylated by proline hydroxylases (PHD), which allows the binding of von Hippel–Lindau tumor suppressor protein (pVHL) and subsequent ubiquitin-mediated proteolytic degradation. Under conditions of hypoxia (<20% O_2) HIF-1α is not merely unhydroxylated and thus not degraded, but it is stabilized, activated, and binds to hypoxic response elements (HREs) in the promoters of its target genes to initiate their transcriptional activation (Fig. 2). Over 100 proven and putative HIF-1 target genes have been identified and, of interest to the control of fracture repair, these include genes associated with anaerobic metabolism, erythropoiesis, vascular regulation, angiogenesis, apoptosis, cell proliferation, cell adhesion, and cellular motility [Semenza, 2009].

We hypothesized that HIF-1α was a vital transcription factor involved in the control of fracture repair. Our first experiment sought to elucidate the spatiotemporal activation of HIF-1α and selected target genes in the course of fracture repair. These studies identified steady increases in HIF-1α mRNA and protein expression that peaked 10 days after fracture, followed by a gradual decline to levels similar to intact bone by day 21 [Komatsu and Hadjiargyrou, 2004]. In addition, the expression pattern of vascular endothelial growth factor (VEGF), a HIF-1α target, and key regulator of angiogenesis precisely correlated with the HIF-1α expression pattern. Furthermore, immunohistochemical analyses localized the origin of both of these signals to the proliferating chondrocytes and young active osteoblasts in zones of fibrocartilage, cartilage, and newly formed bone. With clear evidence of HIF-1α activity during fracture repair, we conducted a fracture repair study using mice with global HIF-1α haploinsufficiency (HIF-1α+/−) and wild-type littermates (HIF-1α+/+) to test the hypothesis that HIF-1 haploinsufficiency results in impaired fracture repair. Contrary to our expectations, the data indicated that fracture calluses from HIF-1α+/− mice were larger, more mineralized, and biomechanically superior to those from wild-type controls [Komatsu et al., 2007]. While callus expression of HIF-1α was expectedly reduced by ~50% in the HIF-1α+/− mice, levels of VEGF and platelet/endothelial cell adhesion molecule (PECAM) showed only modest reductions, suggesting little impairment in angiogenesis. However, further gene expression analyses identified several members of the apoptotic pathway as differentially regulated between genotypes. In particular, significant down-regulation of the pro-apoptotic gene PP2A coupled with significant up-regulation of the anti-apoptotic gene BCL2 were identified in HIF-1α+/− mice. TUNEL staining performed to quantify apoptotic cells within the fracture calluses subsequently confirmed that levels of osteoblast and chondrocyte apoptosis were indeed reduced in HIF-1α+/− mice. Therefore, it was concluded that HIF-1α haploinsufficiency does not significantly affect angiogenesis during fracture repair, but significantly reduces apoptosis leading to prolonged osteoblast and chondrocyte activity and the subsequent development of a larger, more mineralized, and mechanically superior fracture callus.

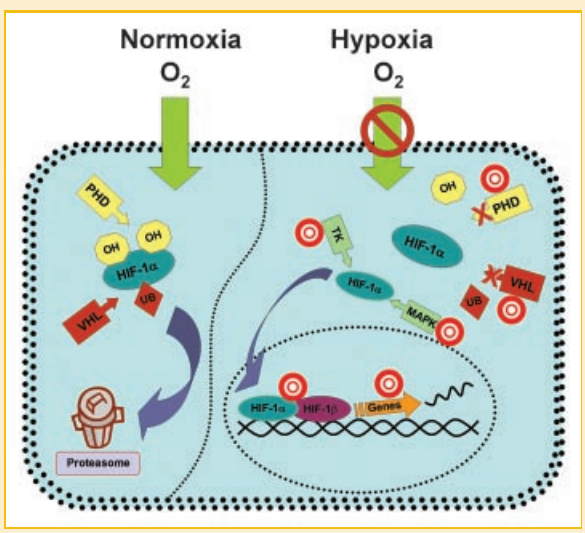

Fig. 2. The HIF-1α signaling pathway. This schematic details the major constituents of the HIF-1α signaling pathway. Under normoxia, two proline residues on the HIF-1α protein are hydroxylated (OH) by proline hydroxylases (PHD), facilitating ubiquitination (UB) by the von Hippel–Lindau tumor suppressor protein (VHL) and resulting in degradation within the proteasome. Under hypoxic condition, proline hydroxylation is prevented, protecting HIF-1α from ubiquitination and degradation. Activity of tyrosine kinases (TK) and map kinases (MAPK) stabilize and activate HIF-1α, subsequent nuclear translocation and dimerization with HIF-1β and then allows for target gene activation. Potential therapeutic targets in this pathway are marked with bulls-eyes and include PHD and VHL inhibitors, TK and MAPK activators, as well as direct modulators of HIF-1α, HIF-1β, and critical target genes.

While our laboratory was the first to publish data on HIF-1α in the context of bone regeneration the use of global heterozygotes made it difficult to test our primary hypothesis that HIF-1α activity is required for successful fracture repair. Several recent studies have used more precise gene targeting and pharmaceutical approaches to better test this hypothesis. Wan et al. [2008] subjected mice with targeted deletion of HIF-1α in osteoblasts to distraction osteogenesis (DO) and noted significant decreases in both angiogenesis and mineralization. They also generated mice with osteoblast-specific pVHL deletion, which resulted in overexpression of HIF-1α, and when subjected to DO these mice were characterized by increased angiogenesis, mineralization, and mechanical properties. Finally, using desferrioxamine (DFO) to pharmacologically inhibit PHD (thereby increasing HIF-1α activity), bone regeneration following DO was significantly enhanced. A recent follow-up to these studies again used DFO to activate HIF-1α, though this time a closed femoral fracture model was used. Consistent with the DO experiments, callus formation and angiogenesis were enhanced, though no gains were seen for biomechanical outcomes [Shen et al., 2009].

The contrasting results achieved by using different approaches to assess how HIF-1α controls fracture repair not only highlight the complexity of this process but also underscore the multitude of specific targets that exist for enhancing fracture repair. Moreover, each of these targets may be suited for specific clinical needs with direct targeting of HIF-1α potentially benefiting fractures with vascular insufficiency and apoptosis inhibition benefiting fractures with mechanical instability.

THE Wnt SIGNALING PATHWAY

The skeletal role of the Wnt signaling pathway has risen in prominence over the past decade and the details of how this pathway influences fracture repair is currently under investigation by multiple research groups. As the essentials of Wnt signaling (Fig. 3) and its role in fracture repair were recently reviewed in this journal [Chen and Alman, 2009] we will only discuss developments that have been reported in the interim.

Prior experiments have shown that modulation of Wnt signaling can affect the process of fracture repair, yet much remains unknown about the function of specific pathway components in the control of this process. In order to ascertain the function of Lrp5 in fracture repair, we performed a study using Lrp5−/− mice and wild-type littermates, as well as normal mice treated with neutralizing antibodies to Dickkopf 1 (Dkk1 Ab). These experiments sought to test the complementary hypotheses that inhibition of Wnt signaling via Lrp5 deletion adversely affects fracture repair and activation of Wnt signaling via removal of Dkk1 inhibition of Wnt ligand binding to Lrp5 enhances this process. The results clearly supported these hypotheses with fractured femurs from Lrp5−/− mice found to be smaller, less mineralized, and biomechanically inferior to those from wild-type littermates [Komatsu et al., 2009b]. Conversely, treatment with Dkk1 Ab increased the size, mineralization, and biomechanical properties of fractured femurs. These data support the consensus that Wnt signaling is required for fracture repair, and the use of Lrp5−/− mice in these experiments indicate that Lrp5 is a critical mediator of Wnt signaling in this process. However, because fracture repair in these mice was not completely abolished, it is likely that other Wnt co-receptors, such as Lrp6, are also involved in this process. Prior researchers have examined Dkk1 modulation in the context of fracture repair and found that overexpression of Dkk1 impedes the normal progression of repair [Chen and Alman, 2009]. Our findings of improved fracture repair following neutralization of Dkk1-mediated Wnt inhibition not only support these data but establish a precedent that such treatment may have efficacy in accelerating fracture repair.

The widely publicized finding by Yadav et al. [2008] that the low bone mass phenotype in Lrp5−/− mice is caused by elevated serotonin synthesis in the duodenum rather than a local osteoblastic response suggests that the impaired fracture repair seen in our Lrp5−/− mice may not be due to direct effects of Lrp5 at the bone cell level. However, the influence of altered circulating serotonin levels on fracture healing has not been adequately explored and whether serotonin accounts for the entire skeletal effects of Lrp5 remains to be confirmed [Warden et al., 2009b].

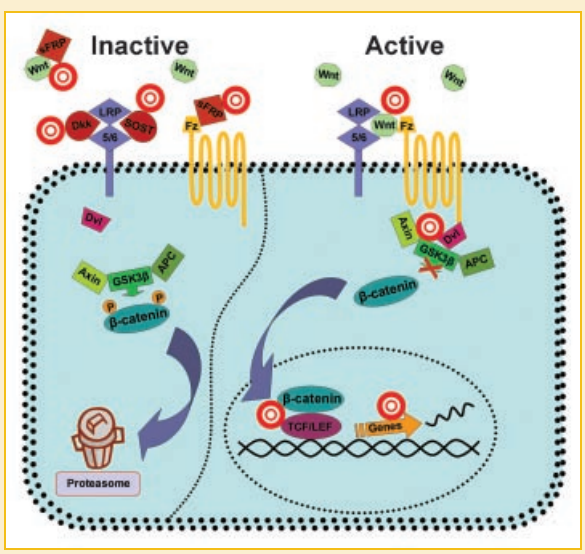

Fig. 3. The Wnt signaling pathway. This schematic details the major constituents of the canonical Wnt signaling pathway. In the absence of Wnt signaling (inactive), glycogen synthase kinase 3β (GSK3β), complexed with Axin and adenomatous polyposis coli (APC), phosphorylates β-catenin, resulting in degradation within the proteasome. In the presence of Wnt signaling (active), Wnt ligands bind to the Wnt co-receptors low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) and Frizzleds (Fz). The intracellular Fz domain then binds and activates disheveled (Dvl) which in turn inhibits GSK3β-mediated phosphorylation of β-catenin. Hypophosphorylated β-catenin then translocates to the nucleus where it binds with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcriptions factors to direct target gene transcription. Potential therapeutic targets in this pathway are marked with bulls-eyes and include the extracellular Wnt inhibitors secreted frizzled-related proteins (sFRP), Dikkopfs (Dkk), and sclerostin (SOST), intracellular pathway components such as GSKβ and Dvl, as well as β-catenin, TCF/LEF proteins, and critical target genes.

THERAPEUTIC TARGETING OF FRACTURE HEALING

BONE MORPHOGENETIC PROTEINS

The BMPs represent the most thoroughly studied bone anabolic biologic agents and the sole clinically approved agents for applications related to fracture repair. The clinical potential of BMPs was first suggested by Marshal Urist in his landmark 1965 report on the osteoinductive potential of decalcified bone [Urist, 1965]. However, it required almost 40 years of further research for the first of these biologics to garner FDA approval for the treatment of fractures. This occurred in 2001 when the FDA granted a humanitarian device exemption for BMP-7, also known as osteogenic protein-1 (OP-1), for the treatment of non-united long bone fractures. BMP-2 followed OP-1 and was approved by the FDA in 2004 for treating open tibial shaft fractures and later for spinal fusions. Clinical data on the efficacy of these products for these indications are generally positive [Kirker-Head et al., 2007], but given the vast number of positive preclinical animal studies for a much broader range of indications, the lack of broader approval and stronger clinical outcomes highlights how much we still need to learn about the control of fracture repair in order to create new therapeutics to improve this process.

OTHER GROWTH FACTORS

In addition to the BMPs, other growth factors have demonstrated efficacy in improving fracture repair in animal models. Of these, PDGF appears to have the greatest potential for regulatory approval in the near future. Animal studies have shown that PDGF can accelerate healing of femoral fractures in diabetic rats and improve bone formation during DO [Al-Zube et al., 2009; Moore et al., 2009]. Clinically, a formulation of PDGF and tricalcium phosphate (GEM OS1) was approved by the FDA in 2005 for the repair of periodontal

bone defects [Hollinger et al., 2008]. Based on a successful pilot study in which this formulation was used as a bone graft material in foot and ankle fusions, a pivotal clinical trial for this indication has been initiated [Hollinger et al., 2008]. In addition, early clinical results show that a similar formulation can accelerate healing in distal radius fractures, suggesting that the pursuit of a fracture indication is probable in the near future.

Following PDGF is an array of other growth factors that have demonstrated efficacy for fracture treatment in preclinical animal studies including growth and differentiation factor-5 (GDF-5, aka BMP-14), TGF-β, insulin-like growth factor-1 (IGF-1), and growth hormone (GH) [Axelrad et al., 2007]. In addition to single proteins, the use of autologous blood products containing multiple growth factors, such as platelet-rich plasma (PRP), have shown limited preclinical efficacy in accelerating fracture repair.

In a femoral fracture study using diabetic rats, a single injection of PRP immediately postfracture increased fracture callus cartilage content and biomechanical properties, as compared to sham injected diabetic controls, but was unable to restore these parameters to levels seen in untreated non-diabetic animals [Gandhi et al., 2006]. However, in a study conducted using normal rats subjected to closed femoral fractures, PRP was unable to improve callus biomechanical integrity and only a small gain in callus formation was seen [Simman et al., 2008]. Furthermore, treatment of ovine tibial segmental defects with PRP delivered in a collagen carrier showed no efficacy in enhancing bone formation [Sarkar et al., 2006]. Despite these mixed results, a small number of clinical studies have been conducted using PRP for a variety of orthopedic applications. Though some clinical successes have been reported, the majority of these studies have been small and poorly controlled, making it difficult to properly evaluate the clinical utility of PRP [Alsousou et al., 2009]. One of the only prospective randomized trials reported compared PRP to BMP-7 for the treatment of long bone non-unions [Calori et al., 2008]. Unfortunately, the results of this study showed that BMP-7 was superior to PRP in terms of time to clinical and radiographic healing as well as overall healing rate. As autologous PRP does not require FDA approval, it is certain that surgeons will continue to utilize it. However, because PRP is essentially an unpatentable treatment, the prospect of well-controlled prospective clinical trials appears remote.

HIF-1α AND ANGIOGENESIS

The previously discussed findings on the control of fracture healing by HIF-1α suggest that timely angiogenesis is critical to successful fracture healing. This hypothesis was first tested in a study that treated murine fractures by either systemic injection with Flt-IgG (a soluble neutralizing VEGF receptor) to block endogenous VEGF or local delivery of VEGF to supplement endogenous VEGF [Street et al., 2002]. Dramatic impairment of fracture repair was observed in the mice treated with Flt-IgG, while those treated with VEGF showed equally dramatic augmentation. Additional studies involving delivery of VEGF encoding plasmids or VEGF protein directly to various defects in rabbits and rats offer further evidence that local increases in VEGF are able to accelerate bone healing [Keramaris et al., 2008].

As a major regulator of VEGF, the HIF-1α pathway presents a variety of therapeutic targets for enhancing fracture healing. To date, the previously discussed study using DFO, as well as dimethyloxalylglycine (DMOG), to inhibit PHD-mediated HIF-1α degradation has shown the strongest direct evidence that such targeting may have clinical utility in enhancing fracture healing [Shen et al., 2009]. In contrast, our own work on HIF-1α and fracture healing suggests that rather than activation of HIF-1α, inhibition of apoptosis (with or without direct targeting of HIF-1α) is itself a valid therapeutic target [Komatsu et al., 2007].

Other fracture healing studies offer circumstantial support that targeting apoptosis may have clinical utility in enhancing this process. Administration of interleukin-1 beta (IL-1β) to rats with tibial defects resulted in increased callus formation due to greater numbers of osteoblasts within the defect resulting from lower rates of osteoblastic apoptosis [Olmedo et al., 1999]. Collectively, the preclinical data derived from studies of HIF-1α, VEGF, PHD, and apoptosis indicate that multiple unique therapeutic targets exist within this signaling complex that have potential clinical utility in enhancing fracture healing (Fig. 2). Despite this promising body of preclinical data, to date the only related treatment to enter clinical trials is the thrombin-related peptide TP508.

TP508 is a synthetic peptide comprising a 23-amino-acid region of the human thrombin receptor-binding domain that exhibits proangiogenic activity in vivo [Wang et al., 2005]. Animal studies performed using TP508 showed it to be efficacious in enhancing the healing of various skeletal injuries, including closed fractures [Wang et al., 2005]. Unfortunately, the inability of TP508 to demonstrate accelerated healing of wrist fractures in a Phase III clinical trial led to the early termination of a similar Phase IIb dose–response study and appears to have halted the pursuit of a fracture repair indication for this compound [Chopack, 2006]. Regardless of the failure of this compound, the multitude of other targets in this signaling complex suggests that future clinical trials of other compounds are likely.

Wnt SIGNALING

As previously discussed, the Wnt pathway looms large in the future of skeletal anabolic therapies, as well as potential modulation of fracture repair (Fig. 3). The simplest Wnt activator is lithium chloride (LiCl), which has been used for decades as an orally active mood stabilizer. In addition to its psychotropic effects, LiCl activates Wnt signaling in bone by its inhibition of glycogen synthase kinase 3β (GSK-3β). Preclinical studies have shown that LiCl treatment can improve fracture repair in mice, and retrospective clinical analyses have identified an association between chronic LiCl administration and reduced fracture risk [Chen and Alman, 2009]. While LiCl is generally safe, its use is associated with several potentially serious and undesirable side effects. These side effects, combined with non-bone-specific effects and a lack of patent protection, indicate that while it may have clinical potential for fracture treatment, the likelihood of clinical trials is remote.

A more precise way to modulate Wnt signaling during fracture repair is to target extracellular Wnt pathway regulators and several antibody-based approaches to such modulation have recently been described. In addition to our previously described experiments with

Dkk1 neutralizing antibodies, neutralizing antibodies to sclerostin (another Wnt inhibitor) are also able to improve fracture repair in rodents [Ke et al., 2009]. Furthermore, a recent fibular osteotomy study conducted in cynomolgus monkeys revealed that treatment with sclerostin neutralizing antibodies enhanced bone formation and biomechanical properties [Ominsky et al., 2009]. These data along with Phase 1 clinical data showing the sclerostin antibodies have an excellent safety profile [Li et al., 2007b] signify that a fracture healing indication will likely be pursued in the future.

The therapeutic potential of other targets in Wnt signaling pathway such as Wnt-1-induced secreted protein 1 (WISP-1) and secreted Frizzled-related protein-1 (sFRP-1) to enhance fracture healing has also been reported [Chen and Alman, 2009]. Several publications relating to inhibition of sFRP-1 during fracture healing suggest that this may be the next clinically evaluated Wnt modulator for this indication. First, the identification of a series of small molecule inhibitors of sFRP-1 was reported. This was followed by a more detailed study that characterized an optimized sFRP-1 inhibitor, WAY-316606, and revealed it to have good potency and selectivity for sFRP-1, as well as ex vivo skeletal anabolic activity, but a poor pharmacokinetic profile [Bodine et al., 2009]. Finally, a study on tibial fracture healing in sFRP-1 knockout mice documented that Wnt signaling is indeed increased, resulting in earlier initiation of intramembranous ossification and acceleration of bony union [Gaur et al., 2009]. Should sFRP-1 inhibitors with better pharmacokinetic profiles be developed, clinical evaluation of their ability to enhance fracture healing seem probable.

PARATHYROID HORMONE

As the only clinically approved systemic skeletal anabolic agent, intermittent parathyroid hormone (PTH), as well as its bioactive fragments and analogs, have been thoroughly researched as potential therapeutic agents for accelerating fracture repair. One of the first studies was reported by Kim and Jahng [1999] and involved inducing tibial fractures in ovariectomized rats and treating them with PTH (1–84). The PTH treated animals demonstrated increased callus diameter and trabecular bone area as well as improved mechanical properties. In the 10 years following this report, the ability of PTH to improve various bone regeneration outcomes has been demonstrated in male and female, young, mature and old, as well as intact and ovariectomized rats [Barnes et al., 2008; Warden et al., 2009a]. In addition to the rat, studies have also been conducted using mice, rabbits, and monkeys; with these species also unequivocally demonstrating that intermittent PTH treatment improves bone regeneration [Barnes et al., 2008]. One flaw in many of these studies is the use of PTH doses that greatly exceed the approved clinical dosage, thus generating results that may not be indicative of clinical utility. We recently performed a dose–response study to test the ability of clinically relevant doses of PTH (1–38) to enhance the healing of femoral drill-hole defects in ovariectomized rats [Komatsu et al., 2009a]. Using a primary outcome of longitudinal quantitative CT imaging, this study revealed clear, dose-dependent enhancement of cortical defect healing over 5 weeks, with the enhanced mineralization seen in the cortices and intramedullary space, but not at the periosteum. However, as only negligible gains were seen at the lowest dosage, this study suggests that clinically relevant doses of PTH may not be sufficient to substantially enhance cortical bone repair.

Clinical data regarding the ability of PTH to enhance fracture repair remain limited. In an uncontrolled trial of three patients with non-healing fractures, daily treatment with PTH (1–34) was associated with successful bony union within 2 months [Schober, 2009]. More persuasive evidence comes from a study that compared the healing of femoral neck fractures between 12 women treated with PTH (1–34) and 12 untreated controls [Corradini et al., 2009]. As judged by decreased time to walking and pain, as well as increased quality of life and hip BMD, PTH treatment was associated with significant benefits. Results from the first prospective, randomized, double-blind clinical trial of PTH (1–34) to accelerate fracture healing were recently reported [Aspenberg et al., 2009]. In this study, 102 postmenopausal women with Colles fractures were randomized to receive placebo, 20 μg PTH (1–34), or 40 μg PTH (1–34) daily for 8 weeks. Despite failing to prove the primary hypothesis that the 40 μg dose would reduce time to healing compared to placebo, a significant improvement in time to healing was seen for the 20 μg dose. Though some benefit was seen in this study, the authors stress caution in interpretation of the results and further trials will be required to resolutely ascertain the specific indications for which PTH may enhance fracture repair.

PROSTAGLANDINS

Prostaglandins are generated by enzymatic modification of polyunsaturated fatty acids and have pleiotropic effects upon multiple organs and physiological processes such as inflammation, vasodilatation, embryo implantation, labor induction, as well as bone formation and healing [Li et al., 2007a]. Preclinical animal studies have shown that systemic administration of prostaglandin E2 (PGE2) is skeletally anabolic at cortical and cancellous sites [Jee and Ma, 1997]. Unfortunately, PGE2 treatment is associated with significant adverse events (e.g., diarrhea, lethargy, and flushing) that preclude its clinical use for treating osteoporosis or fractures. To circumvent these issues, small molecule agonists of the EP2 and EP4 PGE2 receptors have been developed and demonstrated efficacy in animal models of bone healing. Specifically, local delivery of the EP2 agonist CP533,536 was able to induce robust healing in dogs following generation of tibial osteotomies and ulnar segmental defects, with no evidence of adverse effects [Paralkar et al., 2003]. More recently, the EP4 agonist CP-734432 was shown to enhance femoral fracture healing in wild-type mice and rescue the impaired fracture healing phenotype of COX-2-deficient mice [Xie et al., 2009]. Interestingly, this study also examined the efficacy of a different EP2 agonist (CP-463755) in wild-type and COX-2-deficient mice and while a modest benefit was seen in COX-2-deficient mice, no effects were seen in the wild-type mice. Although these data suggest that therapeutic targeting of PGE receptors may be able to avoid the side effects associated with PGE administration and achieve clinical utility in enhancing fracture healing, better understanding of the contributions of each type of receptor, as well as potential species specific variations in receptor/ligand affinity, will be required before these compounds are ready for clinical trials.

STEM CELLS

The potential therapeutics for enhancing fracture healing discussed to this point all function by altering the signaling environment in resident cells. However, in some cases, notably fractures in the elderly or large segmental defects, such alteration may not be sufficient to enhance fracture healing due to a deficit in the number of cells present. The obvious course of treatment in these cases is to deliver osteogenic cells directly to the fracture site. Numerous researchers have approached this issue by following the traditional tissue engineering principles of harvesting and expanding cells, seeding them on scaffolds, and then implanting the cellularized scaffolds [Patterson et al., 2008]. While this approach has some utility, it is time consuming, surgically intensive, and expensive. Moreover, such treatments will face significant regulatory hurdles before approval. In order to simplify this process, a treatment consisting of direct fracture site injection of cell-culture expanded autologous osteoblasts in a fibrin carrier has been developed and was recently tested in a Korean clinical trial [Kim et al., 2009]. Despite a small sample size and the use of varying fracture sites, significant improvements in callus formation were seen. An even simpler approach to cellular therapeutics for enhancing fracture healing has been tested in animals. Researchers first isolated human $CD34^{+}$ peripheral blood cells and then administered them via intravenous injection to nude rats immediately following induction of femoral fractures [Matsumoto et al., 2006]. In the treated animals, fracture healing was enhanced as evidenced by higher rates of radiographic bridging and improved biomechanical properties. Furthermore, angiogenesis and perfusion at the fracture sites improved following treatment, and significant numbers of human cells were found engrafted at the fracture sites. Though preclinical, the simplicity and success of this study highlights the fact that cellular therapeutics have superb clinical potential and will certainly play a role in future treatments to enhance fracture healing.

SUMMARY

The control of fracture repair is a complex process that requires the timely activation of hundreds, if not thousands of genes, and the coordinated migration, proliferation, differentiation, and activity of multiple cell types. Despite this complexity, with proper orthopedic management the vast majority of fractures heal successfully in a timely manner. Perhaps because of this, the pharmaceutical industry has historically shown little interest in developing treatments designed to enhance fracture healing. Advances in our understanding of the molecular mechanisms underlying physiological and pathophysiological fracture healing, combined with an aging society projected to suffer from an ever increasing incidence of difficult to heal fractures, appear to have identified sufficient therapeutic targets and a large enough potential market for these companies to initiate the development of therapeutics designed to enhance fracture healing. Preclinical animal studies have validated the therapeutic potential of numerous targets to enhance fracture healing, but definitive demonstration of clinical efficacy remains lacking. We expectantly await such data and hope to soon see the results of well-designed clinical trials with Level I evidence of enhancement of fracture healing demonstrated not only at the radiographic level but also as measured by improvements in functional outcomes. While the successful development of such new therapeutics will by no means contravene the use of traditional hardware-based approaches for fracture fixation, they will assuredly garner significant clinical adoption. This in turn will improve the success rates not just for difficult to heal fractures, but all fractures, leading to improved patient care and reduced public health costs.

REFERENCES

Alsousou J, Thompson M, Hulley P, Noble A, Willett K. 2009. The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: A review of the literature. J Bone Joint Surg Br 91:987–996.

Al-Zube L, Breitbart EA, O'Connor JP, Parsons JR, Bradica G, Hart CE, Lin SS. 2009. Recombinant human platelet-derived growth factor BB (rhPDGF-BB) and beta-tricalcium phosphate/collagen matrix enhance fracture healing in a diabetic rat model. J Orthop Res 27:1074–1081.

Andersson GBJ, Bouchard J, Bozic KJ, Campbell RM Jr., Cisternas MG, Correa A, Cosman F, Cragan JD, D'Andrea K, Doernberg N, Dormans JP, Elderkin AL, Fershteyn Z, Foreman AJ, Gitelis S, Gnatz SM, Haralson RH III, Helmick CG, Hu S, Katz JN, King T, Kirk R, Kurtz SM, Lane N, Miller A, Novich RL, Olney R, Panopalis P, Pasta DJ, Pollak AN, Puzas JE, Richards BS III, Sestito JP, Siffel C, Sponseller PD, St. Clair EW, Stuart A, Templeton KJ, Thompson G, Tosi L, Ward WG Sr., Watkins-Castillo SI, Weinstein SL, Wright JG, Yelin EH. 2008. United States Bone and Joint Decade: The Burden of Musculoskeletal Diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons. pp 97–161.

Aspenberg P, Genant HK, Johansson T, Nino AJ, See K, Krohn K, García-Hernández PA, Recknor CP, Einhorn TA, Dalsky GP, Mitlak BH, Fierlinger A, Lakshmanan MC. 2009. Teriparatide for acceleration of fracture repair in humans: A prospective, randomized, double-blind study of 102 postmenopausal women with distal radial fractures. J Bone Miner Res. DOI: 10.1359/jbmr.090731 [Epub July 13].

Axelrad TW, Kakar S, Einhorn TA. 2007. New technologies for the enhancement of skeletal repair. Injury 38(Suppl 1):S49–S62.

Bais M, McLean J, Sebastiani P, Young M, Wigner N, Smith T, Kotton DN, Einhorn TA, Gerstenfeld LC. 2009. Transcriptional analysis of fracture healing and the induction of embryonic stem cell-related genes. PLoS One 4:e5393 [Epub May 5].

Barnes GL, Kakar S, Vora S, Morgan EF, Gerstenfeld LC, Einhorn TA. 2008. Stimulation of fracture-healing with systemic intermittent parathyroid hormone treatment. J Bone Joint Surg Am 90(Suppl 1):120–127.

Bodine PV, Stauffer B, Ponce-de-Leon H, Bhat RA, Mangine A, Seestaller-Wehr LM, Moran RA, Billiard J, Fukayama S, Komm BS, Pitts K, Krishnamurthy G, Gopalsamy A, Shi M, Kern JC, Commons TJ, Woodworth RP, Wilson MA, Welmaker GS, Trybulski EJ, Moore WJ. 2009. A small molecule inhibitor of the Wnt antagonist secreted frizzled-related protein-1 stimulates bone formation. Bone 44:1063–1068.

Brighton CT, Krebs AG. 1972. Oxygen tension of nonunion of fractured femurs in the rabbit. Surg Gynecol Obstet 135:379–385.

Calori GM, Tagliabue L, Gala L, d'Imporzano M, Peretti G, Albisetti W. 2008. Application of rhBMP-7 and platelet-rich plasma in the treatment of long bone non-unions: A prospective randomised clinical study on 120 patients. Injury 39:1391–1402.

Chen Y, Alman BA. 2009. Wnt pathway, an essential role in bone regeneration. J Cell Biochem 106:353–362.

Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC. 2003. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 85-A:1544–1552.

Chopack J. 2006. Orthologic announces termination of its phase IIb dose response. Healthpoint Capital. http://www.healthpointcapital.com/research/2006/08/30/orthologic_announces_termination_of_its_phase_iib_dose_response_trial/

Corradini C, Parravicini L, Crapanzano C, Marchelli D, Ulivieri FM, Verdoia C. 2009. Enhanced healing of osteoporotic femoral neck fractures with teriparatide: New therapeutic horizon. J Bone Miner Res 24: A09003283.

Einhorn TA. 1998. The cell and molecular biology of fracture healing. Clin Orthop Relat Res 355:S7–S21.

Gandhi A, Doumas C, O'Connor JP, Parsons JR, Lin SS. 2006. The effects of local platelet rich plasma delivery on diabetic fracture healing. Bone 38:540–546.

Gaur T, Wixted JJ, Hussain S, O'Connell SL, Morgan EF, Ayers DC, Komm BS, Bodine PV, Stein GS, Lian JB. 2009. Secreted frizzled related protein 1 is a target to improve fracture healing. J Cell Physiol 220:174–181.

Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. 2003. Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 88:873–884.

Hadjiargyrou M, Lombardo F, Zhao S, Ahrens W, Joo J, Ahn H, White DW, Rubin CT. 2002. Transcriptional profiling of bone regeneration: Insight into the molecular complexity of wound repair. J Biol Chem 277:30177–30182.

Heiner DE, Meyer MH, Frick SL, Kellam JF, Fiechtl J, Meyer RA, Jr. 2006. Gene expression during fracture healing in rats comparing intramedullary fixation to plate fixation by DNA microarray. J Orthop Trauma 20:27–38.

Hollinger JO, Hart CE, Hirsch SN, Lynch S, Friedlaender GE. 2008. Recombinant human platelet-derived growth factor: Biology and clinical applications. J Bone Joint Surg Am 90(Suppl 1):48–54.

Jee WS, Ma YF. 1997. The in vivo anabolic actions of prostaglandins in bone. Bone 21:297–304.

Ke HZ, Xiang JJ, Li X, Simonet WS, Paszty C, Li G. 2009. Inhibition of sclerostin by systemic treatment with a sclerostin monoclonal antibody enhances fracture healing in mice and rats. Trans Orthop Res Soc 34:0221.

Keramaris NC, Calori GM, Nikolaou VS, Schemitsch EH, Giannoudis PV. 2008. Fracture vascularity and bone healing: A systematic review of the role of VEGF. Injury 39(Suppl 2):S45–S57.

Kim HW, Jahng JS. 1999. Effect of intermittent administration of parathyroid hormone on fracture healing in ovariectomized rats. Iowa Orthop J 19:71–77.

Kim SJ, Shin YW, Yang KH, Kim SB, Yoo MJ, Han SK, Im SA, Won YD, Sung YB, Jeon TS, Chang CH, Jang JD, Lee SB, Kim HC, Lee SY. 2009. A multicenter, randomized, clinical study to compare the effect and safety of autologous cultured osteoblast (Ossron) injection to treat fractures. BMC Musculoskelet Disord 10:20.

Kirker-Head CA, Boudrieau RJ, Kraus KH. 2007. Use of bone morphogenetic proteins for augmentation of bone regeneration. J Am Vet Med Assoc 231: 1039–1055.

Kloen P, Di Paola M, Borens O, Richmond J, Perino G, Helfet DL, Goumans MJ. 2003. BMP signaling components are expressed in human fracture callus. Bone 33:362–371.

Komatsu DE, Hadjiargyrou M. 2004. Activation of the transcription factor HIF-1 and its target genes, VEGF, HO-1, iNOS, during fracture repair. Bone 34:680–688.

Komatsu DE, Bosch-Marce M, Semenza GL, Hadjiargyrou M. 2007. Enhanced bone regeneration associated with decreased apoptosis in mice with partial HIF-1alpha deficiency. J Bone Miner Res 22:366–374.

Komatsu DE, Brune KA, Liu H, Schmidt AL, Han B, Zeng QQ, Yang X, Nunes JS, Lu Y, Geiser AG, Ma YL, Wolos JA, Westmore MS, Sato M. 2009a. Longitudinal in vivo analysis of the region-specific efficacy of parathyroid hormone in a rat cortical defect model. Endocrinology 150:1570–1579.

Komatsu DE, Mary MN, Schroeder RJ, Robling AG, Turner CH, Warden SJ. 2009b. Modulation of Wnt signaling during fracture repair. Trans Orthop Res Soc 34:0436.

Li M, Thompson DD, Paralkar VM. 2007a. Prostaglandin E(2) receptors in bone formation. Int Orthop 31:767–772.

Li X, Warmington KS, Niu Q, Grisanti M, Tan H, Dwyer D, Stolina M, Ominsky MS, Simonet WS, Kostenuik PJ, Paszty C, Ke HZ. 2007b. Anti-sclerostin antibody increases markers of bone formation in healthy postmenopausal women. J Bone Miner Res 22:S36.

Matsumoto T, Kawamoto A, Kuroda R, Ishikawa M, Mifune Y, Iwasaki H, Miwa M, Horii M, Hayashi S, Oyamada A, Nishimura H, Murasawa S, Doita M, Kurosaka M, Asahara T. 2006. Therapeutic potential of vasculogenesis and osteogenesis promoted by peripheral blood CD34-positive cells for functional bone healing. Am J Pathol 169:1440–1457.

Meller Y, Kestenbaum RS, Mozes M, Mozes G, Yagil R, Shany S. 1984. Mineral and endocrine metabolism during fracture healing in dogs. Clin Orthop Relat Res 187:289–295.

Minina E, Wenzel HM, Kreschel C, Karp S, Gaffield W, McMahon AP, Vortkamp A. 2001. BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. Development 128:4523–4534.

Moore DC, Ehrlich MG, McAllister SC, Machan JT, Hart CE, Voigt C, Lesieur-Brooks AM, Weber EW. 2009. Recombinant human platelet-derived growth factor-BB augmentation of new-bone formation in a rat model of distraction osteogenesis. J Bone Joint Surg Am 91:1973–1984.

Olmedo ML, Landry PS, Sadasivan KK, Albright JA, Meek WD, Routh R, Marino AA. 1999. Regulation of osteoblast levels during bone healing. J Orthop Trauma 13:356–362.

Ominsky M, Samadgam R, Jolette J, Vlasseros F, Smith SY, Kostenuik P, Paszty C, Simonet W, Ke HZ. 2009. Sclerostin monoclonal antibody stimulates bone formation and improves the strength and density of the fracture callus and lumbar spine in a primate fibular osteotomy model. J Bone Miner Res 24:A09001495.

Onishi T, Ishidou Y, Nagamine T, Yone K, Imamura T, Kato M, Sampath TK, ten Dijke P, Sakou T. 1998. Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. Bone 22:605–612.

Paralkar VM, Borovecki F, Ke HZ, Cameron KO, Lefker B, Grasser WA, Owen TA, Li M, DaSilva-Jardine P, Zhou M, Dunn RL, Dumont F, Korsmeyer R, Krasney P, Brown TA, Plowchalk D, Vukicevic S, Thompson DD. 2003. An EP2 receptor-selective prostaglandin E2 agonist induces bone healing. Proc Natl Acad Sci USA 100:6736–6740.

Patterson TE, Kumagai K, Griffith L, Muschler GF. 2008. Cellular strategies for enhancement of fracture repair. J Bone Joint Surg Am 90(Suppl 1):111–119.

Phemister DB. 1951. Biologic principles in the healing of fractures and their bearing on treatment. Ann Surg 133:433–446.

Probst A, Spiegel HU. 1997. Cellular mechanisms of bone repair. J Invest Surg 10:77–86.

Sarkar MR, Augat P, Shefelbine SJ, Schorlemmer S, Huber-Lang M, Claes L, Kinzl L, Ignatius A. 2006. Bone formation in a long bone defect model using a platelet-rich plasma-loaded collagen scaffold. Biomaterials 27:1817–1823.

Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS. 2001. Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev 15:2865–2876.

Schober H. 2009. Successful treatment with hPTH for 3 patients suffering from non healing fractures. J Bone Miner Res 24:A09002859.

Semenza GL. 2009. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology 24:97–106.

Shen X, Wan C, Ramaswamy G, Mavalli M, Wang Y, Duvall CL, Deng LF, Guldberg RE, Eberhart A, Clemens TL, Gilbert SR. 2009. Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice. J Orthop Res 27:1298–1305.

Simman R, Hoffmann A, Bohinc RJ, Peterson WC, Russ AJ. 2008. Role of platelet-rich plasma in acceleration of bone fracture healing. Ann Plast Surg 61:337–344.

Street J, Bao M, deGuzman L, Bunting S, Peale FV, Jr., Ferrara N, Steinmetz H, Hoeffel J, Cleland JL, Daugherty A, van Bruggen N, Redmond HP, Carano RA, Filvaroff EH. 2002. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci USA 99:9656–9661.

Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V. 2006. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet 38:1424–1429.

Tsuji K, Cox K, Bandyopadhyay A, Harfe BD, Tabin CJ, Rosen V. 2008. BMP4 is dispensable for skeletogenesis and fracture-healing in the limb. J Bone Joint Surg Am 90(Suppl 1):14–18.

Urist MR. 1965. Bone: Formation by autoinduction. Science 150:893–899.

Wan C, Gilbert SR, Wang Y, Cao X, Shen X, Ramaswamy G, Jacobsen KA, Alaql ZS, Eberhardt AW, Gerstenfeld LC, Einhorn TA, Deng L, Clemens TL. 2008. Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. Proc Natl Acad Sci USA 105:686–691.

Wang H, Li X, Tomin E, Doty SB, Lane JM, Carney DH, Ryaby JT. 2005. Thrombin peptide (TP508) promotes fracture repair by up-regulating inflammatory mediators, early growth factors, and increasing angiogenesis. J Orthop Res 23:671–679.

Warden SJ, Komatsu DE, Rydberg J, Bond JL, Hassett SM. 2009a. Recombinant human parathyroid hormone (PTH 1-34) and low-intensity pulsed ultrasound have contrasting additive effects during fracture healing. Bone 44:485–494.

Warden SJ, Robling AG, Haney EM, Turner CH, Bliziotes MM. 2009b. The emerging role of serotonin (5-hydroxytryptamine) in the skeleton and its mediation of the skeletal effects of low-density lipoprotein receptor-related protein 5 (LRP5). Bone. DOI: 10.1016/j.bone.2009.06.029 [Epub July 8].

Wu X, Shi W, Cao X. 2007. Multiplicity of BMP signaling in skeletal development. Ann N Y Acad Sci 1116:29–49.

Xie C, Liang B, Xue M, Lin AS, Loiselle A, Schwarz EM, Guldberg RE, O'Keefe RJ, Zhang X. 2009. Rescue of impaired fracture healing in COX-2−/− mice via activation of prostaglandin E2 receptor subtype 4. Am J Pathol 175:772–785.

Yadav VK, Ryu JH, Suda N, Tanaka KF, Gingrich JA, Schütz G, Glorieux FH, Chiang CY, Zajac JD, Insogna KL, Mann JJ, Hen R, Ducy P, Karsenty G. 2008. Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. Cell 135:825–837.